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PHARMACEUTICALS IN THE NATION'S DRINKING WATER: ASSESSING POTENTIAL RISKS AND ACTIONS TO ADDRESS THE ISSUE

HEARING

BEFORE THE

SUBCOMMITTEE ON TRANSPORTATION SAFETY, INFRUSTRUCTURE SECURITY, AND WATER QUALITY

OF THE

COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS UNITED STATES SENATE

ONE HUNDRED TENTH CONGRESS

SECOND SESSION

APRIL 15, 2008

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PHARMACEUTICALS IN THE NATION'S DRINKING WATER: ASSESSING POTENTIAL RISKS AND ACTIONS TO ADDRESS THE ISSUE

TUESDAY, APRIL 15, 2008

U.S. SENATE,
COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS,
SUBCOMMITTEE ON TRANSPORTATION SAFETY,
INFRASTRUCTURE SECURITY, AND WATER QUALITY,
Washington, DC.

The subcommittee met, pursuant to notice, at 3 p.m. in room 406, Dirksen Senate Office Building, Hon. Frank R. Lautenberg (chairman of the subcommittee) presiding.

Present: Senators Lautenberg, Boxer, Cardin, Klobuchar, and Vitter.

OPENING STATEMENT OF HON. FRANK R. LAUTENBERG, U.S. SENATOR FROM THE STATE OF NEW JERSEY

Senator LAUTENBERG. This is a hearing on Pharmaceuticals in the Nation's Water: Assessing Potential Risks and Actions, and we are pleased to bring the Committee to order.

First, I want to say thanks to my colleague, the Chairperson of the Committee for her diligence in making sure that we are in constant activity reviewing our responsibilities and assessing what we can do to improve the environmental conditions as they exist in our Country.

I want to welcome everyone to today's critical hearing as we consider the impact of contaminants in our Nation's water supply and the health hazards that they pose to our residents that is asserted, and we want to clear that up.

Every day, all Americans across the Country rely on exactly the same thing, clean and safe water. When it comes to our drinking water, we have a reasonable expectation and that is that the water coming into our homes for ourselves and our families is clean and safe. That is why so many people were concerned about a news article that ran in March. The Associated Press ran a story that told America what many scientists already knew, that there were small amounts of drugs in the water that 41 million Americans drink every day.

The study was conducted on water systems across the Country, including in my home State of New Jersey. While this story captured the public's attention, there is more to it. The untold story is the absence of regulation by the Environmental Protection Agen-

cy and the many hundreds of unregulated chemicals that are permitted to flow into America's water supply.

There are more than 140 chemicals in our drinking water that flows in there without regulation or scrutiny, according to a study by the Environmental Working Group. These includes chemicals that are used in rocket fuel, gasoline additives, and pesticides. These are chemicals that have proven negative effects on people's health. Even the EPA says that some of these chemicals can cause cancer.

For some of these chemicals there is no health information on record in government materials. And yet all of these chemicals are unregulated. We drink them and they end up in our environment. We are already seeing the impacts of some of these contaminants in nature. For instance, it has been noted that we see in male fish carrying female eggs. What a contradictory thing that is in our ecological structure, our species structure.

That is why I am concerned, and I know the public is concerned about the long-term impact of contaminated water. I know many people drink bottled water, but bottled water is not the solution. In fact, it may come as a surprise, but 40 percent of bottled water comes simply from the tap, 40 percent of the water. The cost is not to be ignored. It costs about a penny a gallon to get water from the tap. It costs $10 a gallon when it comes in a bottle.

So it is essential that we work on this by increasing funding for protection from our crumbling water infrastructure, including our wastewater and drinking water facilities. Those facilities are responsible for cleaning up our water and they need the resources to do it. The EPA estimates that there is a $271 billion gap between what our wastewater treatment plants need and what they receive. We have to start closing that gap.

And we need to continue to fund research on this issue. I was disappointed to see that the President's budget cut the funding that we rely on to monitor our rivers and streams for contamination. The story that ran in the Associated Press focused on pharmaceuticals in the water, but I hope it is going to help us focus on the real problem, an EPA that has allows far too many unregulated contaminants in our water.

I look forward to hearing from today's witnesses about how we can better protect America's water and the hundreds of millions of people who drink it every day.

Now I will call on my Ranking Member, Senator Vitter, for his opening remarks.

OPENING STATEMENT OF HON. DAVID VITTER, U.S. SENATOR FROM THE STATE OF LOUISIANA

Senator VITTER. Thank you very much, Mr. Chairman. I will be very brief.

I just want to thank all of our witnesses for coming and being part of this very important discussion. I know several of them have been focused on this issue both studying it, monitoring it, grading levels, and also trying to take proactive steps in mitigating that issue. I will be very eager to hear from them.

And thank you for calling this important hearing.

[The prepared statement of Senator Vitter follows:]

STATEMENT OF HON. DAVID VITTER, U.S. SENATOR
FROM THE STATE OF LOUISANA

Today the Senate Environment and Public Works Subcommittee on Transportation Safety, Infrastructure Security, and Water Quality will focus on pharmaceuticals in our nation's waters. I thank the witnesses for being here.

As we will hear from several of the witnesses today, the issue of pharmaceuticals in our nation's waters is not a new one. I understand that the EPA first reported pharmaceuticals in US waters in 1975.

With technological advances in the last few decades, there have been improvements in analytical methods that have led to the ability to detect trace concentrations in our waters to the parts per trillion (ppt). The ability to measure a tiny concentration of these compounds in the water does not necessarily mean that each of them will be harmful to people. It is likely that most of them would not have adverse health affects on the general population. Some studies have shown that pharmaceuticals measured in the water supply are at very low concentrations that are millions of times lower than a medical dose and at levels that do not pose an acute threat to public health.

However, I do share concern about certain pharmaceuticals in drinking water supplies—endocrine disruptors, for example. There is research that suggests these impact aquatic species, and we should look very closely at any potential long term impacts on people.

Unfortunately, there appears to be very little information about the chronic health effects of trace concentrations of pharmaceuticals in our waters on people, especially for those who are exposed to them for long periods of time. It is important that we better understand what the actual effects are on the public health in the long term.

We need more health effects research on this topic before making sweeping regulatory changes. I look forward to hearing from the witnesses who have experience with the research that has been conducted to date.

Senator LAUTENBERG. Thank you, Senator Vitter.

Senator Boxer.

OPENING STATEMENT OF HON. BARBARA BOXER, U.S. SENATOR FROM THE STATE OF CALIFORNIA

Senator BOXER. Thank you, Senator.

I just wanted to thank Senator Lautenberg for his extraordinary leadership on this and so many environmental issues. I am very proud to have the Subcommittee take the lead on this important matter, and I hope, Senator, you and Senator Vitter will get to the bottom of this. I will be here as long as I can. I have the technical corrections bill on the floor, so I will be going back and forth.

But we are here today to conduct much-needed oversight on the presence of pharmaceuticals in our Nation's water supplies. Clean, safe drinking water is essential to all of us. It is especially important to our children.

The National Academy of Sciences has found that children drink more water and eat more food in proportion to their body weight compared to adults. In addition, children's rapidly developing bodies, including their hormone systems that control their development, are very vulnerable. Pregnant women also undergo a host of similarly delicate changes.

There are particular windows of vulnerability during our development when pregnant women and children may be especially susceptible to very low doses of some toxins. Some pharmaceuticals now being found in our water may affect our hormone systems. Many pharmaceuticals are designed to affect our bodies at very low levels.

This all means that contaminants in water may have a more concentrated impact on pregnant women and children. It is because of the work by the United States Geological Survey, the Associated

Press and others, and I thank them for it, that we now know that some of our drinking water contains a mixture of pharmaceuticals.

Notice I did not thank the EPA. Fish and wildlife that live in our waters are the familiar canaries in the coal mine. Scientific evidence is growing that small levels of contaminants, including pharmaceuticals, can damage reproduction and development in fish and wildlife. Our Chairman made that point about what is happening.

Science is telling us to be careful. What are Federal agencies doing to prevent potentially dangerous exposures to pharmaceuticals in our drinking water? There is an answer, very little. EPA in particular has failed to adequately address this problem. EPA has failed to require the needed testing to determine the effects of these chemicals at low levels.

In 1996, Congress told the EPA in the Safe Drinking Water Act and the Food Quality Protection Act to develop a program to identify and address chemicals that harm the natural balance of hormones in our body. Those are called endocrine-disrupting chemicals. Yet EPA is now nearly 6 years behind the scheduled established in a court settlement to list the endocrine-disrupting chemicals it will test. And EPA still has not even established all of the tests needed to detect these chemicals, much less evaluate the chemicals using those tests.

EPA now says it is not prepared to require drinking water systems to monitor for pharmaceuticals or to set standards for pharmaceuticals in tap water because it doesn't have the data showing harmful effects from low levels of exposure. So it is a real circular reasoning, isn't it? You don't do what Congress tells you. You don't have the information, and when the pharmaceuticals show up and the AP does a front page story, you say, gee whiz, we would love to say something, but we don't have enough information.

This lack of data is in large part a result of EPA's failure to ensure that companies that make these chemicals complete the testing needed to evaluate these effects. Because of these problems, EPA has not set a drinking water standard for a single pharmaceutical. In fact, EPA hasn't even proposed to set a single health standard for any pharmaceutical in drinking water.

The result of these failures is that millions of Americans turn on their kitchen taps and drink low levels of pharmaceuticals in their water every single day. The agency also should be doing much more to prevent these pharmaceuticals from getting into our water in the first place. For example, EPA should better address the disposal of pharmaceuticals and the releases of these chemicals from factories, farms, sewage treatment plants, and sewage sludge.

Now, a White House working group that was supposed to address the pharmaceuticals in the environment and the related antibiotic resistance issues has missed its deadline to issue recommendations. The White House has insisted on keeping many records of this working group secret, which is unacceptable. All of the documents should be released to the public now.

The Associated Press story published in March documenting widespread drinking water contamination with pharmaceuticals highlights the importance and impact of the public right to know. I am sitting next to the man who brought community right to know to environmental legislation. And Mr. Chairman, this is a classic

case in point. When people are told about contaminants in the water, they will start asking questions and maybe we will get something done.

So in closing, I want to lay out five steps that I urge the Bush administration to take starting today. One, immediately release all of the secret records of the White House Working Group on Pharmaceuticals in the Environment; two, immediately start an accelerated testing process for pharmaceuticals and other toxic chemicals for the endocrine-disrupting effects, and you are late on that by years; three, immediately move forward with the process of establishing rules and programs to ensure the safe disposal of waste pharmaceuticals; and four, immediately ask water companies to voluntarily test their water for pharmaceuticals and disclose the results.

Americans a right to expect that their government is ensuring that they can turn on their taps and have water that is safe, safe for their kids, safe for perhaps a sick grandma. And they have a right to know what is in their drinking water. We must protect those who are the most vulnerable, our children, pregnant women, and infants from this problem. It is our moral duty.

Again, thank you very much, Senator Lautenberg, for your leadership.

Senator LAUTENBERG. Thank you, Senator Boxer.

And now our panel of witnesses are prepared to testify: Mr. Benjamin Grumbles, the Assistant Administrator for the Environmental Protection Agency's Office of Water; Dr. Robert Hirsch, Associated Director for Water at the U.S. Geological Survey. I thank you both for joining us here.

And now, Mr. Grumbles, if you would. Limit your testimony please to within 5 minutes, if we can do that.

STATEMENT OF BENJAMIN GRUMBLES, ASSISTANT ADMINISTRATOR FOR WATER, U.S. ENVIRONMENTAL PROTECTION AGENCY

Mr. GRUMBLES. Thank you, Mr. Chairman. It is always an honor to appear before the Committee. I am Benjamin Grumbles, Assistant Administrator for Water at the U.S. EPA.

We are very concerned about this information. We are not alarmed in the sense of a risk to human health, but it does raise a big red flag and we are concerned and we are taking additional steps. We are taking this very seriously.

America's water supplies continue to be among the safest in the world, and we are committed to working with all our partners, including Congress, to ensure that it stays that way. Emerging contaminants are exactly that. They use the phrase emerging because there isn't enough information yet to make a clear determination that there is a clear and present danger from them, but they cause concern. So that is why over the last several years, we and our Federal partners have been making extra efforts. We recognize that we need to continue to do that.

Mr. Chairman, EPA has a four-pronged action plan to respond to the growing concerns about pharmaceuticals in water. The first is to continue to strengthen the science. We are fully committed to doing that. We are working with other Federal agencies. We are

asking the right questions. We are dramatically expanding the scope of the surveys and the studies that the U.S. EPA and other Federal agencies are doing to see what we know and to close the gaps between what we know and what we should know before we can take additional steps.

We are conducting studies of fish tissue, and of sewage treatment plants, influent and the effluent to measure for the occurrence of pharmaceuticals. What we have found to date and also what the AP story has found, is that at truly tiny amounts, there are trace levels of pharmaceuticals in water, truly tiny trace amounts.

The second part of our four-pronged action plan is to increase public understanding and improve risk communication. It is very important for the public to know they shouldn't be rushing away from tap water to purchase bottled water based on alarmist headlines. When you translate it into the parts-per-trillion, it is the equivalent of having an aspirin-size pill in not one, but 100 Olympic-size swimming pools. It is important to provide the context.

It is also important to be diligent and to take this matter very seriously, but improved risk communication is a key component of that, and that is why we have a website. That is why we are coordinating with others in the public sector and other policymakers to get this issue on the radar screen and to communicate clearly and effectively so the public can understand the extent of the risk.

The third prong of our action plan is building partnerships for stewardship, working with other agencies, but also working with the public sector in a variety of ways. Specifically, EPA has several take-back pilot programs that we have been carrying out across this entire Country, not just over the last few weeks or months, but over the last several years—voluntary take-back programs of non-controlled substances at pharmacies, working with institutions, universities, and working with communities. In the Great Lakes this month, there is a week where there will be voluntary take-backs of unused pharmaceuticals at various communities throughout the Great Lakes. It is important to increase product stewardship and pollution prevention through partnership programs, and that is a key component.

The fourth component of our action plan is the regulatory tools, using the regulatory tools that we have when we get the appropriate amount of information, and when it is appropriate to take regulatory steps. We are taking that very seriously. We have developed three methodologies under the Clean Water Act last year, three methods for detection and measurement. We are carrying out a very important Public Health Service study right now under the Clean Water Act, the Effluent Guidelines Program, in hospitals and long-term care facilities, to determine what disposal practices they are using and to improve those disposal practices. We are committed to completing that study and to increasing the stewardship at hospitals.

The other regulatory tool is under the Safe Drinking Water Act, and that is the contaminant candidate listing process. Mr. Chairman, we have a draft list of contaminants candidates for listing. We are taking additional comments. I have written to various organizations, every State in this Country, and every water utility, ask-

ing them for their thoughts about adding pharmaceuticals to that list for potential regulation under the Safe Drinking Water Act.

Mr. Chairman, in conclusion, we are taking several steps. I personally am going to be meeting with representatives of States, cities, utilities, the pharmaceutical industry and environmental groups in the coming weeks. We are looking at expanding our various studies. We are committed to taking action and to working with you to continue to ensure America's drinking water supplies are among the safest in the world.

I would be happy to answer questions from you and your colleagues.

[The prepared statement of Mr. Grumbles follows:]

TESTIMONY OF
BENJAMIN H. GRUMBLES
ASSISTANT ADMINISTRATOR FOR WATER
ENVIRONMENTAL PROTECTION AGENCY
BEFORE THE
TRANSPORTATION SAFETY, INFRASTRUCTURE SECURITY AND WATER QUALITY
SUBCOMMITTEE OF THE
ENVIRONMENT AND PUBLIC WORKS COMMITTEE
UNITED STATES SENATE
April 15, 2008

Good afternoon Chairman Lautenberg and members of the Committee. I am Benjamin H. Grumbles, Assistant Administrator for Water at the United States Environmental Protection Agency (EPA). I appreciate the opportunity to describe EPA's actions to evaluate the potential risks to human health and aquatic life posed by trace amounts of pharmaceuticals in water, and to identify measures to minimize their occurrence in water. The Agency is committed to undertaking a scientific approach in evaluating the risks associated with contaminants in our environment so that we can take the necessary action to ensure clean and safe water.

EPA is concerned about the detection of a number of pharmaceuticals and personal care products in our water. EPA has been actively working with federal agencies and state and local government partners to better understand the implications of emerging contaminants such as pharmaceuticals, endocrine disrupting chemicals, and personal care products detected in drinking water, wastewater, surface water and ground water. We continue to evaluate their routes of exposure, levels of exposure, and potential effects on public health and aquatic life.

Over the last few years, EPA has increased its work in a number of areas to better understand pharmaceuticals and personal care products. We are focused on learning more about the occurrence of pharmaceuticals and personal care products in water. In addition, we are working to better understand what treatment technologies may remove them from wastewater and drinking water. We are developing analytical methods to improve detection capabilities. We are conducting national studies and surveys to help direct our course of action. We are also partnering with government agencies, stakeholders, and the private sector, and increasing public awareness about product stewardship and pollution prevention.

U.S. Drinking Water is Among the Safest in the World

The U.S. has one of the safest drinking water supplies in the world. EPA is committed to keeping our water clean and healthy for the future.

While there is much information about the health effects of pharmaceutical products at the therapeutic doses provided in medication, there is still uncertainty about their potential effects on public health and aquatic life at the extremely low levels observed in drinking water and surface water. Let me give you an example.

Although pharmaceuticals have been detected in a number of U.S. waters (as well as waters worldwide), the amounts at which they are detected -- in the parts per billion and parts per trillion range --are extremely low. For example, in looking at a number of studies, the maximum reported drinking water level for caffeine was 0.12 parts per billion. At that concentration, a person

would have to drink almost 222,000 gallons of water before coming close to ingesting the amount of caffeine that one would get from a six ounce cup of coffee.

We know that collaborating with our partners will be critical so that we can all make the best use of our existing resources and maintain consumer confidence in our drinking water. Keeping public drinking water safe and ensuring that consumers have high confidence in our nation's drinking water is an absolute tenet of EPA's approach to environmental and public health protection. It is also testimony to the daily efforts of thousands of people from the federal officials who develop standards, to the state managers that carry out programs, and the local officials who provide drinking water and treat wastewater.

EPA's Four-Pronged Approach to Emerging Contaminants

EPA is responding to emerging contaminants, with a four-pronged approach aimed at improving science, communicating risks, identifying partnership and stewardship opportunities, and preparing to take regulatory action when appropriate.

Strengthening the Science

Sound science and reliable information must be the foundation for any Agency decision. There is critical work that needs to be done in the area of research and assessment before we can make any decisions as to whether regulatory actions are needed. EPA and other federal agencies are working on projects to evaluate exposure and potential health effects on humans and aquatic life. This is a key, because while we know that pharmaceuticals have health effects at the

therapeutic dose, we are less certain about the health effects associated with long-term exposure to much lower concentrations. Effects may be more likely in aquatic life because they are continually exposed.

Several EPA offices, including my Office and the Office of Research and Development, are working together to better understand potential issues related to exposure pathways and health effects of emerging contaminants including pharmaceuticals. I will describe some of the efforts we are undertaking in my office and have provided a brief summary of efforts in the Office of Research and Development as an attachment to this testimony.

EPA's Office of Research and Development is engaged in a broad portfolio of research efforts, much of which is focused on answering key questions associated with exposure pathways and health effects. EPA is also coordinating research efforts with other federal agencies as part of the Pharmaceuticals in the Environment (PiE) workgroup, under the auspices of the White House's National Science and Technology Council Committee on Environment and Natural Resources Toxics and Risk Subcommittee. The workgroup is co-chaired by EPA, the U.S. Geological Survey, and the Food and Drug Administration.

Other key research areas are associated with assessing the sources of pharmaceuticals. Important to this effort is having analytical methods available to reliably detect these contaminants in water. It is critical to ensure sound collection and analysis of samples because there is a great potential for cross-contamination and thus false identification and incorrect concentrations at the low levels detected in most samples. It is also important to note that the equipment needed to test for pharmaceuticals is highly technical and that analyses are very expensive relative to other contaminants. In December 2007, the Agency released newly developed, cutting edge methods

for the analysis of approximately 100 pharmaceuticals, personal care products, steroids, and hormones in raw sewage, treated wastewater, and biosolids which are some of the most complex samples to test. The new methods, which are continuing to be refined, use high performance liquid chromatography combined with tandem mass spectrometry. The availability of the methods responds to requests for guidance in this area and the need to support studies being conducted by the Agency. We will continue to consider and evaluate additional methods.

EPA, other federal agencies, and academic and private sector researchers are studying the occurrence of pharmaceuticals in wastewater, surface water, ground water and drinking water. We are also evaluating the occurrence of contaminants in fish and other aquatic life. In particular, EPA's Office of Water is conducting:

- a study at nine wastewater treatment facilities to better understand what is going into the plant for treatment and what is coming out in the discharge and in biosolids (sludge). We expect to complete this study by December 2009.
- a Fish Tissue Pilot Study to investigate whether pharmaceuticals and other personal care products may occur in fish from five effluent-dominated streams across the US. Results are undergoing quality assurance review and are expected later this year.
- a survey of biosolids from 74 randomly selected wastewater treatment plants to determine whether contaminants occur in biosolids, and if so at what concentration.

My office has also funded external research projects to assess occurrence. Researchers at the University of Florida are studying the fate and transport of emerging contaminants like triclocarban (an antiseptic widely used in soaps and other products) in biosolids. Researchers at Duke University are studying the presence, fate and treatability of steroid and hormone contaminants in wastewater and biosolids. They are conducting a year-long field study in four municipal wastewater treatment plants and are evaluating the overall potency of the wastewater through laboratory tests.

Additionally, EPA's Office of Research and Development's research agenda includes work to better understand pharmaceuticals in water through several important programs. A brief summary of those activities is attached to this testimony.

We will continue to work within EPA and with our federal, state, and local partners to identify and analyze available information and potential studies to assess the occurrence of pharmaceuticals in our drinking and surface water, along with any potential risks to human health and aquatic organisms.

Another important research area is treatment and removal of pharmaceuticals from wastewater and drinking water. While EPA is active in this area, research foundations representing drinking water and wastewater utilities are key players in determining the removal efficiency of different types of treatment. Research is finding that more sophisticated higher-level treatment strategies are sometimes better at removing certain types of pharmaceuticals. However, it is important to ensure that utilities continue focusing their efforts at removing those contaminants

with known risks (such as pathogens), especially when monitoring and/or treatment efforts aimed at pharmaceuticals at low levels could carry significant cost with unknown risk reduction.

Improving Public Understanding and Risk Communication

One of the most difficult tasks faced by public health and environmental officials is how to communicate risks in the face of uncertainty. At this time we simply do not know whether there is a human health risk of concern from the levels that have been reported in water. Some have argued that it does not make sense to monitor for pharmaceuticals in water if there is limited information about the health effects at the concentrations that could be detected. We disagree. Information about occurrence and health effects is complementary and should be developed in tandem. It would be unfortunate indeed if proactive utilities referenced in media articles made decisions to reduce their research in response to media attention. Furthermore, useful information should be shared with the public in a timely way as it is generated. It is important to communicate with the public so that they can help shape effective public policy in this area and make informed choices. We will continue to work with all of our stakeholders to do the best job possible in communicating available data on both occurrence and health effects for all emerging contaminants, including pharmaceuticals, and any associated uncertainty with that data.

Building Partnerships for Stewardship

One thing we know for certain is that this issue cannot be addressed by EPA alone. Other federal, state, and local agencies and industry will also need to play a role in assessing the occurrence and effects of pharmaceuticals, analyzing their risk, and in determining actions to reduce their concentration in the environment. EPA is already coordinating research efforts with seven other federal agencies as part of the PiE workgroup which is examining current efforts with regard to human and veterinary pharmaceuticals in the environment, , with the goal of avoiding duplication of effort, leveraging existing resources, and better prioritizing Federal efforts.

We also worked with the White House Office of National Drug Control Policy to develop joint guidelines that recommend appropriate disposal methods for unused medication. EPA, other agencies, and the pharmaceutical industry are involved in a number of stewardship activities across the country to communicate the guidelines to the public. While most pharmaceuticals are entering water through natural biological functions, it is also important that the public understand that the toilet is not a trash can for unused medications.

EPA has also been working to develop and promote good stewardship efforts such as take-back programs that would allow consumers to properly dispose unwanted or unused pharmaceuticals. EPA recognizes that any such programs must be consistent with the Controlled Substances Act and regulations with respect to managing medications that are also classified as controlled substances. Toward this end, the Agency will be working with the Drug Enforcement Administration (DEA) to ensure that pilot take-back programs supported by EPA are conducted in a manner that is safe and in compliance with federal and state laws and regulations.

One such program that EPA recently funded involves the Area Resources for Community and Human Services in St. Louis. Last year, EPA's Office of Children's Health Protection and Environmental Education provided grants to this community partnership, which is working on an efficient regional model to responsibly dispose of unwanted non-controlled medications through a regional grocery store chain as the collection point. We are working on the evaluation and development of this pilot return take back programs to allow consumers to return and dispose of medications in a safe manner that complies with federal and state law and are working closely with the Drug Enforcement Administration to ensure applicability to all laws.

Additionally, EPA provided a grant to Villanova University to identify ways to better manage how prescription and non-prescription pharmaceuticals are discarded from university dormitories. The results of Villanova's work can be useful for other universities that are voluntarily taking steps to reduce pollution on their campuses as their commitment to improving the environment.

Using Regulatory Tools

We recognize stewardship activities alone are not always sufficient to manage issues associated with emerging contaminants in water. We are also gathering information that will help us assess whether direct regulatory action is warranted. For example, under the Clean Water Act, EPA establishes technology-based national regulations, termed "effluent guidelines," to reduce pollutant discharges from categories of industrial facilities to waters of the United States. As part of the effluent guidelines planning process, the Agency is reviewing the pharmaceutical disposal

practices of hospitals and long-term health care facilities. We expect to issue a report on our findings in 2009. We view this as an important opportunity to increase product stewardship and proper waste disposal.

Under the Safe Drinking Water Act, the Agency carries out a program to assess contaminants for potential drinking water regulation. On February 21, 2008, the Agency released the draft Contaminant Candidate List (CCL 3) for public review and comment. As part of the process to develop the list, the Agency evaluated pharmaceuticals and personal care products to identify those that have the potential to occur in drinking water provided by public water systems. EPA considered 287 chemicals identified as pharmaceuticals and personal care products; however, only one was included on the Draft CCL 3 because most occurred at levels far below those currently associated with any adverse health effects, based on the best available human health effects data. The Agency is seeking additional data and information on the concentrations of pharmaceuticals in treated or ambient water and adverse health effects that may be posed by their presence. We are also interested in receiving feedback on how we considered pharmaceuticals within the CCL 3 process.

The CCL 3 process evaluated many types of contaminants that can occur in drinking water – including microbial pathogens, pesticides, and chemicals used in industrial practices and consumer products. The 104 contaminants on the Draft CCL 3 include 93 chemicals and 11 microbiological contaminants. In the absence of reliable data indicating potential risks associated with pharmaceuticals in water at the very low levels at which they have been detected, it would be inappropriate to require monitoring and/or treatment that could carry significant cost, with no evidence of significant risk reduction based on currently available data. The Agency needs to

instead focus its regulatory resources on contaminants with known significant risks in order to maximize public health protection.

Looking to the Future

EPA will continue to work on the important issue of pharmaceuticals in water. We regard this as an issue that warrants additional, on-going scrutiny.

I have sent letters to the directors of state environmental and health agencies to inform them about some of our efforts and to request their assistance. In conjunction with the CCL 3 comment period, I have asked them whether they are currently implementing, or planning to implement, a program to monitor for pharmaceuticals and personal care products in wastewater, surface water, ground water, or tap water. This type of information can be very useful to EPA as we carry out our contaminant candidate listing process to identify potential contaminants for unregulated contaminant monitoring and/or drinking water regulation, revise effluent guidelines, and determine which contaminants are the highest priorities for development of new or revised water quality criteria.

As part of EPA's broader strategy to strengthen and expand technical partnerships and information sharing at all levels, I have also asked the states to share information with us with respect to stewardship activities they may be undertaking to manage the presence of pharmaceuticals and personal care products in water within their state. We will compile the information we receive with the goal of sharing best practices and encouraging broad adoption of effective programs across the country.

Some of the additional activities we are considering include:

- expanding our current fish tissue pilot study to ensure a statistically and nationally representative picture of the presence of pharmaceuticals and personal care products in aquatic life;
- improving our public access web site to provide information on the work that we are doing and to communicate our understanding of risks to human and aquatic health;
- working within EPA and across federal agencies to better understand stewardship efforts they are undertaking to both improve coordination and share information; and,
- working to obtain toxicology data from available sources (including other federal agencies) and exploring ways to improve understanding of health effects posed by exposure to low levels of pharmaceuticals in water.

I will also be meeting with a number of key stakeholder groups over the next several weeks – including those representing state programs, the water and wastewater industry, research associations, and the environmental community. It is essential for us to share information and understand specific concerns so that we can work together as effectively as possible in communicating with the public.

Finally, while I have primarily discussed Office of Water activities here today, other offices within EPA and across the EPA Regions have also been carrying out efforts associated with

pharmaceuticals and other emerging contaminants. They are reviewing their efforts to date and assessing what additional activities they may undertake in the future.

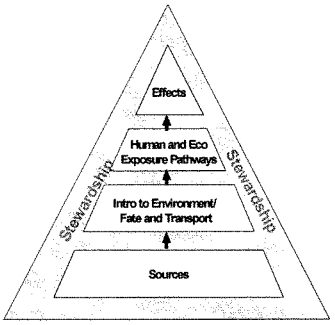
Conclusion

The U.S. continues to have one of the safest drinking water supplies in the world due to our collective long-standing commitment to keep our water clean and healthy. We know that good science and information must continue to drive our decisions. We will continue to evaluate health effects, occurrence, and risk reduction strategies so that we can make sound decisions to protect public health and aquatic life. We recognize the public must be a full partner in our efforts to collect and evaluate data so they can help shape effective public policy in this area and make informed choices. By engaging the full range of public and private partners and by using appropriate regulatory tools, we will continue to ensure the safety of the nation's water.

Thank you Chairman Lautenberg and members of the Committee for this opportunity to describe EPA's important work on pharmaceuticals and other emerging contaminants. I would be happy to answer any questions you may have.

Attachment
EPA Office of Research and Development:
Research Efforts Related to Pharmaceuticals in the Environment

The research related to pharmaceuticals in the environment that is conducted by U.S. EPA's Office of Research and Development (ORD) is organized around the Research Framework under development by the Office of Science and Technology Policy Interagency Working Group on Pharmaceuticals in the Environment (see Figure). EPA has multiple research programs (e.g., Endocrine Disruptors, Drinking Water, Water Quality, Human Health), under which a number of efforts are related to or can be applied to pharmaceuticals in the environment and are helping inform the scientific issues.



In 2008, ORD is projected to commit $2.9 million on research related to pharmaceuticals. Much of the work is carried out by EPA scientists at several of the ORD national laboratories. ORD is also supporting new science on pharmaceuticals and other endocrine disruptors by engaging the broader research community through extramural grant programs. The Science to Achieve Results (STAR) grants program funds targeted research grants and graduate fellowships in numerous environmental science and engineering disciplines through a competitive solicitation process and independent peer review. The Small Business Innovation Research (SBIR) program has funded projects focused on identifying new approaches for detecting contaminants and assessing their endocrine disrupting ability.

The EPA research either completed or currently underway is intended to inform the Agency about the following elements of the framework:

Sources There are many potential sources for pharmaceuticals to enter into the environment. Some potential sources include wastewater treatment plant discharges, concentrated animal feeding operations (CAFOs), land applications (e.g., biosolids) and direct disposal/introduction to environment. Example:

- EPA researchers are collaborating with scientists funded through the Agency's STAR grant program, and from the US Geological Survey to characterize: 1) the extent to which CAFOs are sources of hormones (natural and pharmaceutical) in the environment, 2) their fate and transport and 3) the impact they have on aquatic organisms; and to develop approaches to mitigate exposures, where warranted.

Fate and transport When a pharmaceutical is introduced into the environment, it may undergo chemical reactions to form a new substance (fate) and be physically transported to another location in the environment (transport) with the potential for distribution/deposition into other media. Important considerations include the temporal pattern of introduction (e.g., periodic, persistent) and the potential for bioaccumulation in environmental media (e.g., sediments) or aquatic organisms. Example:

- Through its STAR program, EPA provided awards to six academic institutions to study the occurrence, fate and transport of a variety of pharmaceuticals and personal care products.

Exposure Pathways: Human and Ecological When an individual human comes in contact with a foreign chemical or substance, he or she becomes exposed, whereby the chemical may be transferred from the environment onto/into the individual. The major route of human exposure considered in the draft Framework is ingestion (drinking water or fish), although others may also be considered (e.g., dermal). For aquatic organisms, the main routes of exposure to be considered are dermal and ingestion. Example:

- EPA researchers participated in a study with a team of Canadian scientists who treated an experimental lake with ethynylestradiol (a pharmaceutical commonly found in birth control pills) to produce a chronic exposure to aquatic organisms at a level consistent with that observed in some wastewater treatment effluents. EPA used this opportunity to further develop a molecular assay as an indicator of estrogen exposure and to investigate the effects of long-term exposure to an endocrine-disrupting pharmaceutical in a whole lake ecosystem.

Ecological Assessment The process of determining whether a given chemical (e.g., a pharmaceutical) has an impact on biota or the ecosystem is known as ecotoxicology. This process, when completed for the broader ecosystem scale is an ecological assessment. Example:

- EPA has been conducting research concerning the ecological effects of a subset of human and veterinary reproductive and developmentally toxic pharmaceuticals using multiple small fish and amphibian species. Specific pharmaceuticals tested include the estrogens ethynylestradiol and estradiol (used in humans and, in the latter case, animals as well), the androgenic steroid trenbolone (used for livestock), the anti-androgenic compound flutamide (used for treating prostate cancer) and several drugs that affect natural steroid production such as fadrozole (developed to treat breast cancer), trilostane and ketoconazole. In addition, EPA has been involved in assessing the effects of endocrine-active pharmaceuticals in the field investigating, for example, the occurrence and potential population-level effects (on fish) of trenbolone emanating from CAFOs and estrogenic compounds associated with wastewater treatment effluents.

Human Health Assessment Human health assessment refers to the process of identifying potential health effects associated with a given exposure and then determining the dose-response relationship to characterize the associated risk. Example:

- EPA has ongoing research across multiple programs that are developing the methods, models, and measures that could be applied to the pharmaceuticals issue to assess potential impacts on human health. Some of these activities include: developing multiple short term and longer term assays (*in vitro* and *in vivo* in laboratory animal models) to identify potential endocrine disruptors, studying the effects of long term exposures to low levels of chemicals, and developing methods for cumulative assessments for chemicals that act through similar and different modes of action.

Managing Contaminants If human exposure to contaminants is found to pose a risk, it will be important to identify management approaches for minimizing their occurrence in water. Example:

- In order to establish a baseline understanding, EPA research is determining the effect of different drinking water treatment technologies currently in use on compound removal by examining the occurrence and fate of pharmaceuticals and other wastewater derived compounds through drinking water treatment.

A more complete list of ORD research projects, which includes a description of each project, is available on EPA's web site at www.epa.gov/ppcp/projects.

Question from Senator Benjamin L. Cardin:

1. Given that the EPA has recognized that pharmaceuticals in water are a potential problem, why was the President's request for EPA's FY2009 R&D funding, when adjusted to account for inflation, proposed at the lowest level in more than two decades in real terms?

How does this budget request reflect the priorities of the EPA in keeping air and water clean?

Recognizing the increasing fiscal constraints on Federal spending, EPA's goal is to ensure that it is funding its highest priority research needs. This involves continual review of research progress and programmatic needs. The FY 2009 budget request provides funding for EPA's highest priorities in protecting air and water quality. For example, EPA is actively developing computational toxicology tools to greatly enhance our capability to predict which chemicals are in greatest need of toxicology testing and which endpoints would be the most important to examine. The FY 2009 R&D budget request includes an additional investment in nanotechnology research to improve understanding of the implications of manufactured nanomaterials released into the environment. The FY 2008 budget requested additional funding in Clean Air research to investigate air emissions near roads. The FY 2009 budget request continues that effort.

Questions from Senator Barbara Boxer

The White House has convened an interagency working group titled, "Pharmaceuticals in the Environment." It is my understanding that this group was supposed to make recommendations for addressing this issue last December.

1.A. Please provide a status update of this working group, including a description of all actions that the group is currently undertaking, a timeline for completing each such action, and the individuals – including federal and non-federal employees – involved in each action.

B. In the April 15, 2008 hearing, I requested that EPA provide the Committee with copies of all EPA records related to the Agency's involvement in the "Pharmaceuticals in the Environment" workgroup.

At the hearing, you committed to provide the Committee EPA's records reflecting the agency's role and its involvement in that task force. You said that when you got back from the hearing, you would talk with those who were involved, the Office of Research and Development, and others in the agency that are involved, to provide whatever you could that reflects EPA's involvement. However, the Agency has so far failed to deliver any records.

Provide all EPA records, including all emails, memos, phone logs, calendar entries, letters, notes, and other records, related to the "Pharmaceuticals in the Environment" workgroup by May 12, 2008.

Increased collaborations - The PiE Work Group has provided a catalyst for several Federal Agencies to engage in planning/conducting joint research projects. The PiE effort has also spurred more in-depth discussions among Federal Agency scientists related to the exchange of information on methods and technologies to conduct environmental measurements of pharmaceuticals and other chemicals of emerging environmental concern. The discussions include sharing knowledge and experience on new techniques, evaluating appropriate quality assurance and quality control procedures, and comparison of the performance of different labs and methods to make measurements. These discussions have resulted in joint participation of multiple Federal Agencies in interlaboratory comparisons of analytical performance for pharmaceuticals and hormones, including one with the Global Water Research Coalition.

2.A. Please provide a complete description of all of EPA's investigations and studies, including such ongoing and planned activities, into the human health and environmental effects of pharmaceuticals and emerging contaminants.

B. For each such action, describe the current status of each such study, the expected date of each study's completion, the individuals conducting the study, and whether the study will evaluate the potential adverse effects from low-levels of exposure on vulnerable populations such as pregnant women and children?

The research related to pharmaceuticals in the environment that is conducted by U.S. EPA's Office of Research and Development (ORD) is organized around a research framework under development by the Interagency Working Group on Pharmaceuticals in the Environment. EPA has multiple research programs (e.g., Endocrine Disruptors, Drinking Water, Water Quality, Human Health), under which a number of efforts are related to or can be applied to pharmaceuticals in the environment arid are helping inform the scientific issues.
In 2008, ORD is committing $2.9 million on research related to pharmaceuticals. Much of the work is carried out by EPA scientists at several of the ORD national laboratories. ORD is also supporting new science on pharmaceuticals and endocrine disruptors by engaging the broader research community through extramural grant programs. The

Science to Achieve Results (STAR) grants program funds, targeted research grants, and graduate fellowships in numerous environmental science and engineering disciplines through a competitive solicitation process and independent peer review. The Small Business Innovation Research (SBIR) program has funded projects focused on identifying new approaches for detecting contaminants and assessing their endocrine disrupting ability.

The EPA research either completed or currently underway is intended to inform the Agency about the following elements of the framework:

Sources: There are many potential sources for pharmaceuticals to enter into the environment. Some most common sources include human excretion, wastewater treatment plant discharges, concentrated animal feeding operations (CAFOs), land applications (e.g., biosolids) and direct disposal/introduction to the environment. Example:

- EPA researchers are collaborating with scientists funded through the Agency's STAR grant program and from the US Geological Survey to characterize: 1) the extent to which CAFOs are sources of hormones (natural and pharmaceutical) in the environment, 2) their fate and transport, and 3) the impact they have on aquatic organisms. This work includes the development of approaches to mitigate exposures, where warranted.

Fate and transport: When a pharmaceutical is introduced into the environment, it may undergo chemical reactions to form a new substance (fate) and be physically transported to another location in the environment (transport) with the potential for distribution/deposition into other media. Important considerations include the temporal pattern of introduction (e.g., periodic, persistent) and the potential for bioaccumulation in environmental media (e.g., sediments) or aquatic organisms. Example:

- Through its STAR program, EPA provided awards to six academic institutions to study the occurrence, fate and transport of a variety of pharmaceuticals and personal care products.

Exposure Pathways: Ecological: For aquatic organisms, the main routes of exposure to be considered are dermal and ingestion. Example:

- EPA researchers participated in a study with a team of Canadian scientists who treated an experimental lake with ethinylestradiol (a pharmaceutical commonly found in birth control pills) to produce a chronic exposure to aquatic organisms at a level consistent with that observed in some wastewater treatment effluents. EPA used this opportunity to further develop a molecular assay as an indicator of estrogen exposure and to investigate the effects of long-term exposure to an endocrine-disrupting pharmaceutical in a whole lake ecosystem.

Exposure Pathways: Human: When an individual human comes in contact with a foreign chemical or substance, he or she becomes exposed, whereby the chemical may be transferred from the environment onto/into the individual.

- EPA research is determining the effect of different drinking water treatment technologies on compound removal by examining the occurrence and fate of pharmaceuticals and other wastewater derived compounds through drinking water treatment.

Ecological Assessment: The process of determining whether a given chemical (e.g., a pharmaceutical) has an impact on biota or the ecosystem is known as ecotoxicology. This process, when completed for the broader ecosystem scale, is an ecological assessment. Example:

- EPA has been conducting research concerning the ecological effects of a subset of human and veterinary reproductive and developmentally toxic pharmaceuticals using multiple small fish and amphibian species. Specific pharmaceuticals tested include the estrogens ethynylestradiol and estradiol (used in humans and, in the latter case, animals as well), the androgenic steroid trenbolone (used for livestock), the anti-androgenic compound flutamide (used for treating prostate cancer) and several drugs that affect natural steroid production such as fadrozole (developed to treat breast cancer), trilostane and ketoconazole. In addition, EPA has been involved in assessing the effects of endocrine-active pharmaceuticals in the field by investigating, for example, the occurrence and potential population-level effects (on fish) of trenbolone emanating from CAFOs and estrogenic compounds associated with wastewater treatment effluents. In addition, a STAR grantee has been examining the ecotoxicity of a class of antidepressants, known as selective serotonin reuptake inhibitors (SSRIs), on aquatic organisms (fish and amphibians).

Human Health Assessment: Human health assessment refers to the process of identifying potential health effects associated with a given exposure and then determining the dose-response relationship to characterize the associated risk. Example:

- EPA has ongoing research across multiple programs that are developing the methods, models, and measures that could be applied to evaluating the potential impacts of environmental pharmaceuticals on human health. Some of these activities include: developing multiple short term and longer term assays (*in vitro* and *in vivo* in laboratory animal models) to identify potential endocrine disruptors, studying the effects of long term exposures to low levels of chemicals, and developing methods for cumulative assessments of chemicals that act through similar and different modes of action.

An inventory of ORD research projects, status, timelines for conduct, category of research, and examples of selected products can be found in the table in Attachment A. Each project title is hyperlinked to a more detailed description which is available on EPA's web site at http://www.epa.gov/ppcp/work.html.

A recent publication summarizes findings from over the last decade of the Centers and other children's related research (http://es.epa.gov/ncer/publications/research_results_synthesis/ceh_report_508.pdf).

3. Congress required EPA to implement an Endocrine Disruptor Screening Program (EDSP) by 1999 in order to protect public health from dangerous chemicals. In a settlement agreement, EPA said that it planned to start requiring EDSP testing by 2003.

A. Has EPA completed validation of the full battery of tests and screens under the EDSP?

EPA proposed a Tier 1 battery to be used for screening the initial list of chemicals that included the following assays: uterotrophic, Hershberger, female pubertal, adult male, male pubertal, androgen receptor (AR) binding, aromatase, steroidogenesis, amphibian metamorphosis, fish screen, estrogen receptor (ER) binding. The proposed Tier 1 battery was reviewed by the FIFRA Scientific Advisory Panel (SAP) on March 25-27, 2008. The SAP concluded that, "In summary, the proposed set of Tier 1 assays are appropriate to begin screening for disruptors of the EAT [Estrogen, Androgen, Thyroid] axes." The SAP report is available at http://www.epa.gov/scipoly/sap/meetings/2008/march/minutes2008-03-25.pdf.

Based on the current progress, Tier 2 assays should be validated and ready for use in 2010. If Tier 2 testing is triggered for any of the initial chemicals based on the results of Tier 1 screening, testing can be initiated. The mammalian 2-generation assay is valid for Tier 2 testing. The remaining Tier 2 assays are not yet validated: avian 2-generation, amphibian growth/reproduction, fish 2-generation, and the mysid 2-generation.

In addition to the assays used for screening and testing chemicals, EPA is concurrently pursuing an ambitious research program to develop predictive tools to help prioritize chemicals for future screening and testing. These approaches include cell based assays that can be run in a high throughput mode and computer based predictive systems.

These tools can be applied to both pesticidal and non-pesticidal chemicals so that chemicals that are most likely to interact with the endocrine system can be identified more quickly and using fewer resources, and then considered for screening and testing along with those identified for pesticide registration review (e.g., http://www.epa.gov/comptox/toxcast).

B. Has EPA issued a final list of chemicals to be tested under the EDSP program, and has EPA included any non-pesticide drinking water contaminants on that list pursuant to section 1457 of the Safe Drinking Water Act?

Section 408(p) of the Federal Food, Drug and Cosmetic Act (FFDCA) mandates that EPA screen all "pesticide chemicals," covering active and inert ingredients contained in pesticide products. Both FFDCA section 408(p) and section 1457 of the Safe Drinking Water Act (SDWA) give EPA the discretion to require testing of additional compounds,

including certain contaminants of drinking water. Consistent with the requirement in FFDCA, the Agency has focused its efforts to start screening pesticides.

EPA applied an earlier published methodology to identify a proposed initial list of pesticide active ingredients and pesticide inert ingredients for screening. The methodology used multiple databases to identify pesticide active ingredients and pesticide inert ingredients to which there was relatively more widespread or higher levels of human or environmental exposure compared to other active ingredients and inert ingredients. Application of this methodology resulted in a proposed list of 73 chemicals, which was published in a Federal Register Notice on June 18, 2007 (72 FR 33486). One of the factors that was evaluated in establishing the list was whether a chemical had been detected in surface water monitoring programs. EPA expects to publish the final list of the initial group of pesticide chemicals that will undergo screening using the Tier 1 assay prior to issuing test orders. Eventually the Agency intends to screen all pesticide chemicals, and it may require screening of other chemicals consistent with its authority in FFDCA and SDWA.

C. Has EPA fully screened or tested any chemical under the EDSP?

EPA is currently in the process of completing its final preparation of the packages for the related documents, and intends to expedite the remaining procedural and administrative steps for completing those documents. To date, no chemicals have been fully screened or tested under the EDSP, although the registrants of a number of pesticide chemicals have conducted the Tier 2 mammalian 2-generation assay that provides information on endocrine-related effects, among others. To support pesticide registrations, EPA also requires a large number of additional mammalian and wildlife toxicity studies. Pesticide regulatory decisions ensure protection of human health and wildlife from the most sensitive adverse effects observed in this array of assays. Pesticides that have completed re-registration have been determined to meet required safety standards and to not cause unreasonable risks to the environment, consistent with FFDCA and FIFRA.

D. In a response to questions that my office received on February 15, 2007, EPA informed me that "Tier 1 testing of the initial round of chemicals is anticipated to begin in early 2008." We are now five months into 2008. Has EPA begun such testing on any chemicals?
(1) If so, please provide the chemicals' names and the current status of all such tests.
(2) If not, please provide an explanation for why EPA has not yet begun such tests and the date that EPA now anticipates it will begin such testing, as well as the Agency's schedule for testing chemicals, including the name of the chemicals to be tested and the timeline for completing such tests.

The EDSP was required to develop a battery of validated and scientifically credible endocrine screening assays. Assessing the potential of chemicals to interact with endocrine systems is a new and complex scientific undertaking. When the Food Quality Protection Act (FQPA) was enacted, there was no set of widely accepted, scientifically validated endocrine screening assays. Therefore, because the statute required the use of

validated assays, EPA needed to develop assays, (in some cases where no previous assays existed), and optimize the assays so that they could be sufficiently sensitive to detect the effects for which they were intended. Finally, the Agency needed to ensure that the assays would produce similar results in multiple independent laboratories (i.e., validation). This was a time consuming and technically challenging process, which (as noted in 3A) is nearly complete for all Tier 1 assays and which is continuing for the Tier 2 assays..

On June 18, 2007, EPA published a draft list of the initial chemicals to be tested (72 FR 33486). The final list of the initial chemicals to be screened will be published in the Federal Register Notice prior to issuance of the test orders.

Once Tier 1 testing begins on the initial list of chemicals, the Agency estimates that it will take up to two years to complete the testing (although the results of the shorter, *in vitro* assays are expected sooner). If the results from Tier 1 trigger Tier 2 testing, then the Agency anticipates it would be up to three years from the time at which the Tier 2 tests are requested before the Agency receives any Tier 2 data.

(3) Please describe whether EPA could have already begun tests if it had additional resources.

The Agency does not think that additional resources would have significantly accelerated the initiation of testing. In accordance with the statutory requirements, only "validated" tests could be required as part of the EDSP. Identifying, developing, and validating appropriate assays was a time consuming and technically challenging process. It seems unlikely this process could have been completed more quickly. Some studies can only be redesigned and conducted after the results of earlier studies are known. Thus, such studies can only be conducted sequentially, so additional funds would not have accelerated the validation and availability of the assays. In addition, there are inherent scientific uncertainties associated with the development and validation of the assays.

E. Compared to the fiscal year 2002 funding level for EPA's endocrine disruptor chemical testing program, the Bush Administration's proposed fiscal year 2009 funding request would equal a 35% funding cut, adjusted for inflation. Please inform the Committee when EPA will start and complete full EDSP screening and testing of its initial list of chemicals, and come into full compliance with requirements of the law to implement the EDSP, in light of these cuts.

If EPA were to begin issuing test orders for Tier 1 screening of the initial list of chemicals in early 2009, based on the length of time necessary to conduct the tests and prepare reports, EPA expects to begin receiving the results of Tier 1 assays in 2011. Taking into account the time needed for Agency review of the submitted data, any necessary Tier 2 testing would likely begin in 2012 or 2013. EPA determinations based on Tier 2 testing are not expected before 2015.

The process of validating assays for Tier 1 screening involved significant expenses. Now that validation of Tier 1 is nearly complete, fewer resources are needed in FY09 to continue implementation of the EDSP consistent with the statute.

4. Please provide copies of peer-reviewed studies, including but not limited to studies authored by EPA scientists, available to the Agency that indicate that biological systems, including the reproductive or other endocrine systems, may be affected by exposure to chemical substances in the range of parts per trillion or lower levels.

In order to prepare a response to this question, EPA considered studies conducted in its intramural laboratories, studies conducted through its extramural grants program (Science to Achieve Results or STAR), and studies submitted by industry to the Agency. With few exceptions, we were unable to identify any studies conducted at such extremely low levels of exposure. The exceptions are as follows:

- The "Dioxin Reassessment: NAS Review Draft 2004" estimated that the average body burden of dioxin in the US human population in the late '90s was 25 ppt (TEQ) and that various pathways of intake were in the low ppt (TEQ) range. (http://cfpub.epa.gov/ncea/CFM/recordisplay.cfm?deid=87843)

- In 2007, EPA scientists were co-authors on a paper with Dr. Karen A. Kidd on the results from an experimental whole lake study assessing the impact of an endocrine disruptor on the sustainability of a wild fish population. The seven year study involved the addition of a synthetic estrogen (17α ethinyl estradiol), used in birth control pills, to a natural lake located in the Experimental Lakes Area in northwestern Ontario, Canada. The study showed that chronic exposure of a common species of minnow to low concentrations (5-6 parts per trillion) of the potent hormone led to feminization of male fish and altered reproductive fitness in female fish using molecular, protein, tissue, and individual whole body tests and measurements. Ultimately, these impacts led to a near extinction of this species from the lake. The observations by Kidd and her colleagues demonstrate that the concentrations of synthetic estrogens observed in freshwaters can impact the sustainability of wild fish populations. (Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc National Acad Science May 22, 2007 vol. 104 no. 21 8897–8901). See Attachment B.

Most standard toxicological studies to evaluate the effects of environmental agents in whole mammalian models are conducted at the mg/kg of weight dose range (or part per million). Many pharmaceuticals are typically dispensed in the mg range for therapeutic use. Therefore, it would be extremely rare to find studies conducted in whole mammalian animals at the ppt range.

5. The Agency's testimony stated that EPA is studying 74 randomly selected wastewater treatment plants to determine what types of contaminants occur in their sludge and at what levels.

Please describe the following:
A. The names and locations of the 74 wastewater treatment plants,
See Attachment C. Targeted National Sewage Sludge Survey--*Study Objective and Design.*

B. The date that EPA will finish collecting samples,
EPA completed collecting biosolids samples in March 2007.

C. The date that EPA will finish analyzing collected samples,
EPA is in the process of analyzing the remaining samples. That analysis should be completed by July 2008. Quality assurance and evaluation of the results will then follow and will be completed by September.

D. The review process that EPA intends to use, including any external peer review, and
An external peer review was conducted for the study design. EPA will conduct an internal review of the final report

E. The date that EPA will issue a report on its findings.
A final report will be available by the end of September 2008.

6. Please describe in detail the Agency's plans, with goals and timetables, to include any pharmaceutical or personal care product in the Unregulated Contaminant Monitoring Rule, Contaminant Candidate List, or to otherwise take action to monitor and reduce exposure to these chemicals in drinking water.

The Agency intends to treat pharmaceuticals in a similar manner as the other contaminants that may occur in drinking water. We will continue to implement the processes under the Unregulated Contaminant Monitoring Rule (UCMR) and Contaminant Candidate List (CCL) to address the wide range of contaminants that have the potential to pose a health risk due to their presence in drinking water.

With respect to the CCL process, we described the information on pharmaceuticals that we used to develop the draft list as part of our February 2008 Federal Register notice. We also requested additional information that would help us to determine if other pharmaceuticals should be listed on the CCL. We will review that information, particularly on occurrence in water, to help us to determine if additional pharmaceutical compounds should be included on the list.

With respect to the UCMR process, public water systems initiated monitoring in January for 25 contaminants under the second UCMR round (UCMR2), which they will complete by 2010. We are moving forward to identify additional contaminants that will be

included in the third round of monitoring (UCMR3) for up to 30 new contaminants (per SDWA) which we hope to initiate by 2012. This is one year prior to the date by which the next round of monitoring is scheduled to begin (per SDWA). We are currently evaluating potential contaminants and are evaluating the possibility of including a number of pharmaceutical and/or endocrine disrupting compounds on the list. Before they can be included in UCMR3, we must validate the methods to ensure that they can be widely applied in multiple labs throughout the country. Concurrent with this effort we are working to develop and validate a robust, affordable analytical method for a limited number of pharmaceuticals that could be used by laboratories across the country to support a national drinking water survey. Such methods must be available for any contaminants we include in UCMR3.

We will also continue to evaluate the results of occurrence data collected by other parties, including self-initiated monitoring efforts carried out by public water systems and states and ambient monitoring carried out under EPA's Clean Water Act programs to better understand levels of pharmaceuticals in drinking water sources. We will also continue to collaborate with the U.S. Geological Survey to review the results of monitoring they may carry out as part of the Toxic Substances Hydrology and NAWQA programs or other monitoring efforts. Finally, to reduce possible exposure, we will work within EPA and across Federal Agencies to identify and promote actions that will prevent pharmaceuticals from entering sources of drinking water.

7. Please describe in detail EPA's current and planned regulatory restrictions, enforcement, or other actions to reduce disposal of pharmaceuticals in order to reduce ground and surface water contamination with these products.

EPA is promoting responsible stewardship of unused pharmaceuticals. EPA is working with many of the other Agencies such as the Food and Drug Administration and the Drug Enforcement Administration on this issue.

For example, the Office of National Drug Control Policy (ONDCP), EPA and DHHS (FDA) issued Federal Guidelines for the Proper Disposal of Prescription Drugs in February 2007. The guidelines recommend that consumers remove prescription drugs from their containers and mix them with an undesirable substance, such as used coffee grounds or kitty litter and place them in an impermeable, non-descript containers, such as empty cans, and throw the container in the trash. The guidelines also encourage consumers to take advantage of take-back programs that are in compliance with the Controlled Substances Act. However, there are some drugs (based on their potential for abuse) that the FDA has advised consumers to flush down the toilet in order to quickly and definitively eliminate the possibility for misuse or abuse.

The Agency has funded, through the Office of Children's Health Protection, Environmental Education grants for pilot take-back programs ($150K grant to the University of Maine Center on Aging for a pilot mail-in take-back program that began May 12, 2008; and $150K grant to ARCHS in St. Louis for a pilot program to dispose of

unwanted, non-controlled drugs through a regional grocery store chain as the collection point.)

Additionally, a number of EPA regions are promoting voluntary take-back programs for unwanted or unused pharmaceuticals. For example, in April the Great Lakes Region hosted a voluntary take-back week, where consumers were encouraged to bring in unwanted medicines. This effort funded 24 collection events [medicines, e-waste (e.g., old computers, cellphones, etc.), or both] around the Great Lakes Basin during Earth Week (April 21 – 25, 2008). The goal of collecting 1 million pills was not only met, but exceeded. With only 13 events reporting to date, over 3.8 million pills were collected.

In January 2006, EPA provided a $101K grant to Villanova University to study further ways to reduce the presence of pharmaceuticals in the environment. The project is identifying ways to better manage how pharmaceuticals are discarded from university dormitories as well as developing technology to reduce pollution by preventing pharmaceutically-active chemicals from leaving wastewater treatment plants.

8. Please provide:

A. A list of treatment technologies that can reduce or remove pharmaceuticals from wastewater, and

B. Copies of any studies analyzing the effectiveness of those technologies.

There are thousands of pharmaceuticals and personal care products (PPCP) compounds and only a handful of these have been researched for treatment efficacy. Also, the type and chemical properties of PPCPs vary widely; therefore there is no one treatment that is completely effective for all waste streams. For example, many PPCPs are effectively removed by biodegradation, but some are not. Chemical oxidation including ozonation and chlorination can destroy some PPCPs. Some of the most widely studied treatment technologies for PPCPs include:

- UV disinfection
- Activated Sludge
- Reverse Osmosis/Ultra-filtration
- Ozonation
- Chlorine Disinfection
- Denitrification
- Membrane Bioreactors
- Hydrogen Peroxide Disinfection

There are over a hundred studies dealing with PPCP treatment technologies. Four of these studies have been widely circulated among the scientific community. A short summary of each of the four follows:

1. Stephenson, Roger, Ph.D. and Joan Oppenheimer, M.S.P.H. *Fate of Pharmaceuticals and Personal Care Products through Municipal Wastewater Treatment Processes.* 2007. Water Environment Resources Foundation (WERF) and IWA Publishing.

   This study, sponsored by WERF, was conducted to expand the limited published data on PPCPs from full-scale facilities. Data were collected to measure the removal of 20 PPCPs commonly found in the influent of full-scale wastewater treatment facilities operating in the U.S. Six wastewater treatment systems were sampled in the U.S. for PPCP analytes. The plants employed varying combinations of treatment operations, including: activated sludge, media filtration, chlorine disinfection, ultraviolet disinfection, and reverse osmosis.

2. Drewes, Jorg E., et al. *Removal of Endocrine Disrupting Compounds in Water Reclamation Processes.* 2006. Water Environment Resources Foundation (WERF) and IWA Publishing. 188 p.

   This study, sponsored by WERF, was conducted to develop approaches combining bioassays with chemical analysis to study removal of endocrine disrupting compounds by water reclamation treatment processes. Eleven treatment plants were sampled in the U.S. for Nonyl Phenol/Alkyl Phenol Ethoxylates and other analytes. The plants employed varying combinations of treatment operations, including: activated sludge, media filtration, chlorine disinfection, ultraviolet disinfection, reverse osmosis, membrane bioreactors, and soil-aquifer technology. The study provides information about the influent characteristics (percent of domestic versus industrial) and the sludge retention time at each plant.

3. Snyder, Shane A., et al. *Removal of EDCs and Pharmaceuticals in Drinking and Reuse Treatment Processes.* 2007. American Water Works Association Research Foundation (AwwaRF).

   This AwwaRF sponsored study determined removal of EDCs and PPCPs by drinking water treatment processes. Researchers found that conventional water treatment (coagulation, flocculation, sedimentation) was not effective in removing the majority of target compounds. Chlorine disinfection can remove many compounds, but ozone and other advanced oxidation processes (UV/peroxide and ozone/peroxide) are much more effective. Reverse osmosis and nanofiltration are also highly effective.

4. Ternes, Thomas, Project coordinator Poseidon Project. *Assessment of Technologies for the Removal of Pharmaceuticals and Personal Care Products in Sewage and Drinking Water Facilities to Improve the Indirect Potable Water Reuse, Detailed REPORT related to the overall project duration: January 1st, 2001 – June 30th, 2004.* 2006.

The Poseidon Project was a multi-year research project funded by the European Union. Among other things, wastewater and drinking water treatment technologies were studied to determine their effectiveness in removing PPCPs. In addition to the overall summary report, this project resulted in publication of dozens of technical papers presented at international conferences and published in peer-reviewed journals. One such paper is Clara, M., et al. *The Solids Retention Time--A Suitable Design Parameter to Evaluate the Capacity of Wastewater Treatment Plants to Remove Micropollutants.* 2005. Water Research. 39:97-106.

The Pharmaceutical Research and Manufacturers of America (PhRMA) task force on Pharmaceuticals in the Environment (PiE) has developed a database of the technical literature that refers to aquatic life impacts of pharmaceuticals and fate information. The fate information includes removals by various wastewater treatment processes. Through a partnership between EPA and PhRMA, this database and associated bibliography was shared with EPA. This database includes about 100 references and published removal data for 108 compounds. The published studies represent a variety of experimental designs, sampling programs, and analytical methods. Further review of this literature is required to determine the comparability of the results. This database and bibliography can be provided if so desired.

Questions from Senator James M. Inhofe:

1. The number one reason we are here today is to ensure that our drinking water is safe for our constituents. To your knowledge, is the water safe to drink?

We believe that the U.S. has one of the safest drinking water supplies in the world and we are committed to keeping it that way. Based on what we know and the work we have done under the Clean Water Act and the Safe Drinking Water Act, EPA believes the water is safe to drink. EPA is committed to continue working aggressively to bring good science to bear on the issue so we can find needed answers and continue to ensure that our water is safe to drink.

2. USGS states in their 2002 report that, "measured concentrations were generally low and rarely exceeded drinking-water guidelines, drinking-water health advisories, or aquatic life criteria. Many compounds, however, do not have such guidelines established."

Does that mean that EPA hasn't looked at setting such guidelines or standards, or does that suggest the compounds at such small traces haven't yet warranted a guideline or standard?

In general, many of the studies conducted by USGS monitor for a wide array of contaminants. Because USGS studies often monitor for contaminants that have not previously been studied and/or use analytical techniques that have very low levels of detection, they sometimes find contaminants that have not been previously found in water.

EPA's risk-based, standard-setting processes target contaminants for which there are data to show occurrence in water at frequencies and levels of concern. Therefore, USGS studies can detect contaminants for which there are no EPA drinking water standards, health advisories or water quality criteria, because the previously available data have not shown that a standard is needed. However, when these occurrence data become available, EPA evaluates these data along with health effects information to determine if a guideline or standard needs to be developed.

One of the challenges associated with pharmaceuticals and other emerging contaminants is that our ability to detect them is surpassing our understanding of the potential health effects associated with levels detected in water. EPA has standards for more than 90 microbial contaminants, inorganic contaminants, synthetic and volatile organic chemicals, and disinfectants and disinfection byproducts that have been identified as posing a risk to human health. The Agency has also developed health advisory levels for more than another 160 contaminants that may occur in water, but not widely enough to warrant a national regulation.

3. Based on your October 2006 testimony, isn't it true that EPA has been paying close attention to this issue for years? What progress have you made since the October 2006 hearing?

EPA has made significant progress since the October 2006 hearing. EPA has continued to actively work with Federal Agencies and state and local government partners to better understand the implications of emerging contaminants such as pharmaceuticals and personal care products, and endocrine disrupting chemicals detected in drinking water, wastewater, surface water and ground water. We continue to evaluate their routes of exposure and potential effects on public health and aquatic life.

We are focused on learning more about the occurrence of PPCPs in water. In addition, we are working to better understand what treatment technologies may remove them from wastewater and drinking water. We are developing analytical methods to improve detection capabilities. We are conducting national studies and surveys to help direct our course of action and partnering with government Agencies, stakeholders, and the private sector, to increase public awareness about product stewardship and pollution prevention.

EPA is responding to the issues of PPCPs in water using a four-pronged strategy aimed at:

- strengthening science;
- improving public understanding and risk communication;
- building partnerships for stewardship; and
- using regulatory tools when appropriate.

The Agency has a number of activities underway in each of these areas:

Strengthening Science
Sound science and reliable information must be the foundation for any agency decision. Some of the activities EPA has underway to improve science include:

A) Research
EPA's Office of Research and Development is engaged in a broad research portfolio to answer key questions associated with exposure pathways and health effects. An inventory of the projects developed for the period from 1996 through 2014 is available online. The Agency has also supported and cooperated with research efforts carried out by outside groups, such as the American Water Works Association Research Foundation (AwwaRF) and Water Environment Research Foundation (WERF). For example:

- In support of the Agency's Endocrine Disruptors Screening Program (EDSP), ORD finalized the research, developed/standardized protocols, prepared background materials for transfer, briefed Agency advisory committees, participated on international committees on harmonization of protocols, and/or participated in the validation of 18 different assays.

- ORD participated in a collaboration with the Global Water Research Coalition (GWRC) to apply one of the molecular screening assays it developed to detect estrogenic activity in wastewater treatment effluents from around the world.
- ORD made awards to seven academic institutions to study the impact of hormones released from concentrated animal feeding operations on aquatic organisms and the environment and held a workshop with the awardees and scientists from across EPA and other Agencies (for more details on the solicitation, the awards, and the workshop, see the following sites respectively: http://es.epa.gov/ncer/rfa/2006/2006_star_cafos.html, http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/recipients.display/rfa_id/435, http://es.epa.gov/ncer/publications/workshop/08_20_07_cafo.html)
- EPA, jointly with the US Geological Survey, oversaw the organization of an interagency workshop on the impact of endocrine disruptors on wildlife and the environment. It was an opportunity for the Federal Agencies to share their activities and look for opportunities for improved collaborations. Over 100 scientists from 14 Agencies participated. A summary report is available at: http://es.epa.gov/ncer/publications/workshop/cenr_2202007
- Examples of a few key peer reviewed publications related to pharmaceuticals and personal care products since October 2006 include:
    - Developing an approach to prioritize research to study the risks to aquatic organisms from exposures to human prescription pharmaceutical residues in wastewater effluents[1]
    - Determining that the antibacterial agent found in many consumer products, triclosan, is a thyroid disruptor in a rat model[2]
    - Canadian dosed lake study of the impact of short term environmentally relevant low level exposures to ethynyl estradiol (component of birth control pills) on the long term viability of populations of aquatic organisms[3]
    - Analysis for estrogens in lagoon samples from concentrated animal feeding operations (CAFOs)[4]
- ORD held a mid-cycle review of its Endocrine Disruptors Research Program with a Subcommittee of its Board of Scientific Counselors on September 18, 2007 (http://www.epa.gov/osp/bosc/subcomm-edcs_mid.htm). The Subcommittee determined that the research program had exceeded expectations in its progress toward addressing recommendations from their 2004 review.
- ORD prepared a draft update of the Multi-Year Plan for Endocrine Disruptors that identifies research to be conducted from 2007-2013. The plan includes some efforts related to endocrine-mediated pharmaceuticals (http://www.epa.gov/osp/bosc/subcomm-edcs_mid.htm#documents).

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[1] Kostich MS, Lazorchak JM. Risks to aquatic organisms posed by human pharmaceutical use. Science of the Total Environment. Jan 25, 2008; 389(2-3):329-39. Epub Oct 22, 2007.

[2] Crofton, K.M., Paul, K.B., DeVito, M.J., and Hedge, J.M. (2007). Short-Term In Vivo Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine. *Environmental Toxicology and Pharmacology*, DOI: doi:10.1016/j.etap.2007.04.008.

[3] Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc National Acad Science May 22, 2007 vol. 104 no. 21 8897–8901

[4] Hutchins SR, White MV, Hudson F, Fine DD. 2007. Analysis of lagoon samples from different concentrated animal feeding operations (CAFOs) for estrogens and estrogen conjugates. Environ Sci Technol 41(3):738-744

B) Methods Development

It is important to have analytical methods available to reliably detect PPCPs in water. To fill some current gaps, EPA developed and released cutting edge methods in December 2007, for the analysis of approximately 100 pharmaceuticals, personal care products, steroids, and hormones in water, soil, sediment, and biosolids.

C) Occurrence Studies

The Agency is conducting or funding a number of studies to better understand the potential occurrence of pharmaceuticals in wastewater effluent, biosolids and fish tissue, including:

- Publicly-Owned Treatment Works (POTW) study to better understand what is going into the POTW for treatment and what is coming out both in the discharge and in the biosolids. We expect to complete this study by December 2009.
- Fish Tissue Pilot Study to investigate whether pharmaceuticals and other personal care products may occur in fish from five effluent-dominated streams across the US. Results are expected later this year. We plan to expand the fish tissue study to ensure a statistically- and nationally-representative sample of PPCPs in fish tissue. (2010)
- Targeted National Sewage Sludge Survey to determine whether approximately 100 pharmaceuticals and other personal care products may occur in biosolids. (2008)
- Funding a University of Florida project to evaluate the fate and transport of triclocarban in biosolids. (2009)
- Funding a Duke University project that is monitoring for steroids and hormones in wastewater and biosolids at four wastewater treatment plants. (2009)

Improving Public Understanding and Risk Communication

It is important to communicate with the public about available data and any associated uncertainty with the data. To help in this regard:

- EPA has developed a general website on pharmaceuticals and personal care products, with a primary focus on the Agency's research. EPA plans to improve our web site by providing additional information about work we are doing on PPCPs in water.

Building Partnerships for Stewardship

Federal, state and local agencies, industry and others all have a role to play in better understanding and addressing issues regarding pharmaceuticals in water. Collaboration and building partnerships for stewardship are important components:

- EPA is participating, along with ten other Federal Agencies, on the Pharmaceuticals in the Environment workgroup under the auspices of the National Science and Technology Council, Committee on Environment and Natural Resources, Toxics and Risk Subcommittee, to better coordinate Federal research efforts.

- In conjunction with the White House Office on National Drug Control Policy (ONDCP), EPA issued drug disposal guidelines in early 2007 to help reduce the quantities of pharmaceuticals entering our nation's waterways.
- EPA has also been supporting and promoting good stewardship efforts:
    - Grant to Area Resources for Community and Human Services (ARCHS) in St. Louis ($150K) for take-back of non-controlled, unused medicines at pharmacies
    - Grant to University of Maine ($150K) for mail-back of unused medicines with appropriate involvement of law enforcement
    - Grant to Villanova University in Delaware County, Pennsylvania ($101K) to identify ways to better manage how pharmaceuticals are discarded from university dormitories.
    - Great Lakes Earth Day Challenge to collect one million pills for safe disposal.

Using Regulatory Tools

EPA will use our regulatory tools to take action when sufficient information exists. For example:

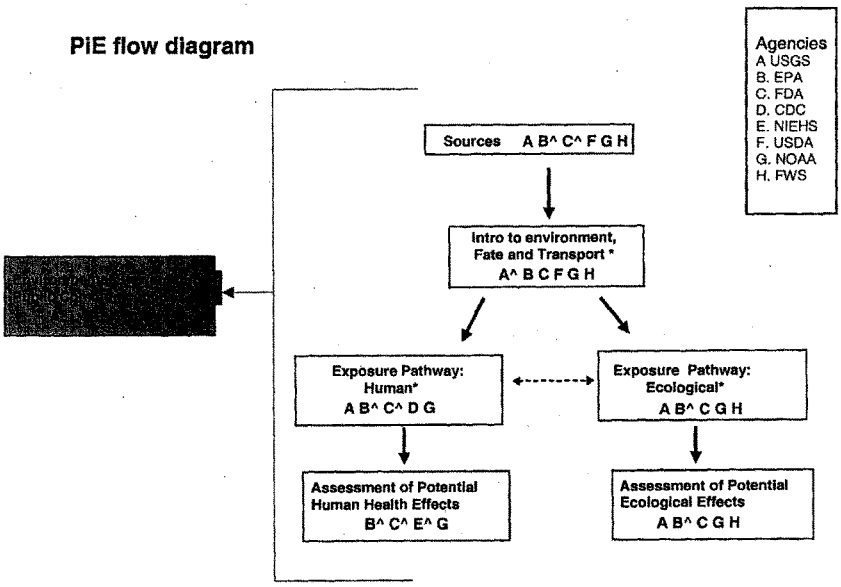
- Health Services Study-- as part of the effluent guidelines planning process, EPA is reviewing the pharmaceutical disposal practices of hospitals and long-term care facilities to identify best practices to minimize pharmaceutical discharges to water.
- Third Contaminant Candidate List (CCL3)—the Agency proposed 104 contaminants it will evaluate to determine if national drinking water regulations are needed to protect public health. Through the public comment process, which concluded May 21, 2008, EPA is seeking additional data on the pharmaceutical concentrations in treated or ambient water and adverse health effects that may be posed by pharmaceuticals in drinking water.

4. Isn't it true that EPA is not the only Agency that must be involved in addressing these contaminants and their pathways into the environment? Can you describe what the other Agencies responsibilities are?

Yes, various Federal Agencies have responsibilities that are relevant to contaminants and their pathways into the environment. The Interagency Workgroup on Pharmaceuticals in the Environment (PiE) was established to identify research gaps and better coordinate Federal research activities on pharmaceuticals. The Workgroup is co-chaired by EPA, USGS, and FDA. The other participating Agencies include United States Department of Agriculture (USDA), the National Institute of Environmental Health Sciences (NIEHS), and the National Oceanic and Atmospheric Administration (NOAA). The Fish and Wildlife Service (FWS), Centers for Disease Control and Prevention (CDC), the Office of Management and Budget (OMB) and the Office of Science and Technology Policy (OSTP) serve in an Ex Officio capacity.

The following flow diagram reflects the major components associated with pharmaceuticals in the environment including those that are of particular interest to a

given Agency. The primary interagency working group leads associated with each component are also indicated.



^PiE Research Report Chapter Leads

The USGS monitors surface water, ground water, and other media. NOAA protects the environmental health of coastal ecosystems and monitors for contaminants in these areas. The FWS is responsible for conserving, protecting and enhancing fish and wildlife and can contribute in this respect. The FDA regulates, among other products, human and veterinary drugs. The USDA looks at issues involving such things as contaminants in food and effects of chemicals used by agriculture. Other Agencies such as the CDC are responsible for promoting health and quality of life by preventing and controlling disease in the human population and dealing with issues of antibiotic resistance. Additionally, the NIEHS (within HHS/NIH) is responsible for research to reduce the burden of environmentally associated disease and dysfunction by determining how environmental exposures affect our health, how individuals differ in their susceptibility to these exposures, and how these susceptibilities change over time.

5. If EPA were to go back and review all pharmaceuticals found in drinking water, how long would that take and what would the cost be?

There are thousands of prescription and over-the-counter drugs registered in the U.S. When developing the draft CCL3, the primary source of health effects information for

pharmaceuticals was the Food and Drug Administration Database on Maximum Recommended Daily Doses (MRDD). This database includes the recommended adult doses for over 1,200 pharmaceutical agents.

A key issue associated with evaluating all of these pharmaceutical agents is that there is limited information about their occurrence in drinking water. Occurrence information from USGS provides ambient water concentration data for 123 contaminants, which include pharmaceuticals. In the absence of water concentration data, EPA uses other information such as the Toxics Release Inventory and chemical production data to identify those agents that have the potential to occur in drinking water.

Due to the size of the pharmaceutical universe and the paucity or lack of environmental occurrence information for pharmaceuticals, it is too difficult to effectively estimate the cost and/or time that would be needed to gather the necessary information.

6. Is it possible that the process of setting a drinking water standard for numerous emerging compounds would delay current efforts to list knowingly harmful drinking water contaminants?

Yes. Note that we evaluated a total of 7,500 potential contaminants when developing our draft CCL3 list. We identified 560 of those potential contaminants that should be further evaluated (the preliminary CCL or PCCL) based on their potential to occur in public water systems and the potential for public health concern. Our final selection of 104 potential contaminants for the CCL was based on more detailed evaluation of occurrence and health effects and expert judgment. Work to further evaluate potential contaminants that have been identified as posing a potential risk in drinking water would be delayed if we had to redirect efforts to look in more detail at pharmaceutical agents. Furthermore, while the Agency has requested further information, at the time of the CCL3 proposal there was unlikely to be sufficient occurrence and health effects information to make regulatory decisions on those pharmaceutical compounds initially considered in the universe of potential contaminants.

ATTACHMENT A
ORD Research Related to Pharmaceuticals in the Environment
May 13, 2008
Draft

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Identification of Non-Target List Analytes in Complex Environmental Samples	In progress	1996-2014	To identify non-target list compounds, including pharmaceuticals and personal care products that enter the environment.	▪ Monitoring and Detection Tools	Grange, A.H., W. Winnik, P.L. Ferguson, and G.W. Sovocool. 2005. "Using a Triple-Quadrupole Mass Spectrometer in Accurate Mass Mode and an Ion Correlation Program to Identify Compounds." Rapid Commun. Mass Spectrom. 19: 2699-2715. Grange, A.H., and G.W. Sovocool. 2003. "Identification of Unanticipated Compounds by High-Resolution Mass Spectrometry." Spectroscopy 18(5): 12-24. Grange, A.H., L.I. Osemwengie, G.M. Brilis, and G.W. Sovocool. 2001. "Ion Composition Elucidation (ICE): An Investigative Tool for Characterization and Identification of Compounds of Regulatory Importance." Environ. Forensics 2: 61-74.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Alkylphenols, Alkylphenolethoxylates and Their Metabolites (APEs) as Potential Great Lakes, Tributaries, and Effluent Dominated Stream Endocrine Disrupters.	Completed	1999-2002	To conduct a survey of the concentration and assess some of the endocrine effect endpoints in the fish of alkylphenols, alkylphenolethoxylates and their metabolites in water of the upper Midwest.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects ▪ Monitoring and Detection Tools	Datta, S., J.E. Loyo-Rosales, and C.P. Rice. 2002. "A Simple Method for the Determination of Trace Levels of Alkylphenolic Compounds in Fish Tissue Using Pressurized Fluid Extraction, Solid Phase Clean-up and HPLC Fluorescence Detection." J. Agriculture and Food Chemistry 50(6): 1350-1354. Schmitz-Afonso, I., J.E. Loyo-Rosales, M. de la Paz Avilés, B.A. Rattner, and C.P. Rice. 2003. "Determination of alkylphenol and alkylphneol ethoxylates in biota by liquid chromatography with detection by tandem mass spectrometry and fluorescence spectroscopy." J. Chromat. A 1010: 25-35.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Investigating Environmental Sinks of Macrolide Antibiotics, and Illicit Drugs, with Analytical Chemistry	In progress	1999-2014	To Develop and apply analytical methodologies to investigate the source apportionments of macrolide antibiotics, and illicit drugs, in the environment.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Jones-Lepp, T.L., and R. Stevens. 2007 . "Pharmaceuticals in Biosolids/Sewge Sludge - The Interface between Analytical Chemistry and Regulation." Invited submission for special issue of Analytical and Bioanalytical Chemistry 387: 1173-1183. Jones-Lepp, T,L. 2006. "Chemical markers of human waste contamination: Analysis of urobilin and pharmaceuticals in source waters." J Environ Monit. 8: 472–478. Jones-Lepp, T.L., D. Alvarez D, J. Petty, and J. Huggins. 2004. "Polar Organic Chemical Integrative Sampling (POCIS) and LC-ES/ITMS for Assessing Selected Prescription and Illicit Drugs in Treated Sewage Effluents." Archives Environ Cont Toxicol 47(4): 427-439.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Outreach and Defining the Scope of the Issues and Concerns Surrounding Pharmaceuticals and Personal Care Products (PPCPs) as Environmental Pollutants	In progress	1999-2014	To (i) define the scope of the scientific issues involved with PPCPs in the environment; (ii) assist those new to the topic with locating published data and with networking; (iii) advance the scientific dialog and catalyze debate in determining the importance of the topic with respect to environmental pollution; (iv) enhance the public's overall awareness and understanding of the origins of chemical pollution and their individual roles (through their everyday activities, actions, and behaviors) in its causes and prevention; (v) foster international research both within and outside government; (vi) create collaborations and networks among scientists, and foster better scientific understanding of the issues among the healthcare communities; (vii) address inquiries from the public, scientists, state and local governments, and municipal water utilities; and (viii) provide advice to federal agencies.	▪ Environmental Stewardship Public Communication	Daughton, C.G.; Ternes, T.A. Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change?" Environ. Health Perspect. 1999, 107(suppl 6), 907-938. Daughton CG. "Cradle-to-Cradle Stewardship of Drugs for Minimizing Their Environmental Disposition while Promoting Human Health. I. Rationale for and Avenues toward a Green Pharmacy. Environ. Health Perspect. 2003, 111(5):757-774. Daughton CG. 2003. "Cradle-to-Cradle Stewardship of Drugs for Minimizing Their Environmental Disposition while Promoting Human Health. II. Drug Disposal, Waste Reduction, and Future Direction. Environ. Health Perspect. 2003, 111(5):775-785. Daughton, CG. "Pharmaceuticals as Environmental Pollutants: the Ramifications for Human Exposure," In: International Encyclopedia of Public Health, (K Heggenhougen & S Quah, Eds), Elsevier, Academic Press; in press; publ targeted for Sept 2008:

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
The Effects of Disinfection on Pharmaceuticals in Drinking Water Supplies	Completed	2000-2003	To use model solutions to examine the effects of chlorination on pharmaceuticals.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Monitoring and Detection Tools	Glassmeyer, S.T., and J.A. Shoemaker. 2005. "Effects of chlorination on the persistence of pharmaceuticals in the environment." Bulletin of Environmental Contamination and Toxicology 74:24-31.
Vitellogenin Gene Expression in Fathead Minnows and Pearl Dace from Reference (Non-dosed) and Lakes Dosed with EE2 in the Canadian Experimental Lakes Area	Completed	2000-2005	To further develop a vitellogenin gene expression assay as an indicator of estrogen exposure and to investigate the effects of long-term exposure to an endocrine-disrupting pharmaceutical in a whole lake ecosystem.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	Kidd K.A., P.J. Blanchfield, K.H. Mills, V.P. Palace, R.E. Evans, J.M. Lazorchak, and R.W. Flick. 2007. "Collapse of a Fish Population Following Exposure to a Synthetic Estrogen." Proceedings of the National Academy of Sciences of the United States of America 104(21):887-8901. Flick, R.W., J.M. Lazorchak, and M.E. Smith. 2004. "Report on Vitellogenin Gene Expression in Fathead Minnows and Pearl Dace from Reference (non-dosed) and Lakes Dosed with EE2 in the Canadian Experimental Lakes Area." EPA 600/R-04/173; EPA APM 273.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Evaluation of Drinking Water Treatment Technologies	In progress	2000-2014	To provide information necessary for the selection of drinking water treatment techniques that provide for optimal EDC removal and subsequently reduce human exposure to EDCs. In order to be able to evaluate the ability of conventional and advanced drinking water treatment processes to remove EDCs, appropriate analytical methods for the selected EDCs in several water matrices must be available or developed. Additionally, the applicability of bioassay(s) to evaluate the removal/presence of compounds with endocrine activity in water samples pre- and post-treatment will be evaluated.	▪ Sources ▪ Monitoring and Detection Tools	USEPA. 2001. "Removal of Endocrine-Disruptor Chemicals Using Drinking Water Treatment Processes." EPA/625/R-00/015. Kathleen Schenck and Thomas Speth, Japan. "Evaluation of Drinking Water Treatment Technologies for Removal of Endocrine Disruptors Chemicals." Japan - US Governmental Conference on Drinking Water Quality Management and Wastewater Control, Tokyo, Japan, October, 2002 and 2002; AWWA Endocrine Disruptors and the Water Industry Symposium, Cincinnati, OH, April 18-20, 2002.
STAR Grant R829004 - Pharmaceuticals and Antiseptics: Occurrence and Fate in Drinking Water, Sewage Treatment Facilities, and Coastal Waters	Completed	2001-2004, extended to 2006	To address data gaps in the occurrence, ecotoxic risk, and fate of pharmaceuticals and antiseptics in drinking water, sewage treatment plant (STP) influent and effluent, and receiving waters.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Abstracts - 1 Journal Articles – 4 Presentations – 13 Proceedings – 4

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R829005 - The Influence of Amphiphilic Molecules on the Environmental Fate and Transport of Pharmaceuticals	Completed	2001-2004, extended to 2005	To evaluate transport processes affecting pharmaceutical movement in the environment, and will study the influence of amphiphiles (e.g., surfactants, phospholipids) on the fate and transport of pharmaceuticals in the environment.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Abstracts – 3 Book Chapters - 1 Journal Articles – 5 Presentations – 7 Kibbey. T., A.C. Hari, R.A. Paruchuri, D.A. Sabatini. 2005. "The effects of pH and cationic and nonionic surfactants on the adsorption of pharmaceuticals to a natural aquifer material." Environmental Science and Technolog, 39: 2592 -2598.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R829006 - The Environmental Occurrence, Fate, and Ecotoxicity of Selective Serotonin Reuptake Inhibitors (SSRIs) in Aquatic Environments	Completed	2001-2004, extended to 2007	To: (1) determine the environmental fate of Prozac (fluoxetine), Luvox (fluvoxamine), Paxil (paroxetine), Zoloft (sertraline) and Celexa (citalopram) in laboratory studies similar to those used for pesticide registration; (2) determine their occurrence in raw wastewater, treated effluent, and downstream receiving waters; (3) determine the acute and chronic toxicity of the five SSRIs and their major environmental metabolites to C. dubia; and (4) determine the developmental effects of chronic SSRI exposure to the mosquitofish and two amphibian species.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Book Chapters - 1 Journal Articles – 8 Presentations – 25 Kwon, J.W., and K.L. Armbrust. 2006. "Laboratory persistence and fate of fluoxetine in aquatic environments." Environmental Toxicology Chemistry 25: 2561-2568.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R829007 - Occurrence and Fate of Pharmaceuticals and Personal Care Products in Groundwater Environments	Completed	2001-2004, extended to 2005	To: (1) determine the distribution of a wide range of highly used or toxicologically significant PPCPs and environmental estrogens in point source discharges and in groundwaters that receive those discharges; and (2) assess the likelihood of migration of PPCPs through sand-gravel aquifers using a combination of field observations and laboratory studies. The secondary objectives are to: (1) develop new HPLC-MS methods to determine conjugated steroid hormone metabolites in wastewater effluent and groundwaters to determine the possible role of these conjugates in solubilizing and transporting hormones in groundwaters; and (2) compare the distribution and likely behavior of PPCPs in subsurface environments with that observed in surface waters.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Book Chapters - 1 Journal Articles – 6 Presentations – 10 Reddy S., C.R. Iden, and B.J. Brownawell. 2005. "Analysis of steroid estrogen conjugates in municipal waste waters by liquid chromatography-tandem mass spectrometry." Anal. Chem. 77: 7032-7038. Reddy S., and B.J. Brownawell. 2005. "Analysis of estrogens in sediments from a sewage impacted estuary using HPLC-time of flight mass spectrometry." Environ. Toxicol. Chem. 24: 1041-1047.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R829008 - Fate and Effects of Fluoroquinolone Antibacterial Agents in Aquatic Ecosystems	Completed	2001-2004, extended to 2006	To assess the fate, attenuation, and ecotoxicity of selected fluoroquinolone (FQ) antibiotics on surface water quality. Emphasis is placed on developing new methods for detecting and quantifying FQs and their degradation products at low concentrations and developing new molecular techniques for monitoring antibacterial resistance to FQs in exposed microorganisms	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Book Chapters - 1 Journal Articles – 16 Presentations – 20 Proceedings – 1 Symposiums - 2 Robinson, A.A., J.B. Belden, and M.J. Lydy. 2005. "Toxicity of Fluoroquinolone Antibiotics to Aquatic Organisms." Environ. Toxicol. Chem. 24: 423-430.
STAR Grant R829014 - Impact of Residual Pharmaceutical Agents and their Metabolites in Wastewater Effluents on Downstream Drinking Water Treatment Facilities	Completed	2001-2004, extended to 2006	To assess the presence and subsequent impact of major use pharmaceutically active compound (25 antibiotics) residues and their metabolites in the environment on drinking water quality. In particular, the project will evaluate the occurrence, fate, and transport of these chemicals from wastewater treatment plant discharges upstream of drinking water treatment plants.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Journal Articles – 3 Presentations – 9 Pereira, V.J.,K.G. Linden, and H.S. Weinberg. 2007. "Evaluation of UV irradiation for photolytic and oxidative degradation of pharmaceutical compounds in water." Water Research 41(19): 4413-4423.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Occurrence, Transport, and Fate of Pharmaceuticals and Other Emerging Contaminants Present in Wastewater	In progress	2001-2014	To determine which chemicals are present in wastewater effluent, and quantify their persistence downstream from wastewater treatment.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Glassmeyer, S.T., E.T. Furlong, D.W. Kolpin, J.D. Cahill, S.D. Zaugg, S.L. Werner, M.T. Meyer, and D.D. Kryak. 2005. "Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination." Environmental Science and Technology 39: 5157-5169. Kolpin D.W., E.M. Thurman, E.A. Lee, M.T. Meyer, E.T. Furlong, and S.T. Glassmeyer. 2006. "Urban contributions of glyphosate and its degradate AMPA to streams in the United States." Science of the Total Environment 354: 191-197.
National Screening Survey of EDCs, Including Some Pharmaceuticals in Municipal Wastewater Treatment Effluents	Completed	2002-2004	To assess whether certain types of domestic wastewater treatment plants or operations are more effective in removing endocrine disrupting chemicals (EDCs) and certain pharmaceuticals.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	Jim Lazorchak. "National Screening Survey of EDCs, including some Pharmaceuticals in Municipal Wastewater Treatment Effluents." EPA Report: EPA/600/R-04/171; EPA APM 201.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Utility of Pharmaceuticals and Other Emerging Contaminants as Chemical Indicators of Human Fecal Contamination	In progress	2002-2009	To determine if the chemicals present in wastewater effluent (including pharmaceuticals) can be used as indicators of human fecal contamination. Even if there is not sufficient evidence to conclude that a chemical can serve as an independent indicator, chemicals may be able to be used in conjunction with microbial indicators to establish the source of fecal pollution.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Monitoring and Detection Tools	Glassmeyer S.T., E.T. Furlong, D.W. Kolpin, J.D. Cahill, S.D. Zaugg, S.L. Werner, M.T. Meyer, and D.D. Kryak. 2005. "Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination." Environmental Science and Technology 39: 5157-5169.
In Vitro Screening of Environment Samples for Estrogenic and Androgenic Activity: Concentrated Animal Feedlot Operations (Beef, Dairy, Poultry).	In progress	2002-2014	To evaluate environmental samples for EDC activities in order to: 1. identify the potency of the samples; 2. attempt to identify specific chemicals in samples responsible for the EDC activity; and 3. try to determine the potential impacts of these chemicals on fish, wildlife and human health.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Conazoles: Application of Omic Technologies to Mode of Action Evaluations	In progress	2003-2014	To apply state-of-the-art molecular tools supported by traditional toxicological methods and develop mechanistic data for risk assessment on this class of pesticides. To examine interspecies relationships, and to develop a model approach for the use of toxicogenomic data in the evaluation of classes of environmental agents for risk assessment.	▪ Monitoring and Detection Tools	Sun, G., S.F. Thai, G.R. Lambert, D.C. Wolf, D.B. Tully, A.K. Goetz, M.H. George, R.D. Grindstaff, D.J. Dix, and S. Nesnow. 2006. "Fluconazole-induced hepatic cytochrome P450 gene expression and enzymatic activities in rats and mice." Toxicol Lett. 64(1): 44-53. Tully, D.B., W. Bao, A.K. Goetz, C.R. Blystone, H. Ren, J.E. Schmid, L.F. Strader, C.R. Wood, D.S. Best, M.G. Narotsky, D.C. Wolf, J.C. Rockett, and D.J. Dix. 2006. "Gene expression profiling in liver and testis of rats to characterize the toxicity of triazole fungicides." Toxicol Appl Pharmacol. 215(3):260-273.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Development of an Tier II Two-Generation Test Guideline for the Sheepshead Minnow	In progress	2004-2008	To develop a guideline for a fish Tier II Test that can be used to determine whether a chemical or chemical mixture adversely affects the organism through endocrine-mediated pathways, and to evaluate those effects with respect to the estrogen, androgen, and thyroid systems. The test includes exposure of multiple generations of the estuarine fish, sheepshead minnow, throughout all life stages	▪ Assessment of Potential Ecological Effects	A transferable guidance document for conducting a two-generation exposure of the estuarine sheepshead minnow will be produced. This guidance is provided to OPPTS/OSCP for Tier II testing of chemicals for endocrine disrupting activity. Document in preparation.
Toxicity Estimation Using the ICE and ACE Modeling Tools	In progress	2004-2008	The development Web-ICE and Web-ACE research includes: (1) expansion of ICE models to wildlife and additional aquatic species and chemicals, (2) rigorous QA/QC and bootstrap validation, (3) inclusion of mode of action (MOA), chemical class, and taxonomic distance	▪ Assessment of Potential Ecological Effects	Currently ICE is used by the Office Water in Biological Evaluations to ensure the protectiveness of national criteria to T&E species. Several technology transfer efforts have put ICE and ACE in the hands of Program Offices and the ecological risk community towards informing assessments in North America and Europe.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Toxicity Pathway-Specific Protein Expression Models for Chemical Screening and Prioritization	In progress	2004-2014	To identify mode of action (MOA)-specific protein biomarkers, develop and optimize exposure protocols and analytical techniques, determine cross-species conservation of such biomarkers, and evaluate the ability of experimentally derived MOA-specific models to accurately classify chemicals by MOA.	▪ Assessment of Potential Human Health Effects ▪ Assessment of Potential Ecological Effects ▪ Monitoring and Detection Tools	Walker, C.C., K.A. Salinas, P.S. Harris, S.S. Wilkinson, J.D. Watts, and M.J. Hemmer. 2007. "A Proteomic (SELDI-TOF-MS) Approach to Estrogen Agonist Screening." ToxSci Advance Access, DOI 10.1093/toxsci/kfl079 Toxicol. Sci. 95: 74-81.
A Systems Approach to Characterizing and Predicting Thyroid Toxicity Using an Amphibian Model	In progress	2004-2014	To develop a sufficient understanding of the amphibian HPT so that screening and testing protocols can be abbreviated, predictive models can be developed, and efforts in inter-species and inter-chemical extrapolation can be improved. As part of this broader research project we have tested several pharmaceuticals. Methimazole and PTU which have therapeutic uses in both human and animal medicine were used as model thyroid axis inhibitors because of their well established mode of action as T4 synthesis inhibitions.	▪ Assessment of Potential Ecological Effects	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Development and Evaluation of Long-term Testing Protocols for Assessing the Effects of EDCs and Reproductive Toxicants in Small Fish	In progress	2004-2014	To develop harmonized medaka multigeneration testing protocols that can be used to evaluate population-relevant endpoints (i.e., fecundity, fertility reproductive behavior, phenotypic and genotypic sex of each generation).	▪ Assessment of Potential Ecological Effects	
An Informatic Approach to Estimating Ecological Risks Posed by Pharmaceutical Use	In progress	2004-2014	To use informatic tools and approaches to answer the question: which drugs are likely to have detrimental effects at environmental concentrations? There are >2000 drugs approved for use in the US with little/no concentration or eco tox data for the vast majority. Chemical analysis and chronic eco toxicity testing are too expensive to perform on all of them. As a result, we need a rational way to 'triage' drugs for chemical monitoring, toxicity testing, and assessment of effectiveness of mitigation strategies.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Human Health Effects ▪ Assessment of Potential Ecological Effects	Presentations have been made to EPA program office – OW, Regional Workgroup on PiE, 3 professional meetings, SETAC Europe and two SETAC NA. The prioritized list pharmaceuticals that have been identified has be provide to USGS to include in their monitoring programs, to the National Risk Management Research Laboratory to include in their WWTP evaluations, and to a newly hired Analytical Chemist to develop water, sediment and tissue LC MS/MS methods.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Development of Analytical Methods for Studying the Environmental Fate of the Veterinary Drug Roxarsone: Part II - Determination of Volatile Arsenic in Chicken Litter Contaminated with Roxarsone.	In progress	2006-2014	To determine whether or not volatile species of arsenic are produced in chicken litter contaminated with Roxarsone. Arsine (AsH3) and methylated derivatives such as mono-, di- and tri-methylarsine are known to be highly toxic to mammals.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Monitoring and Detection Tools	Heithmar, E.M., G.M. Momplaisir, and C.G. Rosal. "Development of sensitive, reliable, and cost-effective elemental speciation methods to measure the toxic and mobile forms of inorganic contaminants in sediments." EPA Internal report EPA/600/X-06/014.
Development of Analytical Methods for Determining PPCPs in Biosolids, Effluent, and Sediment	In progress	2005-2008	To 1) characterize biosolids to understand the matrix, identify matrix components of concern, and characterize the affinity of the matrix for specific analytes; 2) develop methods for fluoroquinolone antibiotics; and 3) develop methods for other analytes that are currently of concern in a variety of matrices to quantify potential exposure.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	Mottaleb, M.A., W.C. Brumley, L.R. Curtis, and G.W. Sovocool. 2005. "Nitro Musks Adducts of Trout Hemoglobin: Dose-response and Toxicokinetics Determination by GC-NICI-MS for a Sentinel Species," Am. Biotechnol. Lab. 23(7): 24, 26-29.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
In Vitro Screening of Environment Samples for Estrogenic Activity: Treated sewage effluent and Global Water Research Coalition (GWRC)	In progress	2005-2014	Use in vitro methods to screen and evaluate environmental samples for EDC activities in order to 1. identify samples with estrogenic activity; 2. examine the potency of the estrogenic activity in the samples; and 3. potentially use the in vitro assays as indicators to aid in the identification of specific chemicals in effluent samples which are responsible for the EDC activity.	▪ Sources ▪ Monitoring and Detection Tools	
Linkage of Exposure and Effects using Genomics, Proteomics and Metabolomics in Small Fish Models	In progress	2005-2014	To utilize two small fish species, the zebrafish and fathead minnow, as a basis for the development of (a) mechanistic biomarkers, and (b) techniques for extrapolation of toxicological effects across endpoints, species and chemicals.	▪ Assessment of Potential Ecological Effects	
Development of Molecular Indicators of Exposures for Pharmaceuticals and EDCs	In progress	2005-2014	To identify molecular indicators (gene expression assays or protein makers) of exposure that can be used to assess existing or past exposures to pharmaceuticals and EDCs in field and laboratory settings.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Assessment of the Occurrence and Potential Risks of EDCs that are Pharmaceuticals or Natural in Discharges from Concentrated Animal Feeding Operations	In progress	2005-2014	To characterize the magnitude and extent of the impact of estrogenic and androgenic hormones and pharmaceuticals in waste from CAFOS and determine the impact of current CAFO waste management strategies on the fate and effects of hormones and pharmaceuticals.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects ▪ Monitoring and Detection Tools	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R832737: Development of Receptor- to Population-Level Analytical Tools for Assessing Endocrine Disruptor Exposure in Wastewater-Impacted Estuarine Systems	In progress	2005-2008	To (1) develop nuclear hormone receptor-affinity extraction techniques as tools for isolating (EDCs) from complex wastewater mixtures; (2) apply these methods in combination with high performance mass spectrometry for activity-directed analysis of EDCs in wastewater and estuarine receiving waters on the SC coast; (3) utilize sensitive vertebrate (zebrafish) and invertebrate (copepod) EDC-exposure laboratory bioassays to link exposure measurements (above) to biological effects; and (4) apply novel biomolecular endpoints to assess EDC exposure in field populations of sensitive meiobenthic invertebrates in wastewater-impacted estuarine environments.	▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects ▪ Monitoring and Detection Tools	Ferguson, P.L., L.C. Kimberley, B.P. Pritchard, and J.C. Clark. 2007. "Novel approaches for mass spectrometric analysis of endocrine-disrupting compounds in the aquatic environment." Presented at the 234th American Chemical Society National Meeting, Boston, MA, August 19-23, 2007.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Cooperative Agreement R832741: Developing Rapid Assessment Tools to Evaluate the Biological Effects of Complex and Biologically Active Chemical Mixture	In progress	2005-2008	To: (1) determine whether synergist interactions increase the potency of endocrine disrupting chemicals in mixture as has been suggested in recent studies; (2) provide improved risk assessment by providing a broad range of reproductive endpoints for mixture exposed aquatic organisms; (3) provide biochemical endpoints that can serve as rapid assessment tools to help regulators determine potential reproductive health consequences for exposed aquatic organisms.	▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects ▪ Monitoring and Detection Tools	Presentations - 5 Working Group/Panel – 1
STAR Grant R832738: Rapid Detection of Trace Endocrine Disrupting Chemicals in Complex Mixtures: A Full-Spectrum Deconvolution Technique with a UV-Transparent Passive Concentrator	In progress	2006-2009	To develop a method for rapid monitoring and detection of endocrine disrupting chemicals at trace concentrations in natural waters and test the method using samples of river water collected from sites where a USGS survey has previously detected multiple EDCs.	▪ Exposure Pathway: Ecological ▪ Monitoring and Detection Tools	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Drug Disposal: Ramifications for the Environment and Human Health	Completed	2006-2008	To (i) define the scope of the scientific issues surrounding the disposal of pharmaceuticals to the environment; (ii) identify all sources and routes leading to drug accumulation and disposal; (iii) identify and quantify all the drug active ingredients accumulated by a particular sub-population in a defined locale; (iv) using these collected data and pharmacokinetic data, predict which drugs hold the greatest potential for disposal and entry to the environment; (v) compare these findings with actual field monitoring data published in the literature; (vi) make recommendations regarding those changes in health care practices that would lessen the accumulation of drugs leading to disposal.	▪ Environmental Stewardship Public Communication	Ruhoy, I.S., and C.G. Daughton. 2007. "Types and Quantities of Leftover Drugs Entering the Environment via Disposal to Sewage - Revealed by Coroner Records." Sci. Total Environ. 388(1-3): 137-148. Ruhoy, I.S., and C.G. Daughton. 2007. "Beyond the Medicine Cabinet: An Analysis of Where and Why Medications Accumulate." Sci. Total Environ. Manuscript in Preparation Ruhoy, I.S. 2008. "Examining Unused Pharmaceuticals in the Environment," Doctoral Dissertation, University of Nevada, Las Vegas, Department of Environmental Studies. In Preparation. Glassmeyer ST, Hinchey EK, Boehme SE, Daughton CG, Ruhoy IS, Conerly O, Daniels RL, Lauer L, McCarthy M, Nettesheim TG, Sykes K, and Thompson VG. "Disposal Practices for Unwanted Residential Medications in the United States," Environment International, submitted, April 2008.

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Drug Disposal: Ramifications for the Environment and Human Health (cont.)					Daughton, C.G. and Ruhoy, I.S. "The Afterlife of Drugs and the Role of PharmEcovigilance," Drug Safety, submitted, April 2008. Ruhoy, I.S., and C.G. Daughton. 2007. "Pharmaceutical Disposal and the Environment." U.S. EPA, Las Vegas, NV. Illustrated poster, October 2007. Ruhoy, I.S., and C.G. Daughton. 2007. "Disposal as a Source of Pharmaceuticals in the Environment." U.S. EPA, Las Vegas, NV. Illustrated poster, October 2007. Daughton, C.G. "Pharmaceuticals in the Environment: Sources and Their Management," Chapter 1, 1-58, *In* Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle (M. Petrovic and D. Barcelo, Eds.), Wilson & Wilson's Comprehensive Analytical Chemistry series (D. Barcelo, Ed.), Volume 50, Elsevier Science; 2007, 564pp; available: http://www.epa.gov/nerlesd1/bios/daughton/Chap1_Petrovic&Barcelo.pdf

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Persistence of Contaminants from Wastewater Discharges During Drinking Water Treatment: Identification of Compounds and Degradation/ Disinfection Byproducts, Evaluation of Removal, and Potential Exposure	In progress	2006-2014	To determine the effect of different drinking water treatment technologies on compound removal by examining the occurrence and fate of pharmaceuticals and other wastewater derived compounds through drinking water treatment.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Human ▪ Assessment of Potential Human Health Effects ▪ Environmental Stewardship Public Communication ▪ Monitoring and Detection Tools	
Regional Methods Initiative: Examination of Enzyme-Linked Immunosorbent Assay (ELISA) for Endocrine Disrupting Compounds (EDCs), Estrogen and Alkyl Phenol Ethoxylates	In progress	2006-2014	To evaluate the reliability and reproducibility of this ELISA technique to analyze environmental samples in different matrices for EDCs.	▪ Monitoring and Detection Tools	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Report on the Transport and Fate of Selected EDCs During the Land Application of Biosolids	In progress	2006-2014	To determine the major sources and environmental fates of EDCs natural and/or pharmaceutical additives, to evaluate existing risk management strategies for the disposal of biosolids, and begin to evaluate alternative risk management options.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Monitoring and Detection Tools	
Fate of Selected EDCs Under Redox Conditions Typical of Wastewater and Sediments	In progress	2006-2014	To characterize the degradability and rates of degradation of selected natural and pharmaceutical hormones and alkylphenols under redox conditions typical of wastewater treatment, receiving waters, and sediments.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Monitoring and Detection Tools	
Natural and Synthetic EDCs from Wastewater Treatment– Source Characterization, Environmental Fate, and Risk Management	In progress	2006-2014	To determine the efficacy of existing risk management approaches to minimize exposure to suspected EDCs and develop new risk management tools where needed.	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Monitoring and Detection Tools	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Analytical Methods Development for 65 Ecologically Relevant Pharmaceuticals and Metabolites	In progress	2006-2014	To develop the analytical methods to detect 65 ecologically relevant pharmaceuticals and metabolites in water, sediment, and tissue for use in assessing laboratory and environmental exposures	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	
Identification of Ecological and Molecular Indicator Responses to Pharmaceuticals Under Simulated Field Conditions at the EPA Experimental Stream Facility	In progress	2006-2014	To identify community structural and functional responses of macroinvertebrate, periphyton and fish and a select number or molecular markers (gene expression assays or protein markers) to environmental relevant concentrations of pharmaceuticals and EDCs at EPA's Experimental Stream Facility (ESF).	▪ Sources ▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
Hydrolytic Transformation of Emerging Contaminants	In progress	2007-2009	To assess the fate of emerging contaminants, with emphasis on pharmaceuticals and their abiotic hydrolytic transformations, at environmentally relevant conditions.	▪ Intro to Environment, Fate and Transport ▪ Exposure Pathway: Ecological ▪ Assessment of Potential Ecological Effects	
STAR Grant R833417: Fate of Hormones in Tile-Drained Fields and Impact to Aquatic Organisms Under Different Animal Waste Land-Application Practices	In progress	2007-2010	To quantify the hormone load in manure and lagoon effluents from tile-drained fields, evaluate hormone persistence in fields after manure application, assess the effects of the discharged hormones on aquatic organisms, and determine the impacts of different types of manure management practices on hormone discharge.	▪ Intro to Environment, Fate and Transport	
STAR Grant R833418: Transport/Fate/ Ecological Effects of Steroids from Poultry Litter & Evaluations of Existing/Novel Management Strategies	In progress	2007-2010	To determine the abundance and fate of steroids associated with poultry litter and their impacts on ecological systems, and to evaluate current management practices in this context.	▪ Intro to Environment, Fate and Transport	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R833421: Assessing Occurrence, Persistence, and Biological Effects of Hormones Released from Livestock Waste	In progress	2007-2010	To (1) characterize the environmental transport and fate of natural and synthetic steroid hormones that accompany discharges and the disposal of animal wastes from CAFOs in Wisconsin; (2) evaluate how various animal waste handling/management strategies (e.g., lagoon storage and spraying of liquid manure vs. deep-stacking and field application of solid manure) impact the transport, fate, potential exposure, and associated effects of steroid hormones discharged from CAFOs; and (3) investigate the ecological effects associated with steroid hormones in animal waste from CAFOs.	▪ Intro to Environment, Fate and Transport	

Project Title	Status	Active Dates	Purpose	Research Category	Products (partial list)
STAR Grant R833422: Transport and Transformation of Natural and Synthetic Steroid Hormones at Beef Cattle and Dairy Concentrated Animal Feeding Operations (CAFOs)	In progress	2007-2010	This project will assess the occurrence, fate, and transport of synthetic steroid hormones used for beef cattle production and endogenous steroids produced by cattle and cows from concentrated animal feeding operations (CAFOs). We hypothesize that the most important pathways for steroid releases from CAFOs are the discharge of contaminated stormwater runoff and migration in groundwater recharged through animal waste lagoons and animal feeding areas.	▪ Intro to Environment, Fate and Transport	
STAR Grant R833423: Effects of Cattle Manure Handling and Management Strategies on Fate and Transport of Hormones in the Feedlot and the Field	In progress	2007-2010	To (1) quantify hormones in various stages of the manure pathway in cattle feedlots; (2) determine the effects of different handling practices of cattle feedlot wastes on the stability and availability of hormones; (3) determine the effects of different land application strategies on the fate and transport of hormones used in beef cattle production; and (4) determine if grasses from conservation buffers assimilate hormones.	▪ Intro to Environment, Fate and Transport	

Attachment B

Collapse of a fish population after exposure to a synthetic estrogen

Karen A. Kidd*†, Paul J. Blanchfield*, Kenneth H. Mills*, Vince P. Palace*, Robert E. Evans*, James M. Lazorchak‡, and Robert W. Flick‡

*Fisheries and Oceans Canada, Freshwater Institute, 501 University Crescent, Winnipeg, Manitoba, Canada R3T 2N6; and ‡Molecular Indicators Research Branch, United States Environmental Protection Agency, 26 West Martin Luther King Drive, Cincinnati, OH 45268

Edited by Deborah Swackhamer, University of Minnesota, Minneapolis, MN, and accepted by the Editorial Board March 29, 2007 (received for review October 27, 2006)

Municipal wastewaters are a complex mixture containing estrogens and estrogen mimics that are known to affect the reproductive health of wild fishes. Male fishes downstream of some wastewater outfalls produce vitellogenin (VTG) (a protein normally synthesized by females during oocyte maturation) and early-stage eggs in their testes, and this feminization has been attributed to the presence of estrogenic substances such as natural estrogens [estrone or 17β-estradiol (E2)], the synthetic estrogen used in birth-control pills [17α-ethynylestradiol (EE2)], or weaker estrogen mimics such as nonylphenol in the water. Despite widespread evidence that male fishes are being feminized, it is not known whether these low-level, chronic exposures adversely impact the sustainability of wild populations. We conducted a 7-year, whole-lake experiment at the Experimental Lakes Area (ELA) in northwestern Ontario, Canada, and showed that chronic exposure of fathead minnow (*Pimephales promelas*) to low concentrations (5–6 ng·L^{-1}) of the potent 17α-ethynylestradiol led to feminization of males through the production of vitellogenin mRNA and protein, impacts on gonadal development as evidenced by intersex in males and altered oogenesis in females, and, ultimately, a near extinction of this species from the lake. Our observations demonstrate that the concentrations of estrogens and their mimics observed in freshwaters can impact the sustainability of wild fish populations.

endocrine disrupters | fathead minnow | municipal wastewaters | population-level effects | whole-lake experiment

There is considerable evidence that fishes inhabiting waters that receive untreated municipal wastewaters or effluents from municipal wastewater treatment plants (MWTPs) are exposed to chemicals that affect reproductive endocrine function. Male fish downstream of some wastewater outfalls produce vitellogenin (VTG) mRNA and protein, associated with oocyte maturation in females, and early-stage eggs in their testes (1–3). This feminization has been linked to the presence of estrogenic substances such as the natural estrogen 17β-estradiol (E2) and the synthetic estrogen 17α-ethynylestradiol (EE2) (4). Natural and synthetic estrogens are not completely broken down in current MWTP processes (5), and, as a result, are discharged into receiving waters in both treated and untreated wastewaters and found in the aquatic environment at low parts per trillion concentrations (typically <5 ng·L^{-1}; refs. 6–8). These effluents contain mixtures of individual estrogens and their mimics that differ in their ability to elicit estrogenic responses (e.g., ref. 9). For this reason, total estrogenicity (expressed as E2 equivalents) of an effluent or water sample is determined either by summing concentrations of individual compounds after adjusting these concentrations by the compound's estrogenic potency (relative to E2; ref. 10) or with bioassays (e.g., refs. 10–12). Using these approaches, total E2 equivalents of up to 147 and 17 ng·L^{-1} have been measured in final effluents and surface waters, respectively (10–12). Within this group of substances, the estrogen used in birth-control pills, EE2, is one of the more potent estrogens present (9) and has been linked to the feminization of male fishes in rivers receiving municipal wastewater (4, 6).

Despite growing documentation on the feminization of male fishes in waterways receiving municipal effluents, a critical question in the field of endocrine disruption research is whether these low-level, chronic exposures adversely impact wild populations (13). Although laboratory studies have shown decreased reproductive success of fish exposed to <1–5 ng·L^{-1} of EE2 (14, 15), it is unknown whether this response would be observed in wild populations and whether it would result in a subsequent decline in abundances. To assess the ecological risk posed by this class of compounds, we must understand population-level effects of estrogens and their mimics on aquatic organisms.

The fathead minnow (*Pimephales promelas*) is a common species in North America, and its range extends from the southern United States to northern Canada (16). It is an important food source for numerous game fish species, such as lake trout (*Salvelinus namaycush*), walleye (*Sander vitreus*), and northern pike (*Esox lucius*). In the lakes used for this study, fathead minnow have a lifespan of ≈4 years, but few individuals live past 2 years of age (17). Asynchronous spawning starts in early summer and extends for a period of ≈2 months; multiple females will typically spawn in the nest of a single male, who will care for developing eggs until hatching. Sexual maturity occurs during the second year of life, so most fish spawn for only one season. Therefore, 2 consecutive years of reproductive failure cause catastrophic declines in abundance (18, 19). Fathead minnow have been widely adopted by the scientific community for toxicity testing because of their widespread distribution, ease of laboratory culture, well characterized biology and life history, and the large family (Cyprinidae) of fishes that they represent (20–22). It is a freshwater equivalent of the "miner's canary."

We describe the results of a 7-year, whole-lake study at the Experimental Lakes Area (ELA) in northwestern Ontario, Canada, to assess the subcellular-level through population-level effects of the potent synthetic estrogen EE2 on fathead minnow. The concentrations of EE2 achieved in the experimental lake, Lake 260, during the 3 years of additions were within the range of those observed in untreated and treated municipal wastewaters (5, 6) and below the total E2 equivalents (with EE2 having

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Abbreviations: CPUE, catch-per-unit-effort; E2, 17β-estradiol; EE2, 17α-ethynylestradiol; VTG, vitellogenin.

†To whom correspondence should be sent at the present address: Canadian Rivers Institute and Biology Department, University of New Brunswick, Saint John, New Brunswick, Canada E2E 4P1. E-mail: kidck@unbsj.ca.

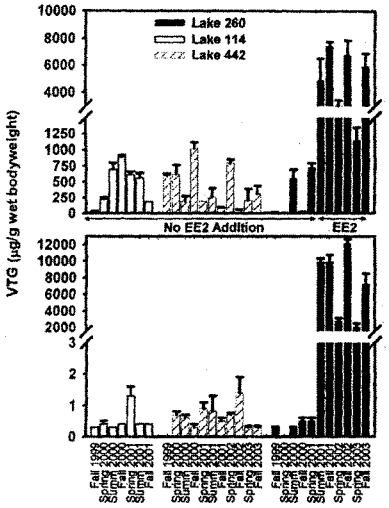

Fig. 1. Mean ± SE (n = 4–7) VTG concentrations in whole-body homogenates of male (*Lower*) and female (*Upper*) fathead minnow captured in 1999–2003 from reference Lakes 114 and 442 and from Lake 260 before and during additions of 5–6 ng·L^{-1} of EE2 (low catches of fish in Lake 260 in 2004 and 2005 did not allow for these analyses in the latter 2 years of the study).

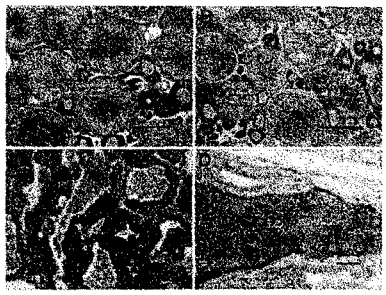

Fig. 2. Gonadal tissue sections from fathead minnow sampled in early May 2003. (*A*) Ovary from reference Lake 442 with small, dark, primary-stage oocytes situated between cortical alveolar-stage oocytes and large vitellogenic oocytes. (*B*) Ovary from EE2-amended Lake 260 demonstrating an atretic follicle (arrow). (*C*) Testis from reference Lake 442. (*D*) Testis from EE2-amended Lake 260 demonstrating intersex; arrows indicate primary-stage oocytes among the remnants of testicular tissue. (Scale bars: *A*, *B*, 300 μm; *C*, *D*, 80 μm.)

an E2 equivalent of 0.19–1.9; ref. 23) measured in effluents and receiving waters (10–12).

Results and Discussion

Measures of VTG mRNA and protein are used to assess exposure of fishes to estrogens and estrogen mimics, and elevated concentrations in males are indicative of the presence of these substances in effluents or surface waters (1, 3). We used these endpoints to assess the subcellular responses of fathead minnow from Lake 260 to EE2 additions. Seven weeks after the first estrogen additions to Lake 260 began in 2001, VTG concentrations in male and female fathead minnow were elevated when compared with preaddition or reference lake data (Fig. 1). Whole-body homogenates of males from Lake 260 had concentrations of VTG that were three orders of magnitude greater than reference samples, and this response was sustained in each of the 3 years of EE2 additions. Female fathead minnow also produced more VTG after exposure to EE2 when compared with reference samples (reference lake fish had 2.5% of the VTG concentrations observed in treated fish from Lake 260; Fig. 1), although their response was less dramatic than that observed for male fish. Liver VTG mRNA was measured in samples collected from the EE2-amended and reference lakes concurrently with those used for VTG protein analyses. Results from these mRNA analyses showed patterns similar to the VTG protein expression. Over the 3 years of dosing, male fathead minnow from Lake 260 had mean ± SD normalized liver VTG mRNA values ranging from 0.422 ± 0.685 to 1.22 ± 0.181. Liver VTG mRNA values for males from the two reference lakes were on average <0.1–1.6% of those for the EE2-exposed males. In addition, males from Lake 260 had VTG mRNA values (0.904 ± 0.437) that were more than an order of magnitude higher than female fish (0.045 ± 0.048) collected from the reference lakes during the same time periods. Females collected from Lake 260 in the spring and mid-summer of each year of EE2 additions had gene expression that ranged from 0.834 ± 0.879 to 0.378 ± 0.053, and this response was 82–152% of the values in females from the reference lakes. These among-lake differences were highest in the fall; for example, females collected in 2002 from Lake 260 exhibited levels of VTG mRNA expression (0.849 ± 0.793) that were more than an order of magnitude higher than those of reference females (0.045 ± 0.048). This trend was also observed in the accompanying protein data, demonstrating that EE2 amendments prolonged the expression of VTG mRNA and protein in males and females well past the breeding season.

Wild fishes exposed to municipal wastewater effluents and fishes exposed to estrogens in laboratory studies have shown disruption in gonadal development as evidenced by the presence of both male and female gonadal tissues (i.e., intersex) and decreased gonadosomatic indices (GSIs) (2, 14, 15). In this study, testicular tissues of all of the male fathead minnow collected during the first spring after EE2 additions displayed delayed spermatogenesis, widespread fibrosis, and malformations of the tubules (see ref. 24). Testicular germinal tissue from all EE2-exposed males consisted primarily of spermatogonia instead of the spermatocytes that were normally observed at this time of year in the reference lakes and in Lake 260 before EE2 additions. In the spring of 2002, the GSI for males from Lake 260 averaged 0.40 ± 0.21% (n = 10). This mean was well below the GSI values of 1.39 ± 0.38% (n = 15) and 2.27 ± 0.41% (n = 10) recorded for males from reference Lakes 114 and 442, respectively. This arrested testicular development continued in 2003 and 2004, and four of nine males captured in the spring of 2003 had ova-testes with the presence of primary-stage oocytes (Fig. 2). It has been shown previously that male fish with intersex have reduced fertilization success because of decreased sperm production and mobility (25).

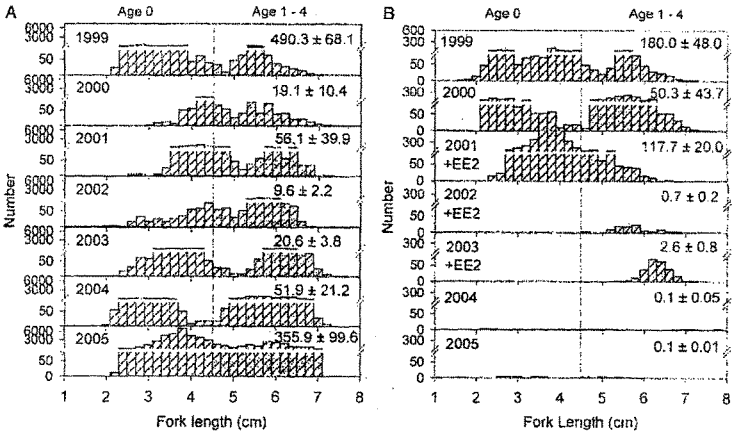

Fig. 3. Length frequency distributions of fathead minnow captured in trap nets in reference Lake 442 (*A*) and Lake 260 (*B*) (amended with 5–6 ng·L^{-1} of EE2 in 2001–2003) during the fall of 1999–2005. Distributions for each fall have been standardized to 100 trap-net days. Mean ± SE daily trap-net CPUE data for adults and juveniles for the fall catches are shown in the panels.

High VTG production in female fathead minnow from Lake 260 was followed by delayed ovarian development in individuals collected the next spring (Fig. 2) (ref. 24), which is consistent with observations for the other dominant minnow species in this lake, the pearl dace (*Margariscus margarita*) (26). Oocytes in female fathead minnow collected from Lake 260 the first spring after EE2 amendments began were at a much earlier stage of development (cortical alveolar versus vitellogenic) than those from fathead minnow in nearby pristine lakes or in Lake 260 before EE2 additions. However, delayed oocyte maturation was not evident in ensuing spring samples nor were there any consistent effects on gonadosomatic indices during the 3 years of EE2 amendments (data not shown). Each year subsequent to 2002 there was an increasing number of fish ovaries that contained a few atretic follicles, which were rarely observed in reference and preaddition fish (Fig. 2).

Index trap netting [adults and young-of-the-year (YOY)] and minnow traps (adults) were used to examine changes in the fathead minnow population size and structure in the fall during each of the 7 years in both Lake 260 and Lake 442 (Fig. 3). Trap-net catch-per-unit-effort (CPUE) data showed that the mean abundance of this species varied considerably (9.6 ± 2.2 to 490 ± 68) in reference Lake 442 from 1999 to 2005 (Fig. 3*A*). In contrast, the CPUE of fathead minnow decreased consistently after EE2 additions to Lake 260 from 180 ± 48 and 50 ± 43 (before additions) to 0.1 ± 0.01 in the fifth year after additions began (Fig. 3*B*). Lake 260 and Lake 442 fathead minnow CPUE data derived from fall catches using minnow traps (data not shown) showed the same trends as the trap-net data and, overall, the two methods were highly comparable (Spearman rank correlation: $t_{12} = 6.45$, $R = 0.88$, $P < 0.0001$). Although fathead minnow populations typically fluctuate in size from year to year because of variable recruitment, year class failures are not observed in unmanipulated lakes (17, 27).

The fathead minnow population in Lake 260 collapsed in the fall of 2002, after the second season of EE2 additions, because of a loss of young-of-the-year (Fig. 3*B*). This reproductive failure was also observed in the third season of amendments and continued for an additional 2 years after the EE2 additions had ceased, although a few small individuals were caught each year, indicating some reproduction was occurring. The loss of smaller size classes of fathead minnow was not observed in reference Lake 442 (Fig. 3*A*), a system with similar species composition, water volume, and trophic status (24, 28). In addition, the mean ± SE size of adult fish from Lake 260 increased from 50 ± 0.28 mm ($n = 300$) in 2001, just after the first EE2 additions, to 62 ± 0.18 mm ($n = 263$) in the fall of 2003, because of a shift in the age structure of the remaining population. Population-level effects documented in this study mimicked what was observed during a long-term acidification study at the Experimental Lakes Area (18). At lower pH, there was near total extinction of fathead minnow and an increase in the median size of the remaining adult population (19). The reproductive failure and near extirpation of fathead minnow in Lake 260 has not yet been observed in the longer-lived pearl dace in this system (26), and this suggests that life-history characteristics such as lifespan are important determinants of a species' risk from exposure to estrogens and estrogen mimics.

The results from this whole-lake experiment demonstrate that continued inputs of natural and synthetic estrogens and estrogen mimics to the aquatic environment in municipal wastewaters could decrease the reproductive success and sustainability of fish populations. Chronic exposure of fathead minnow to a potent synthetic estrogen led to feminization of males through production of VTG mRNA and protein, continued production of VTG

in females beyond the normal breeding season, impacts on gonadal development, as evidenced by intersex in males and altered oogenesis in females, and a near extinction of this species from the lake. Likely because of its short life-cycle, the fathead minnow was the first to show population collapse in this experiment. This response implies that short-lived fish species may generally be at greatest risk from exposure to estrogens and their mimics, but chronic exposure of longer-lived species to these substances may result in the loss of these populations as well.

Materials and Methods

The experimental system, Lake 260, is located in an undisturbed watershed at the Experimental Lakes Area, has a surface area of 34 ha and a maximum depth of 14 m, and contains naturally reproducing populations of lake trout, white sucker (*Catostomus commersoni*), pearl dace, and fathead minnow, as well as several other small-bodied fish species. We studied Lake 260 and two nearby reference systems, Lakes 114 and 442, for 2 years before EE2 additions, 3 years during the amendments to the experimental lake, and 2 years after the additions were discontinued. The physical, chemical, and biological characteristics of Lake 260 and the reference lakes are detailed in refs. 24 and 28.

We added EE2 (Schering AG, Berlin, Germany) three times weekly to Lake 260 starting in May 2001, after the lake had stratified, and additions were continuous during the open-water seasons of 2001–2003 for a period of 20–21 weeks. For each EE2 addition, the required mass [calculated by using its predetermined half-life in the water column (K.A.K., unpublished data), the volume of the epilimnion, and the mean concentration of EE2 measured the previous week] was dissolved in 30 ml of DIG-grade methanol (Caledon Laboratories, Georgetown, ON, Canada) and added to the propeller wash of the boat as it was driven in transects across the lake. Mean ± SD epilimnetic concentrations of EE2 were quantified weekly by using replicate samples collected at each of five sites around the lake. EE2 was extracted onto preconditioned C-18 columns (Supelco, Oakville, ON, Canada), eluted with 100% DIG methanol, and quantified along with the internal standard testosterone by using standard RIA techniques and GC-MS confirmation for 10% of the samples. Additional details on extraction methods and EE2 concentrations have been described in refs. 24 and 28. Seasonal mean ± SD EE2 concentrations in Lake 260 were 6.1 ± 2.8 (n = 189), 5.0 ± 1.8 (n = 169), and 4.8 ± 1.0 (n = 170) ng·L^{-1} in 2001–2003, respectively.

For VTG mRNA and protein analyses, fathead minnow were collected by using live-minnow traps in the spring and fall of 1999–2003 and in the summer of 2000 and 2001 from Lake 260 and two reference lakes (n = 4–10 individuals per sampling date for each analysis). Each fish was handled according to Canadian Animal Care protocols (approved by the Freshwater Institute Animal Care Committee) and individually euthanized in pH-buffered tricaine methanesulfonate (250 mg·L^{-1}). For VTG analyses, whole fish were placed in sterile plastic bags, immediately frozen on dry ice, and kept at −90°C until analyzed. Whole-body homogenates were used to quantify the VTG by using the indirect competitive ELISA methods detailed previously (24). Reagent VTG from fathead minnow was obtained from the Core Biomarker Facility (University of Florida, Gainesville, FL). The primary antibody used for the analysis was a mouse anti-carp VTG monoclonal antibody (ND-2D3; Biosense Laboratories, Bergen, Norway) raised against VTG from common carp (*Cyprinus carpio*). Fish captured for VTG mRNA analysis were euthanized and dissected immediately after capture, and their livers were placed in RNA*later* (Ambion, Austin, TX) for transport to the laboratory. Liver VTG mRNA was measured by using quantitative real-time PCR and normalized to 18S ribosomal gene expression, an invariant ribosomal gene, by using methods detailed previously (29).

Impacts on fathead minnow gonadal development in Lake 260 were best examined in the spring of each year when gonads were mature and not affected by the asynchronous spawning season. Fish were collected from Lake 260 and the two reference lakes each spring from 1999 to 2003 as described above; whole fish were euthanized, immersed in Bouin's solution, and processed by using standard histological techniques as described previously (30).

Index trap netting (adults and young-of-the-year) and minnow traps (adults only) were used to examine changes in the fathead minnow population size and structure in the fall during each of the 7 years in both Lake 260 and reference Lake 442. Each September, at least three trap nets were set in each lake and fished from 7 to 33 consecutive days; nets were emptied every 2–3 days. Lake 260 and Lake 442 CPUE data (not shown) were also derived from fall minnow trapping (30 traps set per lake for 10–11 consecutive days and emptied daily) and compared with CPUE data from fall trap netting (shown in Fig. 3).

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Attachment B

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ATTACHMENT C

Targeted National Sewage Sludge Survey: Study Objective and Design

1. Study Objective

The objective of the 2006-2007 Targeted National Sewage Sludge Survey (TNSSS) is to obtain national estimates of percentiles of concentrations of selected pollutants in sewage sludge. EPA selected 80 publicly owned treatment works (POTWs) in 37 States to be sampled, using a stratified random sampling design.

1.2 Target Population

EPA defined the target population for the 2006-2007 TNSSS as POTWs that met the following criteria:

- Existed in 2002 or 2004
- With flow rates greater than or equal to 1 million gallons per day (MGD)
- Employ at least secondary treatment
- Produce a final treated biosolids product
- Are not known to employ a pond or lagoon as the final stage of treatment, and
- Located in the contiguous United States.

EPA included the limitation regarding lagoons or ponds because materials collected in lagoons continue to undergo physical and chemical changes and do not represent a "final product" until they are removed from the lagoon. EPA chose to control sampling costs through the geographic limitation.

Beginning with a list of 16,255 POTWs, EPA narrowed the list to 3,337 POTWs that meet the definition of the target population above. EPA selected a national sample of 80 POTWs from that list of 3,337 facilities in the target population.

1.3 Stratification

EPA selected POTWs using a random sampling design stratified for flow. EPA divided the 3,337 facilities into three categories, or strata, based on their design flow:

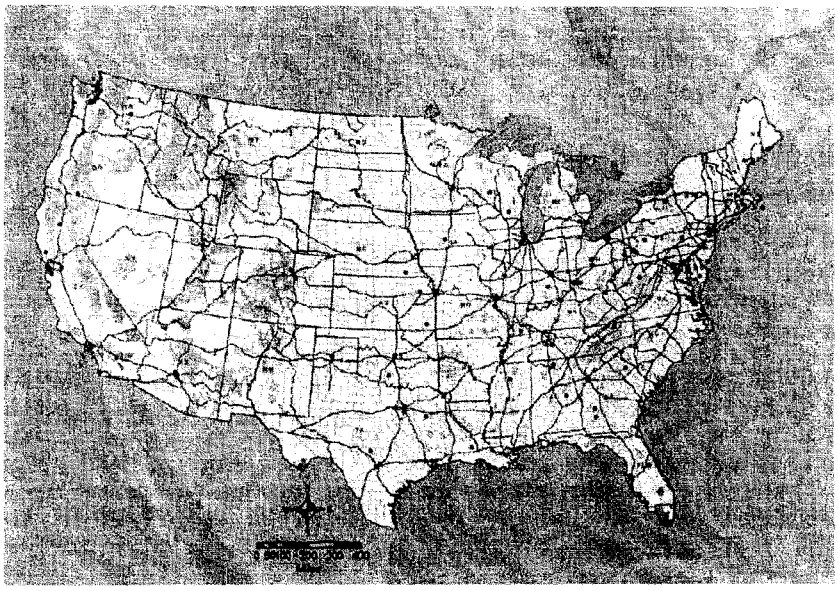
- 1 to 10 MGD, representing approximately 75% of the POTWs nationwide
- 10 to 100 MGD, representing approximately 15% of the POTWs nationwide
- >100 MGD, representing approximately 10% of the POTWs nationwide

After EPA determined the total number of facilities to be included in the study, it selected a proportionate number of POTWs from each stratum at random.

1.4 Final Selection

Figure 2 presents a map of the contiguous United States showing the approximate locations of the 80 POTWs original randomly selected for this survey. The purpose of this figure is to illustrate the national scope of the survey. It does not indicate locations of specific wastewater discharges.

Figure 2. Geographic Distribution of 80 POTWs Originally Selected for Sampling



EPA sent each facility a formal written invitation, which was followed by a telephone call from either EPA or CSC. The invitation outlined the nature of the study, the analytes of interest, the timeframe for completion, and assured the facilities that all samples would be submitted for analyses "blind" (i.e., the results from a given sample could not be associated with a particular facility).

These initial telephone contacts identified a small number of facilities that provided only partial treatment or employed a lagoon. Initially, EPA dropped such facilities from consideration. However, as the list of potential problem facilities grew, EPA worked to find suitable replacement facilities.

In partial treatment situations, EPA used the POTW receiving the partially treated material as the replacement, if possible. One facility with a lagoon provided contact information for a nearby facility that did not use a lagoon and that facility was willing to

participate. In two other instances of facilities with lagoons, EPA obtained samples during routine dredging operations to remove the final sludge product and send it to disposal. Those two instances required EPA to rapidly mobilize sampling personnel to collect samples in a narrow timeframe. As one facility noted, the alternative would be to wait 5 years until the next dredging operation.

The result of these adjustments was that EPA contacted 85 POTWs and conducted sampling at 74 of them. Table 2 includes all 85 facilities. Those that were dropped or replaced are listed at the end of the table, with a brief explanation of the rationale.

Table 2. Original and Replacement POTWs Selected for Sampling

Facility Name	City	ST	Status
Sugar Creek WWTP	Alexander City	AL	Replacement for Coley Creek
Aldridge Creek WWTP	Huntsville	AL	Original selection
Phoenix WWTP	Phoenix	AZ	Original selection
Valley Sanitary District STP	Indio	CA	Original selection
San Francisco	San Francisco	CA	Original selection
El Estero WWTP	Santa Barbara	CA	Original selection
Santa Rosa	Santa Rosa	CA	Original selection
Stockton Water Quality Plant	Stockton	CA	Original selection
Los Angeles County Sanitation District	Whittier	CA	Original selection
Boulder WWTP	Boulder	CO	Original selection
South Windsor	South Windsor	CT	Original selection
Three Oaks WWTF	Estero	FL	Original selection
Orange County Northwest WRF	Orlando	FL	Original selection
Tampa	Tampa	FL	Original selection
Albany	Albany	GA	Original selection
Americus-Mill Creek	Americus	GA	Original selection
Boone STP	Boone	IA	Original selection
Calumet Water Reclamation Plant	Chicago	IL	Replacement for North Side WRP
Plainfield WWTP	Plainfield	IL	Original selection
Lake County DPW, New Century STP	Vernon Hills	IL	Original selection
Dupage County-Knollwood STP	Wheaton	IL	Original selection
Blucher Poole WWTP	Bloomington	IN	Original selection
William Ross Edwin WWTP	Richmond	IN	Original selection
Parsons	Parsons	KS	Original selection
Topeka	Topeka	KS	Original selection
Mayfield WWTP	Mayfield	KY	Original selection
Eunice	Eunice	LA	Original selection
Jefferson Parish East Bank WWTP	Marrero	LA	Original selection
Nantucket	Nantucket	MA	Original selection
Salisbury	Salisbury	MD	Original selection
Mechanic Falls Treatment Plant	Mechanic Falls	ME	Original selection
Benton Harbor-St. Joseph WWTP	St. Joseph	MI	Original selection
Wixom WTP	Wixom	MI	Original selection
Festus Crystal City STP	Crystal City	MO	Original selection
Elizabeth City WWTP	Elizabeth City	NC	Original selection
Hillsborough WWTP	Hillsborough	NC	Original selection
Beatrice	Beatrice	NE	Original selection
Wildwood Lower WTF	Cape May Court House	NJ	Original selection
Middlesex County Utility Authority WRC	Sayreville	NJ	Original selection
Verona TWP DPW	Verona	NJ	Original selection
Buffalo	Buffalo	NY	Original selection
Canajoharie WWTP	Canajoharie	NY	Original selection
Geneva A-C Marsh Creek STP	Geneva	NY	Original selection
NYC DEP - Jamaica WPCP	New York City	NY	Original selection
North Tonawanda STP	North Tonawanda	NY	Original selection
Clermont County Commissioners	Batavia	OH	Original selection
Bedford	Bedford	OH	Original selection
Metropolitan Sewer District Little Miami WWTP	Cincinnati	OH	Original selection
Northeast Ohio Regional S D Southerly WWTP	Cleveland	OH	Replacement for Easterly
Delaware County Alum Creek WWTP	Delaware	OH	Original selection
Mingo Junction STP	Mingo Junction	OH	Original selection
Duncan Public Utilities Authority	Duncan	OK	Original selection
City of Klamath Falls WWTF	Klamath Falls	OR	Replacement for South Suburban
Western Westmoreland Municipal Authority	Irwin	PA	Original selection
Allegheny County sanitary Authority	Pittsburgh	PA	Original selection
Greater Pottsville Area Sewer Authority	Pottsville	PA	Original selection
Punxsutawyney	Punxsutawyney	PA	Original selection
South Kingstown WWTF	Narragansett	RI	Original selection
Plum Island WWTP	Charleston	SC	Original selection
Lawson Fork WTP	Spartanburg	SC	Original selection
Elizabethton	Elizabethton	TN	Original selection
Amarillo	Amarillo	TX	Original selection
Dallas Southside WWTP	Dallas	TX	Replacement for Dallas Central

C) The proposed and enacted funding levels for 2002-2009 are as follows:

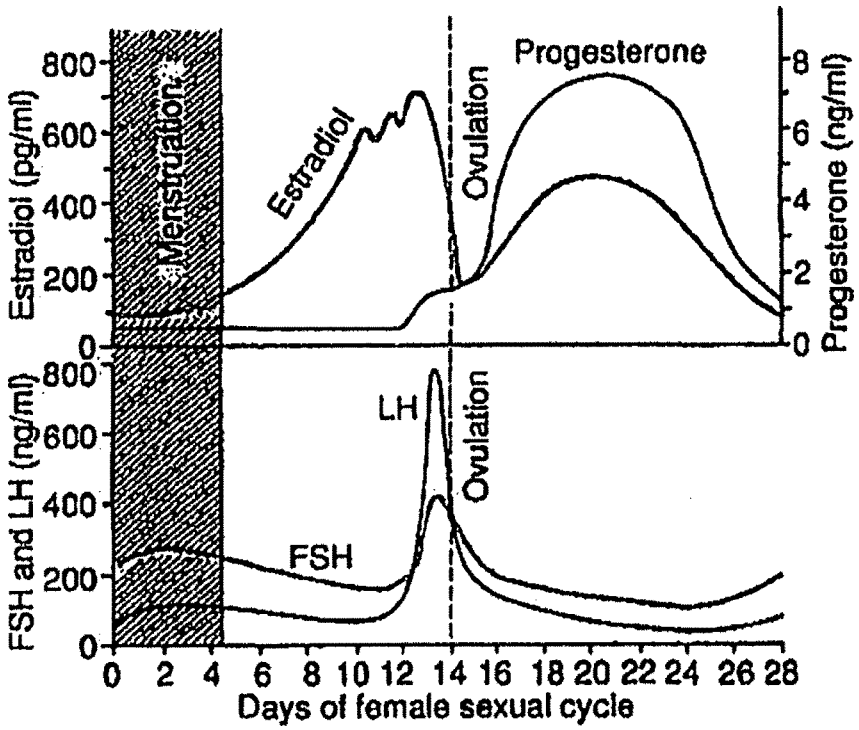
USGS Program Funding: Proposed & Enacted, 2002 - 2009 (Dollars in Thousands)

Fiscal Year	Toxics		NAWQA		CWP		Contaminants		Fisheries	
	Proposed	Enacted	Proposed	Enacted	Proposed	Enacted	Proposed	Enacted	Proposed	Enac
2002	3,919*	13,919	44,579	63,986	64,318	64,318	11,900	11,900	16,200	16,2
2003	0	13,437	57,312	63,217	64,339	64,433	12,000	10,800	16,200	27,4
2004	11,065	14,902**	63,818	63,285	64,536	63,995	10,200	10,700	24,900	26,7
2005	12,638	14,476**	62,506	61,645	63,007	62,337	9,800	9,600	25,800	24,9
2006	13,120	14,386**	63,132	62,203	63,770	62,833	10,700	9,700	26,700	24,2
2007	13,215	13,293	62,571	62,818	62,171	64,345	8,900	9,100	21,900	23,8
2008	13,730	13,516	64,925	63,912	62,381	62,849	8,400	8,700	21,700	23,2
2009	10,704***	TBD	54,113	TBD	62,285	TBD	8,500	TBD	22,800	T
Total '02-'08	**67,687**	**97,929**	**418,843**	**441,066**	**444,522**	**445,110**	**71,900**	**70,500**	**153,400**	**166,4**

* 10,000 proposed to be redirected to the National Science Foundation.

** Earmark for Tar Creek contamination site / University of Oklahoma: 2004 - 1,482; 2005 - 1,460; 2006 - 1,213.

*** 2,257 represents a funding shift for Priority Ecosystem Studies from Toxics to Biological Research & Monitoring


Estradiol (pg/ml)
800
600
400
200
0
Menstruation
Estradiol
Ovulation
Progesterone
8
6
4
2
0
Progesterone (ng/ml)
FSH and LH (ng/ml)
800
600
400
200
0
LH
Ovulation
FSH
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days of female sexual cycle

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Serum hormone levels in humans with low serum concentrations of 2,3,7,8-TCDD

ES JOHNSON,[a] C SHORTER,[a] LL BESTERVELT,[b] DG PATTERSON,[c] LL NEEDHAM,[c] WN PIPER,[b] G LUCIER[d] AND CJ NOLAN[b]

[a]*Department of Epidemiology, Tulane University School of Public Health and Tropical Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112, USA*

[b]*Toxicology Program, Department of Environmental and Industrial Heath, School of Public Health, University of Michigan, 1420 Washington Heights, Ann Arbor, Michigan 48109, USA*

[c]*Division of Environmental Health and Laboratory Sciences, Centers for Disease Control and Prevention, Mail Stop F17, 4770 Guilford Highway NE, Atlanta, Georgia 30341, USA*

[d]*Division of Environmental Toxicology, National Institute of Environmental Health Sciences, 111 Alexander Drive, Research Triangle Park, North Carolina 27709, USA*

We measured current serum hormone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentrations in 37 men who sprayed 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in the State of Victoria, Australia. TCDD levels were consistently significantly inversely related to prolactin levels in all analyses. In correlation analyses, TCDD levels were also inversely related to triiodothyronine (T3), thyroid-stimulating hormone (TSH), and testosterone levels, and positively associated with glucagon levels. The mean serum TCDD concentration in these sprayers was between 2.6 and 8.1 parts per trillion (ppt). Since such TCDD levels are commonly found in the general population in countries such as the US, the results could suggest that background levels of TCDD in the general population could have an effect on hormone levels. The findings are preliminary and need to be replicated in order to evaluate their full public health significance. *Toxicology and Industrial Health* 2001: **17**, 105–112.

Key words: *dioxin; hormones; prolactin; sprayers; TCDD; 2,4,5-T*

Introduction

The substance, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), occurs in the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a contaminant formed during its manufacture. It is one of the most potent chemical carcinogens known in animals (International Agency for Research on Cancer (IARC), 1987). Human studies of the carcinogenic potential of TCDD have not been conclusive (Zober et al., 1990; Fingerhut et al., 1991; Manz et al., 1991; Johnson, 1992; 1993; Pesatori et al., 1993; IARC, 1997). The IARC classifies TCDD as a human carcinogen based on the overall assessment, even though it is concluded that there is limited evidence for its carcinogenicity in humans (IARC, 1997).

Several animal studies have shown that endocrine organs are targets of dioxin toxicity. In general, administration of TCDD results in decreased serum or plasma levels of testosterone and thyroxine (T4). For example, rats fed a single oral dose of 4.5 μg of TCDD/kg body weight showed a significant decrease in serum androgen concentrations (Moore et al., 1985; Jones et al., 1987; Sewall et al., 1995). Triiodothyronine (T3) is generally not affected in animals (Jones et al., 1987; Sewall et al., 1995). An increase or decrease of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) has also been inconsistently reported, depending on the time of administration of TCDD and the time of sample collection (Ruangwises et al., 1991; Mably, Moore, Peterson, 1992; Mably, Moore, Goy, et al., 1992; Mably, Bjerke, et al., 1992; Li et al., 1995). In the study by Ruangwises et al. (1991), rats fed a single oral dose of 50 μg of TCDD/kg showed a decrease in serum LH concentrations. Serum LH levels were decreased to 60% of controls as from the first day of TCDD administration, and returned to control values by Day 5, in spite of continued depression of serum

1. Abbreviations: ACTH, adrenocorticotropin; CDC, Centers for Disease Control and Prevention; 2,4-D, 2,4-dichlorophenoxyacetic acid; FSH, follicular-stimulating hormone; GH, growth hormone; IARC, International Agency for Research on Cancer; LH, luteinizing hormone; NIOSH, National Institute for Occupational Safety and Health; PCDD, polychlorodibenzo-*p*-dioxins; 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TSH, thyroid-stimulating hormone; T4, thyroxine; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; T3, triiodothyronine

2. Address all correspondence to: Dr. Eric S. Johnson, Department of Epidemiology SL18, Tulane University School of Public Health and Tropical Medicine, 1430 Tulane Avenue, New Orleans, LA 70112, USA E-mail: meatjoh@tulane.edu

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testosterone concentrations. TCDD also induces tumors of the thyroid, adrenal, and pituitary glands in animals. The thyroid gland in rats is the most sensitive organ to its carcinogenic action in a two-year bioassay, with tumors induced in animals with mean adipose tissue TCDD concentrations of 540 parts per trillion (ppt) (Kociba et al., 1978; National Toxicology Program, 1982). Disruption of hormonal function of the pituitary gland in rats occurs following the administration of a single oral dose of 50 μg of TCDD/kg body weight (Bestervelt, Cai, et al., 1993; Bestervelt, Pitt, et al., 1993). Plasma adrenocorticotropin (ACTH) levels are increased for at least up to 14 days, while plasma corticosterone concentrations are increased during the first five days, but are depressed later. Corticosterone secretion by TCDD-exposed adrenal glands is also depressed following stimulation with ACTH (Bestervelt, Cai, et al., 1993). There is evidence also that TCDD decreases the bioactivity of ACTH (Bestervelt, Pitt, et al., 1993).

Epidemiologic studies of the effects of exposure to TCDD on the human endocrine system are scarce. Egeland et al. (1994) studied the effects on serum testosterone and gonadotropin levels in men with high exposure to TCDD during the manufacture of phenoxyacetic acid herbicides and chlorophenols. The highest serum TCDD levels in these workers were extrapolated to be up to 30,000 ppt at the time occupational exposure ceased. They found that those who were exposed exhibited elevated serum LH and FSH, and decreased serum testosterone. The decrease in serum testosterone is consistent with observations seen in rats (Ruangwises et al., 1991).

Higher levels of polychlorinated dibenzo-*p*-dioxins PCDD were observed to correlate with lower plasma levels of maternal total T3 and T4 (Koopman-Esseboom et al., 1994). However, this study did not examine the effect of TCDD specifically.

The only other study to have examined serum hormone levels in human subjects exposed to TCDD was one in which the serum concentrations of eight hormones were measured in the 1992 follow-up of US Air Force Ranch Hands who sprayed Agent Orange in Vietnam. The hormones were thyroid-stimulating hormone (TSH), T4, LH, FSH, insulin, glucagon, testosterone, and estradiol (see below). The findings were not published in a scientific journal, but are available in the Internet and are considered because this group also sprayed herbicides as did our study group (Ranch Hand Report, 1992)

The purpose of the current paper is to examine serum hormone concentrations in subjects with low level occupational exposure to TCDD, which occurs during the spraying of 2,4,5-T. Subjects in this study sprayed both 2,4,5-T and 2,4-dichlorophenoxyacetic acid (2,4-D), and their exposure to TCDD has been previously characterized in detail (Johnson et al., 1992).

Methods

A total of 37 subjects were randomly selected from a total of 654 men who had sprayed 2,4,5-T and 2,4-D for at least 12 months in the State of Victoria, Australia (Johnson et al., 1992). Data and sample collection for the study followed a protocol that was approved by the Human Subjects Committee of the National Institute of Environmental Health Sciences, National Institutes of Health, USA. Fasting blood samples were collected in the morning (8:00 a.m. to 11:00 a.m.) according to a procedure developed by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. Samples were analyzed by high-resolution gas chromatography and high-resolution mass spectrometry at CDC, according to methods previously described (Patterson et al., 1987). Attempts were made to collect 180 mL of blood from each of the 37 subjects.

For 4 of 37 sprayers, because of markedly insufficient blood collected, the assay for TCDD was not able to give a reliable estimate; hence, these subjects were excluded from this report. Another subject who had the highest serum TCDD concentration of 34 ppt was excluded because there was not sufficient blood to test for hormone levels. The results presented below are therefore limited to a total of 32 male subjects who sprayed 2,4,5-T, all of whom had serum TCDD of <20 ppt. The mean age of the 32 sprayers was 48 years ranging from 29 to 76 years. For 9 of 32 workers, because of marginally insufficient volume of blood collected, TCDD levels could not be reported exactly, and instead for these nine samples, the assay was only able to report that the subject's true serum TCDD concentration was below a certain value (censored value). The serum TCDD concentrations for these nine subjects were reported as <3, <3, <4, <5, <13, <14, <17, <17, and <18 ppt. (In one type of statistical analysis conducted (see below), these censored values were assumed to be the real values.) It is to be noted that background TCDD exposure in the general population varies depending on geographic region. In some populations, serum levels are not detectable at 1 ppt, whereas in the US, average serum TCDD levels in the general population are around 4–7 ppt with the levels in some individuals approaching 20 ppt (Fingerhut et al., 1991; Egeland et al., 1994; Johnson et al., 1994). The State of Victoria in Australia, where our study was performed, appears to be one of those areas with very low background TCDD exposure in the general population, probably not exceeding 2 ppt on average.

Serum samples were assayed using commercially available radioimmunoassay kits for human hormones provided by Diagnostic Products (Los Angeles, CA). The assays were performed as described in the manufacturer's protocol. The following hormones were measured: T4, T3, glucagon, insulin, estradiol, progesterone, testosterone, prolactin, growth hormone (GH), LH, FSH, and

TSH by competitive binding radioimmunoassay. Normal expected ranges for the assays used to quantify the hormones are as follows: T4, 0.045–0.125 μg/ml; T3, 0.86–1.87 μg/ml; glucagon, 40–130 pg/ml; insulin, non-detect (ND) – 30 μIU/ml; estradiol, ND – 44 pg/ml; progesterone, ND – 0.6 ng/ml; testosterone, 2.7–10.7 ng/ml; prolactin, ND – 15 ng/ml; GH, ND – 7.0 ng/ml; LH, 0.4–3.7 mIU/ml; FSH, 3.0–15.0 mIU/ml; TSH 0.3–5.0 μIU/ml.

Statistical analyses

We examined the relationship between serum TCDD and individual serum hormone levels in four ways.

1) *An analysis in which correlation coefficients were determined for the relationship between individual serum TCDD levels and corresponding hormone levels.* These analyses were deemed the more sensitive, since they were based on individual rather than grouped data. This supposition is vindicated by the observation that there were no results statistically significant in any of the group analyses that were not significant in the correlation analyses, while conversely some comparisons for which a statistically significant result was obtained in the correlation analyses were not significant in the group analyses. Since we examined 12 different hormones, for clarity of presentation, in analyses other than the correlation analyses, we present only the results for five hormones (prolactin, glucagon, T3, TSH, and testosterone), since only these were statistically significant in the correlation analyses. The remaining seven hormones results were not significant in any analyses and are presented in Table 2 only.

Because of the small sample size, while some specific hormone data appeared roughly normally distributed, others and the TCDD data were clearly not so. Accordingly in the correlation analyses, we present results for untransformed and log-transformed data separately.

Also, in these analyses, because the exact serum TCDD levels were not known for nine subjects, only the fact that their levels were below a certain number (censored concentrations), we conducted three types of analyses in which assumptions were made about the censored values: a) one in which the nine censored TCDD results were assigned the value of 2 ppt (*background*); b) one in which all nine censored TCDD results were each assigned the value at the point of censoring, e.g., if reported as <3 ppt then the concentration is taken as 3 ppt (*censored*); c) one in which the serum TCDD value was back-calculated to what it was estimated to have been at the time exposure ceased (*historical*).

2) *Analyses in which for each hormone in turn, subjects were divided into two groups: those with a serum hormone concentration at or less than the 33rd percentile, and those with a value above the 33rd percentile.* The mean serum TCDD levels in both groups were then compared.

3) *Analyses in which for each hormone in turn, subjects were divided into two groups: those with a zero serum hormone concentration (i.e., below the detection limit of the assay), and those with a measurable hormone concentration.* The mean serum TCDD levels in both groups were then compared. Only the results for prolactin are presented, since this is the only hormone of the five significant in the correlation analyses for which zero values were recorded.

4) *We divided study subjects according to whether they sprayed herbicides in areas where 2,4,5-T was used in large amounts (high 2,4,5-T area) or whether they worked in low 2,4,5-T-use areas.* We then for each hormone in turn compared mean hormone levels in the two groups.

Table 1. Correlation coefficients for serum hormones showing statistically significant correlations with serum TCDD concentrations in sprayers.

Hormone	Censored TCDD values handled as	Log-transformed TCDD values		Original untransformed TCDD values	
		Original hormone values (Spearman)	Hormone values log-transformed (Pearson)	Original hormone values (Spearman)	Hormone values log-transformed (Pearson)
Prolactin	Background	−0.4*	−0.4	−0.4*	−0.4
	Censored	−0.4*	−0.4	−0.4*	−0.0
	Historical	−0.1	−0.0	−0.1	0.0
Glucagon	Background	0.4*	0.3	0.4*	0.2
	Censored	0.4*	0.3	0.0	−0.1
	Historical	0.1	0.0	0.0	−0.1
T3	Background	−0.1	−0.2	−0.1	−0.1
	Censored	−0.1	−0.2	−0.4*	−0.2
	Historical	−0.5**	−0.4*	−0.5**	−0.2
TSH	Background	0.2	−0.0	0.2	−0.1
	Censored	0.2	−0.0	−0.3	−0.2
	Historical	−0.4*	−0.4*	−0.4*	−0.2
Testosterone	Background	−0.3	−0.3	−0.3	−0.4*
	Censored	−0.3	−0.3	−0.2	−0.2
	Historical	−0.1	−0.1	−0.1	0.0

*Statistically significant at 0.05 level.
**Statistically significant at 0.01 level.

Table 2. Correlation coefficients for serum hormones showing no correlation with serum TCDD concentrations in sprayers.

Hormone	Censored TCDD values handled as	Log-transformed TCDD values		Original untransformed TCDD values	
		Original hormone values (Spearman coefficient)	Log-transformed hormone values (Pearson coefficient)	Original hormone values (Spearman coefficient)	Log-transformed hormone values (Pearson coefficient)
Estrogen	Background	0.1	−0.1	0.1	−0.2
	Censored	0.1	−0.1	−0.2	−0.3
	Historical	−0.3	−0.2	−0.3	−0.1
FSH	Background	−0.0	0.1	−0.0	0.2
	Censored	−0.0	0.1	0.2	0.1
	Historical	0.2	0.3	0.2	0.1
LH	Background	−0.0	−0.3	−0.0	−0.3
	Censored	−0.0	−0.3	0.2	−0.1
	Historical	0.2	0.4	0.2	0.2
GH	Background	0.0	−0.3	0.0	−0.4
	Censored	0.0	−0.3	0.2	0.5
	Historical	0.3	0.5	0.3	0.6
Progesterone	Background	−0.1	−0.2	−0.1	−0.3
	Censored	−0.1	−0.2	−0.1	−0.2
	Historical	−0.2	−0.1	−0.2	−0.1
T4	Background	0.1	0.0	0.1	0.0
	Censored	0.1	0.0	−0.0	0.1
	Historical	−0.0	0.1	−0.0	0.0
Insulin	Background	0.1	0.0	0.1	−0.1
	Censored	0.1	0.0	−0.2	−0.3
	Historical	−0.0	−0.1	−0.0	−0.2

Subjects with a hormone value of zero are excluded from the analysis of that hormone.

In the correlation analyses, Spearman or Pearson coefficients were estimated where appropriate, as well as associated *P* values, using the BMDP Program 6D. In the grouped data analyses, two-sample *t*-tests using BMDP Program 3D were performed to test for differences in mean values between groups (BMDP Statistical Software Manual).

Results

The results for hormones showing a statistical correlation with TCDD concentrations are given in Table 1, and those not showing one in Table 2. As seen in Table 1, there was a consistent inverse correlation between serum prolactin levels and serum TCDD levels. In contrast, a

Table 3. Comparison of mean serum TCDD levels in 2,4,5-T sprayers with a hormone value at or less than the 33rd percentile, and in subjects with higher values.

Hormone	Censored TCDD values handled as	Number of subjects		Mean serum TCDD concentration in ppt		
		Subjects with a hormone value at or less than the 33rd percentile	Subjects with a hormone value greater than the 33rd percentile	Subjects with a hormone value at or less than the 33rd percentile	Subjects with a hormone value greater than the 33rd percentile	*P* value
Prolactin	Background	10	22	7.6	3.4	0.06†
	Censored	10	22	10.3	5.7	0.02*
	Historical	10	22	79.8	51.2	NS
Glucagon	Background	10	22	2.7	5.7	0.01*
	Censored	10	22	7.7	6.9	NS
	Historical	10	22	116.4	34.6	NS
T3	Background	10	22	4.1	5.0	NS
	Censored	10	22	7.3	7.0	NS
	Historical	10	22	100.7	41.7	NS
TSH	Background	10	22	5.3	4.5	NS
	Censored	10	22	8.5	6.5	NS
	Historical	10	22	81.6	50.4	NS
Testosterone	Background	10	22	6.6	3.9	NS
	Censored	10	22	8.3	6.6	NS
	Historical	10	22	32.5	72.7	NS

NS, not statistically significant at the 95% level.
†*P* value borderline statistically significant.
**P* value significant at the 95% significance level.

Table 4. Comparison of mean serum TCDD levels of sprayers with a zero hormone level, and of subjects with a measurable hormone value.

Hormone	Censored TCDD values handled as	Number of subjects		Mean serum TCDD concentration in ppt		
		Subjects with a hormone value of zero	Subjects with a hormone value greater than zero	Subjects with a hormone value of zero	Subjects with a hormone value greater than zero	*P* value
Prolactin	Background	7	25	8.89	3.58	0.09
	Censored	7	25	12.80	5.45	0.001
	Historical	7	25	99.50	49.11	NS
FSH	Background	2	30	9.20	4.45	NS
	Censored	2	30	9.20	7.00	NS
	Historical	2	30	60.10	60.14	NS
LH	Background	6	26	4.67	4.76	NS
	Censored	6	26	7.30	7.10	NS
	Historical	6	26	100.12	50.90	NS
GH	Background	26	6	4.82	4.42	NS
	Censored	26	6	7.10	7.35	NS
	Historical	26	6	46.81	117.85	NS
Progesterone	Background	1	31	2.00	4.83	NS
	Censored	1	31	4.60	7.20	NS
	Historical	1	31	148.40	57.28	NS

NS, not statistically significant at the 95% level.

somewhat consistent positive correlation was observed between TCDD and glucagon. A negative correlation between serum TCDD levels and T3 was observed particularly for the analyses in which estimated *historical* TCCD levels were employed. The negative correlation observed for testosterone was quite consistent also, although only one result was statistically significant. The results for TSH were not consistent although they seem to suggest an inverse relationship when *historical* TCDD values were used.

The results for analyses based on grouped data are given in Tables 3–6, and they, by and large, follow the same pattern observed in Table 1, especially for prolactin. As expected, analyses based on grouped data were not as clear-cut as those in the correlation analyses based on individual data.

In Table 7, as a partial check on the validity of the findings, serum concentrations of individual hormones were correlated with each other. The results obtained were as expected, e.g., very strong positive correlations were observed between serum FSH and LH, between LH and prolactin, between TSH and T3, between estrogen and testosterone, while again, as expected, negative correlations were observed between glucagon and insulin, and between insulin and progesterone. The fact that these results follow the expected patterns gives some credence to the overall results obtained.

Discussion

The negative correlation between serum TCDD and prolactin was consistently evident in the different analyses performed, and therefore could be real, since in animal experiments, TCDD has been shown to cause not only a similar decrease in serum prolactin, but also a decrease in prolactin receptor (PRLR) mRNA, and a decrease in PRLR binding (Jones et al., 1987; Moore et al., 1989; Lu et al., 1996). There are no human data on the effect of TCDD on serum prolactin.

Interestingly, from our Table 6, it is observed that the depressed prolactin levels were of the same magnitude irrespective of whether exposure had last occurred 15 years earlier (prior to 1975) or whether exposure was current. Possible explanations include: 1) that this is an artifact; 2) that current serum prolactin levels are only related to the current levels of serum TCDD irrespective of whether those levels could have been the result of a previous high-dose exposure; 3) that the effect of TCDD on prolactin involves receptors that are rapidly saturated at low doses of TCDD, so that further exposures at much higher doses do not cause a further incremental effect. In support of this hypothesis is the observation in the NIOSH study (Egeland et al., 1994) that the effect seen for FSH, LH, and testosterone in subjects in the lowest category of TCDD exposure (just

Table 5. Comparison of serum TCDD levels in sprayers in high and low 2,4,5-T areas by epoch.

Censored TCDD handled as	All time periods			Pre-1975 epoch			1975 and after epoch		
	High 2,4,5-T area (ppt)	Low 2,4,5-T area (ppt)	*P* value	High 2,4,5-T area (ppt)	Low 2,4,5-T area (ppt)	*P* value	High 2,4,5-T area (ppt)	Low 2,4,5-T area (ppt)	*P* value
Background	6.0	2.6	0.01	7.2	2.4	0.06	5.4	2.8	0.17
Censored	8.1	5.5	0.20	9.8	6.1	0.33	7.2	5.1	0.39

Table 6. Comparison of serum hormone levels in sprayers in high and low 2,4,5-T areas by epoch (whether workers were last exposed prior to 1975 or later).

Censored TCDD handled as	Pre-1975 epoch			1975 and after epoch		
	High 2,4,5-T area (n=7)	Low 2,4,5-T area (n=5)	P value for hormone difference in high versus low 2,4,5-T areas	High 2,4,5-T area (n=13)	Low 2,4,5-T area (n=7)	P value for hormone difference in high versus low 2,4,5-T areas
Prolactin	6.2 ng/ml	10.1 ng/ml	0.24	6.6 ng/ml	10.5 ng/ml	0.09
Glucagon	134.2 pg/ml	133.8 pg/ml	0.97	126.9 pg/ml	126.4 pg/ml	0.97
T3	1.1 μg/ml	1.3 μg/ml	0.08	1.2 μg/ml	1.6 μg/ml	0.15
TSH	1.9 IU/ml	2.0 IU/ml	0.85	2.8 IU/ml	2.2 IU/ml	0.40
Estrogen	42.7 pg/ml	44.5 pg/ml	0.82	53.0 pg/ml	39.6 pg/ml	0.07
Testosterone	6.0 ng/ml	6.6 ng/ml	0.42	7.2 ng/ml	6.1 ng/ml	0.40

above background levels) was no different from that seen for subjects with the highest serum TCDD levels of up to 30,000 ppt at the time exposure ceased. Furthermore, the change in levels of these hormones attributable to TCDD was very small. For example, subjects with serum TCDD of 2980 ppt had a mean change of FSH, LH, and testosterone above background levels of only 2.5 IU/l, 3.26 IU/l, and 2.29 nmol/l, respectively (Egeland et al., 1994). These values represent a change within the normal range for these hormones of only 8.9%, 14.2%, and 9.1%, respectively. In our study, a difference of 3.4 or 2.6 ppt (depending on how censored TCDD values were handled) in mean serum TCDD between subjects in high 2,4,5-T and low 2,4,5-T areas resulted in a mean change in sprayers serum prolactin of 3.84 ng/ml (P<0.03), i.e., a change equivalent to 25.6% of the normal range for prolactin. A change in any body constituent of 25% for a given individual is a significant change, which conceivably may be detrimental to that individual, irrespective of whether the value lies within the normal range for the general population.

With regards to the results for hormones other than prolactin, as can be seen in Table 1, a positive relationship was observed between serum TCDD levels and glucagon levels in correlation analyses, although this finding was not consistently observed in grouped data analyses. To our knowledge, no animal study has reported on glucagon levels following TCDD exposure. Given the known opposite effects of glucagon and insulin on blood glucose levels, and the decreased serum prolactin levels associated with TCDD exposure observed in this study, the observed positive association between serum TCDD and glucagon levels is not inconsistent. The only other study in humans with information on glucagon is the Ranch Hand study. In that study, a positive significant association between serum dioxin and glucagon was recorded in some models, and no association in others, and a negative significant association within the subgroup of enlisted officers. Serum dioxin showed a significantly positive association with diabetes, and also with insulin levels in nondiabetics, but not in diabetics.

We observed a significant negative correlation between individual serum TCDD levels and individual testosterone levels in these workers (Table 1). This association was also somewhat evident when mean levels were investigated (Table 3), although not as clear-cut. The results for testosterone in this study are consistent with both reported human and animal data (Moore et al., 1985; Ranch Hand Report, 1992; Egeland et al., 1994).

In our correlation analyses, T3 and TSH levels were significantly negatively associated with TCDD levels, the association being strongest when *historical* serum TCDD levels but not current serum levels were considered. This pattern was not observed in the group analyses in which censored TCDD levels for nine subjects were treated as *background*. Depression of serum T4 levels, but not T3 levels, have been reported in animals following TCDD exposure (Jones et al., 1987; Sewall et al., 1995). Higher levels of PCDD have been observed to correlate with lower plasma levels of maternal total T3 and T4 (Koop-

Table 7. Spearman and Pearson correlation coefficients for hormones related and not related to each other.

Coefficient	LH/ Prolactin	FSH/ Prolactin	Insulin/ Prolactin	Insulin/ Proges-terone	LH/ FSH	TSH/ T3	TSH/ T4	FSH/ Estrogen	FSH/ Testos-terone	LH/ Estrogen	LH/ Testos-terone	Estrogen/ Testos-terone	Glucagon/ Insulin
Spearman	0.39*	0.31	0.34	−0.42*	0.49**	0.26	0.29	−0.13	0.05	0.03	0.13	0.52**	−0.14
Pearson	0.36*	0.28	0.17	−0.39*	0.61**	0.21	0.15	−0.24	−0.05	−0.03	0.15	0.55**	−0.16

S, Spearman coefficient (hormone values untransformed); P, Pearson coefficient (hormone values log-transformed).
*Significant at the 95% confidence level.
**Significant at the 99% confidence level.

man-Esseboom et al., 1994). On the other hand, the Ranch Hand Report found no relationship between TSH and T4 and serum dioxin concentrations.

We did not find statistically significant relations between serum dioxin concentrations and serum concentrations of estrogen, LH, and FSH. These findings were also confirmed in the Ranch Hand study.

This is only the second published study of hormonal effects of above background TCDD exposure in humans. To our knowledge, no study in animals or humans has examined the effects of TCDD on as many as 12 different hormones simultaneously. This decidedly is an advantage. The fact – that in Table 7, the patterns of correlations observed between the different hormones themselves are what would be expected – adds some credence to the possibility that the results obtained could be real.

Importantly, the mean levels of between 2.6 and 8.1 ppt current serum TCDD concentration, which we observed in this TCDD-exposed sprayer population, are comparable to the means of 4–7 ppt seen in occupationally *unexposed* members of the US general population. Hence, the findings are directly relevant for the general US population. This study provides evidence for the first time that blood levels of dioxin in 2,4,5-T sprayers in Australia, which are similar to those found in the general population in the US, may be associated with changes in the levels of at least one hormone in men, which is prolactin, and possibly others also.

Factors supporting that the findings could be real include the following: 1) the observed negative association between TCDD and prolactin persisted in all analyses even in the presence of extreme assumptions about censored TCDD values; 2) the results for other hormones were also consistent with findings in other human and animal studies; 3) no correlation was observed between any hormone level and 2,4-D exposure (results not shown); and 4) the presence of the expected correlations between one hormone and the other. On the other hand, there are factors which should temper any claim from this study of a real association between serum TCDD and hormone concentrations; these include the following: 1) it is possible that some other pesticides these sprayers may have used, or some other factors not taken into account, could be responsible for these findings; 2) the sample size was small, and we had made many comparisons; hence, the results could have happened by chance; 3) the fact that exact serum TCDD levels were not available for some subjects. For these reasons, caution should be exercised in interpreting the findings. The results should be regarded as preliminary, but they clearly merit further investigations. There is, therefore, need to replicate them in other populations for their full significance to be evaluated.

Acknowledgments

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Priority Report

Bisphenol A Induces a Profile of Tumor Aggressiveness in High-Risk Cells from Breast Cancer Patients

Shanaz H. Dairkee,[1] Junhee Seok,[2] Stacey Champion,[1] Aejaz Sayeed,[1] Michael Mindrinos,[2] Wenzhong Xiao,[2] Ronald W. Davis,[2] and William H. Goodson[1]

[1]California Pacific Medical Center Research Institute, San Francisco, California and [2]Stanford Genome Technology Center, Stanford University School of Medicine, Palo Alto, California

Abstract

Breast cancer outcome is highly variable. Whether inadvertent exposure to environmental xenobiotics evokes a biological response promoting cancer aggressiveness and a higher probability of tumor recurrence remains unknown. To determine specific molecular alterations which arise in high-risk breast tissue in the presence of the ubiquitous xenoestrogen, bisphenol A (BPA), we used nonmalignant random periareolar fine-needle aspirates in a novel functional assay. Early events induced by BPA in epithelial-stromal cocultures derived from the contralateral tissue of patients with breast cancer included gene expression patterns which facilitate apoptosis evasion, endurance of microenvironmental stress, and cell cycle deregulation without a detectable increase in cell numbers. This BPA response profile was significantly associated with breast tumors characterized by high histologic grade ($P < 0.001$) and large tumor size ($P = 0.002$), resulting in decreased recurrence-free patient survival ($P < 0.001$). Our assays show a biological "fingerprint" of probable prior exposure to endocrine-disrupting agents, and suggest a scenario in which their presence in the microenvironmental milieu of high-risk breast tissue could play a deterministic role in establishing and maintaining tumor aggressiveness and poor patient outcome. [Cancer Res 2008;68(7):2076–80]

Introduction

The challenges encountered in studies of breast cancer and the environment include a lack of reliable exposure and risk assessment tools. Issues related to individual differences in breast cancer susceptibility pose critical difficulties in the experimental design of such studies. Despite these obstacles, epidemiologic evidence suggests a strong association between breast cancer and prolonged estrogen exposure (1). This raises concerns regarding the increasing prevalence of xenoestrogens, such as bisphenol A (BPA), in the environment, leading to a higher exposure burden over time.

BPA is widely found in polycarbonate plastics, which enter human consumption by leaching out from food and beverage containers, and in epoxy resins used as dental sealants (2, 3). It mimics endogenous estrogen and has a reasonably high affinity for estrogen receptors (ER), thereby eliciting profound effects, including the development of preneoplastic mammary lesions in rodents within a few months of exposure (4). Although reports on cancer cells themselves have described the growth-stimulating effects of several xenoestrogens, particularly in estrogen-dependent breast cancer cell lines (5), the direct effects of such exposure on cancer-prone human breast tissue and its role in further malignant development and behavior has not been fully addressed by such studies. To capture early changes induced by continuous BPA exposure of susceptible breast tissue, we have evaluated its effects on global gene expression in clinical samples of random periareolar fine-needle aspirates (RPFNA) propagated in an *in vitro* setting in which epithelial-stromal feedback is facilitated as this is reportedly a more sensitive approach for the detection of both direct and indirect estrogenic effects (6). To test the hypothesis that the biological response of high-risk cell cultures to BPA recapitulates the path followed by tumor cells in the clinical tissue of similarly exposed individuals, a search was conducted for subsets of breast cancer which mirrored the gene expression changes induced *in vitro*. Confirmed demonstrations of such a parallel compel serious consideration of the consequences of BPA exposure in susceptible individuals.

Materials and Methods

Cell culture and hormone exposure. Using a minimally invasive procedure, nonmalignant RPFNAs were collected from unafflicted, contralateral breast tissue of patients at the time of surgical resection of the primary breast lesion with written informed consent and institutional review board approval. Nonmalignant samples derived from eight patients receiving surgical treatment only were used in the experiments described here. Patients were diagnosed with invasive ductal carcinoma (four cases: ages 43, 52, 66, and 79 years), ductal carcinoma *in situ* (one case: age 47 years), atypical intraductal papilloma (one case: age 41 years), and fibroadenoma (two cases: age 47 years).

After 2 to 3 weeks of propagation in optimized growth medium (7), cells were cocultured in 0.4 μm inserts with hanging geometry (Becton Dickinson) with nonmalignant breast tissue fibroblasts at a 3:1 ratio. Continuous 7-day treatments consisted of luteal phase concentrations of 17-β-estradiol (E2, Sigma) and progesterone (PG, Sigma), and 10^{-7} mol/L of BPA (Sigma). For immunofluorescence, cell monolayers fixed with 1:1 acetone/methanol were tested for reactivity with mouse monoclonal pan-cytokeratin (34BE12) and vimentin (AMF17b) primary antibodies, in conjunction with Alexa 488–conjugated antimouse (Invitrogen), and evaluated using confocal microscopy.

Gene expression analysis. Epithelial cell RNA derived from duplicate wells of each treatment using the RNeasy kit (Qiagen) was labeled and hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix) and genome-wide expression profiles were generated as previously described (8). Data from the CEL files of 59 independent arrays was normalized using dChip, and the expression level was modeled using the perfect match–only model.

Gene set analysis was used to assess the significance of predefined gene sets (9), which identified differentially activated or repressed genes in one of

Requests for reprints: Shanaz H. Dairkee and William H. Goodson, California Pacific Medical Center Research Institute, 475 Brannan Street, San Francisco, CA 94107. Phone: 415-600-1653; Fax: 415-600-1725; E-mail: dairkes@cpmcri.org (S.H. Dairkee) and Phone: 415-923-3925; Fax: 415-776-1977; E-mail: goodsow@cpmcri.org (W.H. Goodson).

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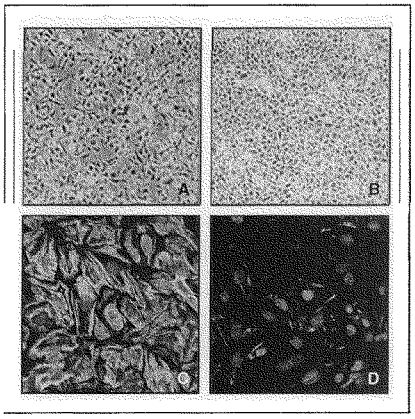

Figure 1. Authentication of the epithelial phenotype of high-risk RPFNA samples (*A* and *B*). Bright-field microscopic images of 2-week-old primary epithelial cultures from two independent cases. Immunolocalization of cytokeratin (*C*) and vimentin (*D*). Note the strong expression of the cytoplasmic cytokeratin network only. Nuclei were counterstained with propidium iodide.

the two following sample groups comparing no-treatment versus E2, E2 versus E2 + PG, and E2 + PG versus E2 + PG + BPA. The C2 functional gene sets of Subramanian et al. (10) were evaluated based on a two-class paired test using significance analysis of microarrays (11). Significant gene sets were chosen from 100 permutation tests at <5% estimated false discovery rate (FDR). Within each gene set, some genes were activated whereas others were repressed. A breast cancer classifier was derived for each treatment by using all activated genes in activated gene sets and repressed genes in repressed gene sets.

Class prediction. The classifier trained on the expression profiles of E2, PG, and BPA-exposed cell cultures was applied to breast cancer array data publicly available via the National Center for Biotechnology Information Gene Expression Omnibus. Data sets GSE5460 and GSE6532 were selected for matching the Affymetrix platform which we used, and the availability of patient follow-up and/or clinicopathologic information on >100 patients. The training and test sets were first normalized so that the within-set average was 0 and within-set SD was 1 for the expression values derived as follows:

$$xt_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{\mathrm{var}(x_i)}}$$

$\bar{x}_i$ and $\mathrm{var}(x_i)$ is average and variation of expression values of gene i along the samples within a set. We calculated the feature w_{kj} of gene set S_k of sample j by taking average expression values of associated genes (selected as described above) to the gene set k. n_k is the number of genes associated with the gene set S_k.

$$w_{kj} = \frac{1}{n_k} \sum_{i \in S_k} xt_{ij}$$

The nearest shrunken centroid classifier was used to train and classify test samples. This algorithm estimated the class probabilities by analogy to Gaussian linear discriminant analysis. The likelihood score of a tested sample was defined as the ratio of the probability of the predicted class to the probability of the opposite class. Comparisons for tumor size and grade were done for all samples of GSE5460 and GSE6532. ER status variation (available only for GSE5460) and patient outcome (available only for GSE6532) were evaluated in samples with likelihood scores >2. *P* values were determined by Fisher test.

Results and Discussion

RPFNA is a simple procedure, resulting in cells with optimal cytomorphologic evaluability (reviewed in ref. 12). By optimizing *in vitro* propagation, the limitations of low cell yield, particularly of samples derived from nonmalignant cancer-prone tissue, were circumvented. An experimental setup comprised of RPFNA-derived pure epithelial cells (Fig. 1), in coculture with breast fibroblasts, enabled cross-exposure to secreted gene products in the presence of physiologic hormone levels. At these concentrations, E2, as well as endocrine-disrupting agents are known to induce proliferation in established breast cancer cell lines. However, in our nonmalignant cocultures, cell numbers were consistent for all treatments relative to control for the entire experimental duration.

Global gene expression analysis of the high-risk epithelial component of the cocultures identified 11, 37, and 38 differentially expressed gene sets in gene set analysis–based comparisons of control versus E2, E2 versus E2 + PG, and E2 + PG versus E2 + PG + BPA expression profiles, respectively. Most significantly, PG and BPA exposure induced dramatically opposing patterns of expression for six independent gene sets in these cells. The combined pattern of expression of these six sets, designated as the cancer-prone response profile (CPRP), depicts a distinctive biological response of high-risk breast epithelial cells to E2, PG, or BPA (Fig. 2). These gene sets are curated in the Molecular Signatures Database C2 functional collection[3] as: IGF_VS_PDGF_UP (73 genes), IGF1_MTOR (20 genes), MTOR (23 genes), FERRANDO_MLL_T_ALL_DN (87 genes), BLEO_MOUSE_LYMPH_HIGH_24HRS_DN (34 genes), and CANTHARIDIN_DN (52 genes).

Consistent with previous reports in breast cancer cell lines (13, 14), the overall transcriptional profiles of E2 and BPA treatment were strikingly similar in primary cultures of nonmalignant breast epithelial cells as well. Within the six gene sets, E2, PG, and BPA response was distinctive for 28, 123, and 52 genes, respectively (Fig. 3). The distinctive E2-activated CPRP genes were associated with stress response, glucose metabolism, antiapoptosis, cell cycle regulation, and DNA replication and repair. Although these were significantly repressed in epithelial cells exposed to PG, the genes in pathways associated with cell differentiation, muscle development and contraction, and motility were significantly activated. Notably, the addition of BPA to growth medium supplemented with the other two hormones resulted in a dramatic reversal of PG-induced effects. Thus, in the presence of BPA, nonmalignant epithelial cells were programmed to override differentiation-inducing signals. Moreover, they were committed to a phenotype of increased oxidoreductase activity, fatty acid β-oxidation, tricarboxylic acid cycle, and the respiratory electron transport chain, in addition to overexpression of genes facilitating cell cycle progression and multidrug resistance.

To determine whether the biological response to BPA described above in nonmalignant breast epithelial cells of patients with cancer was reflected within pathologically identified carcinoma

[3] http://www.broad.mit.edu/gsea/msigdb

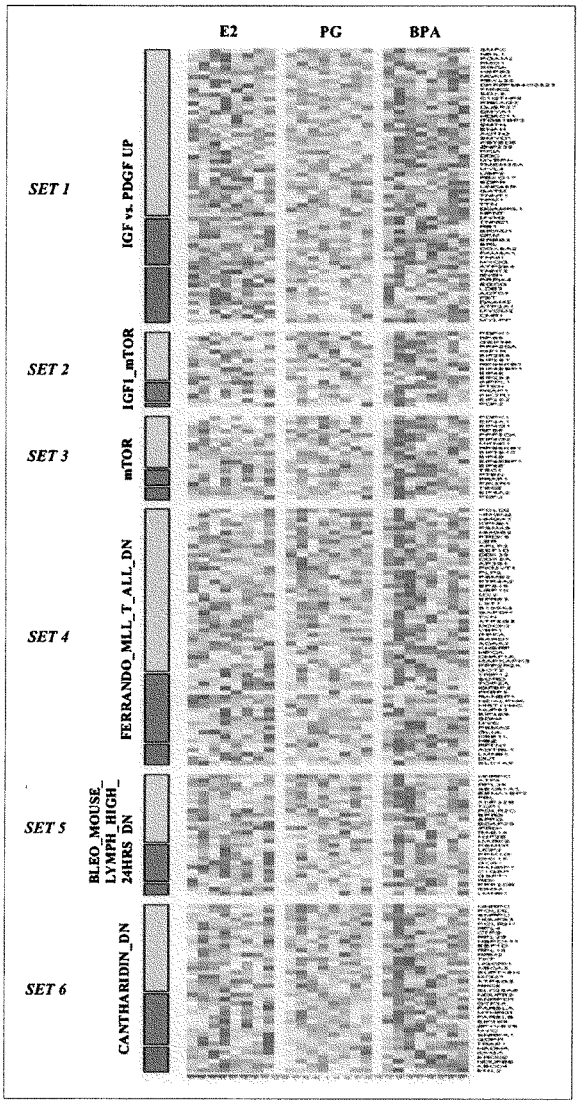

Figure 2. Derivation of the CPRP using nonmalignant epithelial-fibroblast cocultures. Heat map of response to E2, PG, and BPA in breast epithelial cells of women at high risk. *Columns under each treatment,* independent cases; *rows,* genes. Six gene sets identified by gene set analysis display similarities between E2- and BPA-induced profiles which contrast with PG-induced effects. *Yellow side bars,* genes with similar expression patterns; *non-yellow side bars,* opposing expression patterns in E2- versus BPA-exposed cells. *Green side bars,* genes down-regulated by BPA in set 1, vice versa in sets 2 to 6. *Blue side bars,* genes down-regulated by E2 in set 1, vice versa in sets 2 to 6.

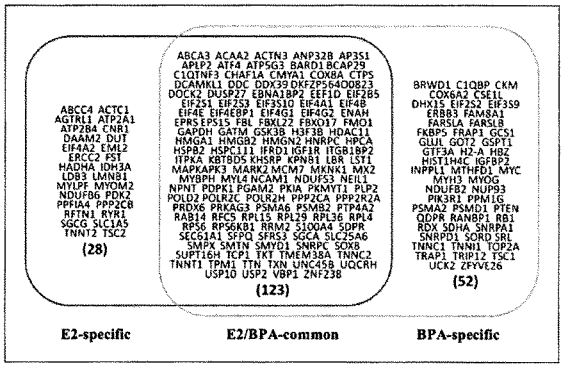

Figure 3. Distinctive E2- and BPA-associated changes in RPFNA cultures of patients at high risk of breast cancer. E2 gene profile (*left*), the BPA profile (*right*), and genes representing expression patterns common to E2 and BPA (*middle*), but reversed in the presence of PG.

from a similar cohort of patients, CPRPs associated with E2, PG, and BPA were individually applied towards class prediction in primary breast cancer data sets: GSE5460 (n = 125) and GSE6532 (n = 414; ref. 15). First, the cell culture–based classifiers were used to stratify ER status, tumor size, and tumor grade. CPRP-BPA was more prevalent in ER-negative tumors (P = 0.0055), which generally reflect the most aggressive disease subset. This profile was also displayed by tumors >2 cm in size (P = 0.0022), and higher histologic grade (grade 1 versus 3, P = 0.000002; grade 1 versus 2 versus 3, P = 0.000009; Fig. 4*A* and *B*). Histologic grade is the most consistent indicator of breast cancer aggressiveness. One-third of all breast cancers display a high-grade phenotype leading to disease-related mortality (16, 17). Clinical follow-up data (GSE6532), revealed that tumors which displayed CPRP-E2, or CPRP-BPA, conferred a significantly poor prognosis compared with those that displayed CPRP-PG (P = 0.0014 and P = 0.00057, Fig. 4*C* and *D*, respectively).

In terms of its striking association with tumor aggressiveness, it is to be noted that in contrast to global gene signatures preselected for correlation with breast cancer prognosis (18, 19), CPRP was derived from a direct functional cellular response restricted to six well-known biological pathways implicated in tumorigenesis. Another important point of distinction from other predictive signatures is that in the derivation of CPRP, major cell proliferation genes did not attain significance. This is likely due to the fact that gene expression profiles were compared here between cell cultures of equivalent growth rate. A similar observation was made in the identification of a cancer cell immortalization signature based on the comparison of finite life (but proliferating) and immortalized primary breast tumor cells (20). Thus, the association of CPRP-BPA with breast cancer aggressiveness does not merely reflect a correlation between proliferating cells *in vitro* and those in high-grade tumors. Instead, it reveals cellular alterations that may play an underlying role in the induction and maintenance of dysregulated cell proliferation in cancer, thereby influencing patient outcome. The robust similarity observed here between the phenotypes of BPA-exposed nonmalignant cell cultures and full-blown aggressive breast tumors provides a conceptual framework for the detection of a poor prognosis signature even at the earliest disease stages (18).

Unlike classical methods of evaluating endocrine-disrupting agents by *in vitro* ER ligand–binding and transactivation assays, our comprehensive genomics-based evaluation of BPA exposure in conjunction with a paired study design that minimizes the effects of confounding factors has successfully tracked early causal changes in the context of nonmalignant breast tissue. Such assays which yield data that is biologically relevant to tumor-promoting alterations could be invaluable for determining which chemicals might pose a long-term threat to human health, enabling a meaningful payoff from focused epidemiologic studies of breast cancer and the environment.

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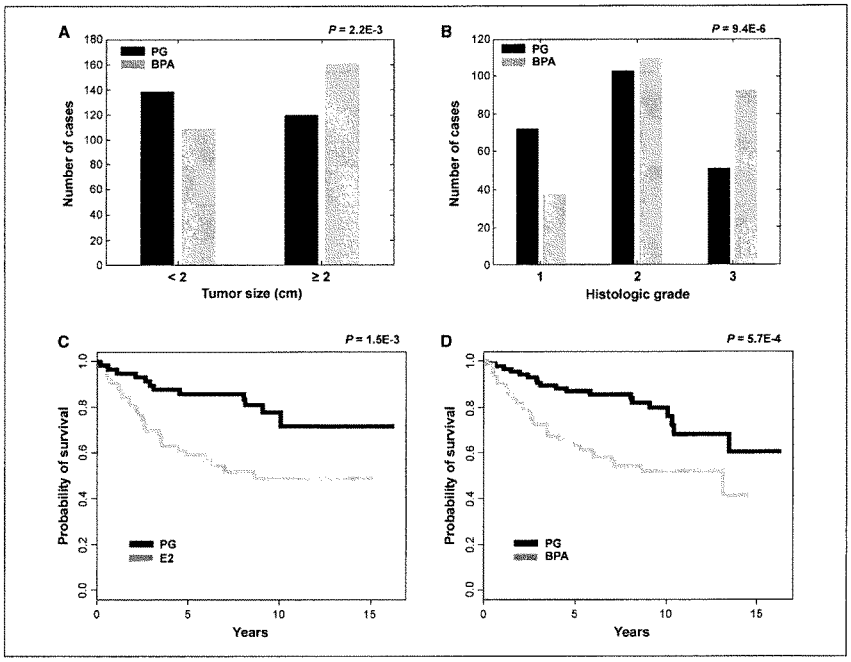

Figure 4. Application of the CPRP towards class prediction of primary breast cancer. *A* and *B*, the BPA-induced profile is significantly associated with larger tumor size and higher histologic grade in comparison with the PG-induced expression profile. *C* and *D*, Kaplan-Meier survival curves for subgroups with high correlation to profiles induced by E2, PG, and BPA. Compared with CPRP-PG, both CPRP-E2 and CPRP-BPA independently predict a worse prognosis for patients with breast cancer.

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Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra

Barry G. Timms*, Kembra L. Howdeshell†, Lesley Barton*, Sarahann Bradley*, Catherine A. Richter†, and Frederick S. vom Saal*‡

*Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, Vermillion, SD 57069; and †Division of Biological Sciences, University of Missouri, Columbia, MO 65211

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Exposure of human fetuses to man-made estrogenic chemicals can occur through several sources. For example, fetal exposure to ethinylestradiol occurs because each year ≈3% of women taking oral contraceptives become pregnant. Exposure to the estrogenic chemical bisphenol A occurs through food and beverages because of significant leaching from polycarbonate plastic products and the lining of cans. We fed pregnant CD-1 mice ethinylestradiol (0.1 μg/kg per day) and bisphenol A (10 μg/kg per day), which are doses below the range of exposure by pregnant women. In male mouse fetuses, both ethinylestradiol and bisphenol A produced an increase in the number and size of dorsolateral prostate ducts and an overall increase in prostate duct volume. Histochemical staining of sections with antibodies to proliferating cell nuclear antigen and mouse keratin 5 indicated that these increases were due to a marked increase in proliferation of basal epithelial cells located in the primary ducts. The urethra was malformed in the colliculus region and was significantly constricted where it enters the bladder, which could contribute to urine flow disorders. These effects were identical to those caused by a similar dose (0.1 μg/kg per day) of the estrogenic drug diethylstilbestrol (DES), a known human developmental teratogen and carcinogen. In contrast, a 2,000-fold higher DES dose completely inhibited dorsolateral prostate duct formation, revealing opposite effects of high and low doses of estrogen. Acceleration in the rate of proliferation of prostate epithelium during fetal life by small amounts of estrogenic chemicals could permanently disrupt cellular control systems and predispose the prostate to disease in adulthood.

bisphenol A | ethinylestradiol | urogenital sinus

More than 60 years ago, there was speculation that exposure of male fetuses to elevated estrogen levels during fetal life could predispose men to have an enlarged prostate in old age. This hypothesis was proposed because the prostate derives from a portion of the embryonic urogenital sinus (UGS) that differentiates into the estrogen-responsive vagina in females (1). In contrast to this prediction, numerous studies have shown that high doses of diethylstilbestrol (DES) and other estrogenic chemicals inhibit prostate development in mice and rats (2). These studies were conducted because pregnant women in the 1950s and 1960s were prescribed high doses of DES based on the mistaken assumption that DES would prevent spontaneous abortion. Maternal DES administration resulted in cancer and other abnormalities of the reproductive organs in offspring, which was not detected until subsequent adulthood and after millions of human fetuses had been exposed (3–5). It is now well known that hormones can have opposite effects at low vs. high doses. Studies that include only very high doses of drugs or chemicals can miss unique effects that are observed only within a physiologically relevant low dose range (6).

We examined whether very low doses of estrogenic chemicals in drugs and consumer products (7) could affect the development of prostate ducts in male mouse fetuses. Androgen is required for differentiation of the prostate, and our objective was to examine the modulating effect of estrogenic chemicals on the initial differentiation and growth of primary ducts in the fetal prostate. There are many possible opportunities for exposure of fetuses to estrogenic chemicals (7). An unexpected source of estrogen exposure by human fetuses is the drug ethinylestradiol, which is the estrogenic chemical used in oral contraceptives. It is estimated that each year almost 2 million of the more than 60 million women in the United States and Europe who use oral contraceptives become pregnant accidentally, primarily because of missed pills; the average is three missed pills per month and a 3% pregnancy rate per year for this population (8). Oral contraceptive pills often are taken for many months until the unplanned and unexpected pregnancy is discovered (9). Even though oral contraceptives have been used for decades, relatively little research has been conducted in experimental animals to assess effects on offspring of maternal administration of ethinylestradiol at or below the clinically relevant dose of 0.4–0.8 μg/kg per day, based on 30 μg of ethinylestradiol per pill and body weights ranging from 37 to 75 kg (10, 11).

Bisphenol A was shown to have full activity (efficacy similar to estradiol) as an estrogenic drug in 1936 (12). Since the 1950s, bisphenol A has been used as the monomer that is polymerized to manufacture polycarbonate plastic, certain dental sealants, and the resin lining of most food and beverage cans. Bisphenol A also is used as an additive in many other products, with global capacity at >6 billion pounds per year (13). Polycarbonate is less durable than commonly believed, because the ester bond linking polymerized bisphenol A molecules can be hydrolyzed, and hydrolysis increases dramatically at high or low pH and as temperature increases. Bisphenol A thus leaches into food and beverages under the normal conditions of use of tin cans and polycarbonate plastic containers (14–16), and when polycarbonate is scratched and discolored, the rate of leaching can be very high (16). There is significant exposure of pregnant women to bisphenol A, because mean blood levels of biologically active (unconjugated) bisphenol A in human fetuses at parturition are in the range of 2–3 ng/ml (≈10 nM) (17), and levels in human amniotic fluid during reproductive tract differentiation in fetuses are even higher at 8 ng/ml (18).

We examined the morphology (by using computer-assisted 3D reconstruction) and cytology (by using histochemical analysis) of the fetal mouse prostate after maternal exposure for 5 days to estrogenic chemicals during the initial period of development of the primary prostate ducts; this begins on gestation day (GD) 17 in mice and occurs during the 10th week of gestation in humans.

Freely available online through the PNAS open access option.

Abbreviations: DES, diethylstilbestrol; UGS, urogenital sinus; GD, gestation day; PCNA, proliferating cell nuclear antigen; MKS, mouse keratin 5.

‡To whom correspondence should be addressed. E-mail: vomsaalf@missouri.edu.

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The dose of bisphenol A used in this study results in average levels of parent (unconjugated) bisphenol A in mouse fetuses throughout the 24 h after administration (19) that are below average levels reported in blood from human fetuses at parturition (17). The dose of ethinylestradiol that we administered is ≈4- to 8-fold lower than the average dose in oral contraceptives taken by adult women (8). A low dose of DES also was examined as a positive control estrogen, and a high dose of DES was included for comparison to the large literature on developmental effects of high, pharmacological doses of estrogenic chemicals.

Materials and Methods

Animals. CD-1 mice (*Mus musculus domesticus*) were purchased from Charles River Laboratories. Pregnant and lactating females were housed in standard (11.5 × 7.5 × 5 in) polypropylene mouse cages on soy-based Purina 5008 chow. Drinking water was available ad libitum in glass bottles. The drinking water was purified by ion exchange, followed by passage through a series of carbon filters to remove chlorine as well as hydrophobic molecules, and flowed through copper pipes into our bottle-filling apparatus. Rooms were maintained at 25 ± 2°C under a 12-h/12-h light/dark cycle, with lights on at 1000 hours. All animal procedures were approved by the University of Missouri Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals.

Administration of Chemicals to Pregnant Females and Delivery of Pups. Adult virgin females were placed with a stud male for 4 h, beginning 2 h before the end of the dark phase of the light/dark cycle. Females with a vaginal plug were housed three per cage. We randomly assigned 21 pregnant mice to four different treatment groups, and on GD 14–18 (mating = GD 0), we fed pregnant mice oil vehicle (oil controls, $n = 5$); either ethinylestradiol (0.1 μg/kg per day) ($n = 5$) or DES (0.1 μg/kg per day) ($n = 5$); or bisphenol A (10 μg/kg per day) ($n = 6$). The chemicals (Sigma) were dissolved in tocopherol-stripped corn oil (ICN) and delivered through a pipette placed into the animal's mouth. Based on prior findings, DES (0.1 μg/kg per day) was included as a positive control (20, 21). Our dose level of bisphenol A was based on prior results suggesting that bisphenol A is ≈100-fold less potent than DES in terms of stimulating a permanent increase in prostate size in mice (20, 22, 23).

A separate study was conducted in which pregnant mice were fed either the oil vehicle ($n = 4$) or a high dose (200 μg/kg per day) of DES ($n = 4$) by using the same procedures described above. Our objective was to compare these results with those of other studies that examined effects on adult prostate size of exposure during development to high doses of DES, similar to doses that had been prescribed to pregnant women (3, 4, 20).

Just before normal parturition on GD 19, we removed the fetuses from the uterus by cesarean section. We recorded the intrauterine position of male fetuses relative to adjacent male and female fetuses as they were removed from the uterus. We reduced variation in background fetal blood levels of the sex hormones estradiol and testosterone due to the sex of the adjacent fetuses by examining only one male fetus per litter that developed *in utero* between a male and a female fetus, because intrauterine position influences serum hormone levels during fetal life, subsequent prostate size, and many other traits in litter-bearing species. The observation that male mouse fetuses with the highest serum levels of estradiol (due to developing between two female fetuses) had an enlarged prostate beginning in fetal life and persisting through adulthood (24–27) provided the basis for studying developmental effects of low doses of man-made estrogenic chemicals.

3D Reconstruction. Beginning on GD 17 in the mouse, the primary prostate ducts emerge from the UGS as solid cords of cells. The primary ducts elongate, and subsequent to the time examined in this study, branch, form lumens, and eventually become the functional glandular component of adult prostate. Prostate morphology on GD 19 was determined by a 3D computer reconstruction technique (26–28). Briefly, the UGS was removed, fixed in 4% paraformaldehyde, and sectioned. Digital images were used for 3D reconstruction and morphometric analysis of the developing prostate ducts, coupled with immunohistochemical analysis. We determined the number and volume of epithelial outgrowths (primary ducts) from the UGS. The individual primary ducts in each region (dorsal, lateral, and ventral) were counted. Prostate duct volume was determined as the sum of all of the individual cross-sectional areas for all of the ducts in a specific region.

Proliferating Cell Nuclear Antigen (PCNA) and Mouse Keratin 5 (MK5) Immunocytochemistry. Sections for PCNA staining were heated twice for 5 min in 50 ml of deionized water in a microwave oven at 600 W and then allowed to cool to room temperature before continuing. Immunocytochemical staining for PCNA was performed by using the avidin–biotin peroxidase method as follows. Endogenous peroxidase activity was blocked by using 3% hydrogen peroxide in methanol for 5 min, and slides were washed in PBS-TX [PBS containing 0.03% Triton X-100 (Fisher)]. Sections were then incubated for 30 min in a blocking serum (2% normal goat serum made in PBS-TX). All incubations were done in a humid chamber at room temperature. Excess serum was blotted from the slides, which were then incubated overnight with the primary antibody to PCNA (PC-10 clone; Santa Cruz Biotechnology) at a dilution of 1:600. After washing in PBS, sections were incubated for 30 min in the biotinylated secondary antibody from the Vectastain Elite ABC kit (Vector Laborato-

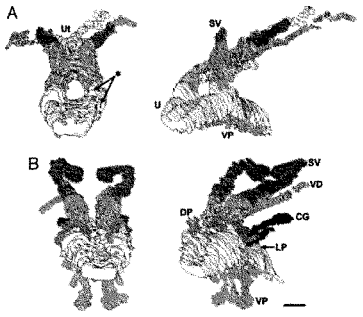

Fig. 1. 3D serial section reconstruction of the UGS from GD-19 mice exposed to a high dose (200 μg/kg per day) of DES from GD 14–18 (*A*), compared with vehicle-treated controls (*B*). Reconstructions are shown from the caudal–dorsal perspective in the left images and caudal–dorsolateral on the right. Exposure of pregnant females to this DES dose resulted in the inhibition (+) of dorsal (DP, green) and lateral (LP, yellow) prostate ducts and coagulating glands (CG, dark blue). There is an abnormal pattern of development in ventral duct formation (VP, light blue). The utricle (UR, pink) was present in oil controls only as a small remnant of the Müllerian ducts, whereas these ducts did not regress in the DES-exposed males. DES also resulted in a decrease in the size of the seminal vesicles (SV, purple). VD, vas deferens, U, urethra. (Scale bar, 100 μm.)

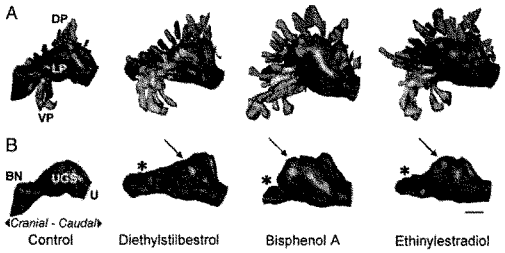

Fig. 2. 3D serial section reconstruction of the UGS from GD-19 mice exposed to low doses of estrogenic chemicals, DES, bisphenol A, and ethinylestradiol, during fetal development. The UGS depicted for each treatment was closest to the group mean for prostate duct number and size. All images are viewed from a left-lateral perspective. (*A*) Shown are the differences in patterns of prostate ductal development after fetal exposure to these chemicals, compared with oil-treated controls. There is a significant increase in the total number of ducts in estrogen-treated animals and a corresponding increase in overall prostate volume. (*B*) Shown is the marked alteration in the shape of the urethra (U, red) in the region of the bladder neck (BN), which is markedly constricted (*) in the mice exposed to the estrogenic chemicals, compared with controls. In addition, the region of the UGS (the prostatic sulcus or colliculus, arrow) associated with the development of the dorsal (DP, green) and lateral (LP, yellow) prostate ducts is enlarged, particularly by bisphenol A, compared with controls. Ventral prostate (VP, light blue). (Scale bar, 100 μm.)

ries) at a dilution of 1:200 in PBS-TX. Sections were washed in PBS and incubated in the avidin reagent as described in the ABC kit. Slides were washed again in PBS before immersion in the chromogen, diaminobenzidine, at a concentration of 0.025% made in PBS with 0.0025% hydrogen peroxide. Sections were washed in water and counterstained with hematoxylin. Slides were dehydrated, cleared, and mounted with Permount (Fisher Scientific). For MK5 staining with rabbit anti-MK5 (Covance Research Products, Berkeley, CA), antigen retrieval was performed by using procedures described by DAKO.

To determine the percentage of cells labeled for PCNA within the ducts of each region of the developing prostate, cell counts were performed on dorsal, lateral, and ventral ducts as well as the urethra. A total of 500–1,000 cells were counted in each region. Care was taken to ensure that sections selected for analysis included the entire length (both proximal and distal portions) of a duct. Sections were viewed with a BX60 microscope (Olympus, Melville, NY), and digital photographs of alternate sections throughout the entire developing gland were captured by using a DVC1301C camera (Digital Video Camera, Austin, TX). Images were analyzed by using IMAGE-PRO PLUS (Media Cybernetics, Silver Spring, MD). To determine cell counts in an area of interest, nonconvolution filters were used to enhance the outline of individual cells and differentiate between stained and unstained cells. We counted the number of cells within each area and calculated the percent of stained vs. unstained cells within each area.

Statistical Procedures. ANOVA was conducted by using the Statistical Analysis System general linear model procedure (SAS Institute, Cary, NC). Planned comparisons of differences between controls and treatment groups were made by using the Fisher's least-squares means test in SAS if the overall analysis was statistically significant. The confidence level for rejecting the null hypothesis was $P < 0.05$. All procedures were conducted blind to ensure the absence of bias.

Results

A High Dose of DES Inhibits Prostate Morphogenesis. As predicted, administration of the high, 200-μg/kg per day, dose of DES to pregnant mice completely inhibited the formation of ducts in the dorsal and lateral prostate in male fetuses, including the coagulating glands, which form from dorsal ducts that develop in the most cranial region of the UGS. The Müllerian ducts also were clearly evident in the DES-treated animals, suggesting that this treatment interfered with the action of Müllerian-inhibiting hormone. Relative to the oil-treated controls, this high dose of DES caused a very different pattern of outgrowths in the ventral UGS. Numerous abnormally short outgrowths were apparent throughout the entire length of the prostatic urethra, compared

Table 1. Data from reconstruction of the prostate and urethra and immunochemistry in control and estrogenic chemical-treated male fetuses

	No. of prostate ducts			Prostate duct volume, μm^2			PCNA staining, %		Cranial urethra
Treatment	DL	V	DLV	DL	V	DLV	DL	V	volume, μm^3
Control	45.4 ± 6.3	7.2 ± 1.3	53.0 ± 6.7	25,616 ± 4,773	26,531 ± 5130	25,921 ± 3561	36.3 ± 3.2	35.0 ± 7.8	35,033 ± 8126
DES (0.1 μg)	57.2 ± 2.5*	7.8 ± 0.9	65.0 ± 2.8	48,321 ± 5,156***	56,576 ± 2692***	51,073 ± 3589***	64.2 ± 2.7**	42.1 ± 7.2	25,556 ± 2911
BPA (10 μg)	64.2 ± 4.6*	10.0 ± 1.4	74.1 ± 5.2**	50,886 ± 6,921***	47,112 ± 4726*	49,592 ± 4790***	52.4 ± 2.5*	34.1 ± 7.5	22,767 ± 2875*
EE (0.1 μg)	56.4 ± 3.9*	7.6 ± 1.2	64.0 ± 5.1	45,508 ± 5,215***	49,369 ± 3947*	46,795 ± 3688***	69.2 ± 3.0**	40.9 ± 9.0	21,748 ± 2047*

All results are presented as mean ± SEM. Shown are the number of developing epithelial ducts in the entire prostate (DLV) and for the individual dorsolateral (DL) and ventral (V) regions of the prostate on GD 19. For control, DES, and ethinylestradiol (EE) treatments, $n = 5$ fetuses per group; for bisphenol A (BPA) $n = 6$ fetuses. Prostate volume on GD 19 was calculated as the sum of all the individual cross-sectional areas based on all of the ducts in a specific region in histological sections as described in ref. 27. Urethral volume is of the initial 200 μm of the urethra beginning at the bladder neck, cranial to the region of prostate duct formation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ vs. controls.

with 7–10 elongated ducts that normally develop in the ventral region of the prostatic urethra. This high dose of DES also reduced the size of the seminal vesicles relative to controls (Fig. 1). Because of the gross abnormalities caused by this dose of DES, no attempt was made to quantify differences relative to controls.

Low Doses of Estrogenic Chemicals Stimulate Prostate Duct Development. In sharp contrast with the high dose of DES, the low doses of DES, ethinylestradiol, and bisphenol A significantly increased the total number of ducts in the dorsal, lateral, and ventral prostate relative to controls (ethinylestradiol, 24%; DES, 26%; and bisphenol A, 41%) and also increased total volume of these ducts (ethinylestradiol, 67%; DES, 68%; and bisphenol A, 81%). A significant increase in volume of the paired coagulating glands (that consist of two to three ducts per side) also was caused by the low dose of each chemical (ethinylestradiol, 40%; DES, 47%; and bisphenol A, 56%). These effects were thus apparent within the first 48 h after the initiation of prostate duct development (Fig. 2*A* and Table 1).

To determine whether an increase in cell proliferation accounted for differences in the size of prostate ducts in fetuses exposed to estrogenic chemicals, tissue sections were labeled with an antibody to PCNA, a marker of proliferative activity (29). All of the estrogenic chemicals resulted in a significant increase in the number of proliferating epithelial cells (Table 1 and Fig. 3 *A–D*). A significant increase in proliferative activity was observed only in the dorsolateral ducts but not in the ventral ducts of the mice exposed to the estrogenic chemicals. The pattern of PCNA staining was similar to the pattern of staining in cells that expressed a marker, MK5, of basal cells (Fig. 3*E*).

Low Doses of Estrogenic Chemicals Cause Urethral Malformations. Fetal exposure to a low dose of DES, ethinylestradiol, and bisphenol A also caused a malformation (an abnormal narrowing or urethral stricture) in the portion of the urethra associated with the neck of the bladder (Table 1 and Fig. 2*B*). The internal sphincter is associated with this region and is involved in the control of the retention of urine in the bladder. Our findings show that this malformation is observed in a region cranial to the region of the UGS where these chemicals result in prostate duct epithelial proliferation. Fig. 2*B* also shows a distinct malformation (enlargement) caused by low doses of all of the estrogenic chemicals in the region of the paired prostatic sulci that form the lateral walls of the colliculus (a protuberance on the posterior wall of the prostatic urethra).

Discussion

The findings of this study demonstrate that the differentiating UGS in male mouse fetuses is exquisitely sensitive to low-dose bisphenol A and ethinylestradiol exposure via the pregnant female, which can result in male fetuses beginning life with a greater number of primary ducts in the dorsolateral prostate, increased epithelial proliferation in these ducts, an overall increase in prostate volume, constriction of the urethra at the bladder neck, and malformation of the prostatic sulci that form the colliculus. These effects are virtually identical to effects of the low dose (0.1 μg/kg per day) of DES (Table 1).

Studies have demonstrated that there is a permanent "imprinted" increase (up to 7-fold) in prostate androgen receptors, associated with an increase in prostate size, in adult male mice due to fetal exposure to low doses of estradiol, ethinylestradiol, DES, and bisphenol A, whereas fetal exposure to much higher doses reduces adult prostate androgen receptors and prostate size (10, 20, 23, 30). Bisphenol A over a 1,000-fold dose range (between 100 pM and 100 nM) also showed an inverted-U dose–response curve for the stimulation of proliferation of human LnCAP prostate cancer cells (31). Bisphenol A stimulates a wide range of effects through binding to estrogen receptors in the nucleus (6, 32). However, extremely low doses (1 pM or 0.23 pg/ml) of bisphenol A also stimulate a rapid response (calcium influx within 1 min) in rat pituitary tumor cells resulting in prolactin secretion. This response occurs through binding of bisphenol A or estradiol to receptors associated with the cell membrane and the rapid activation of kinases, and the dose–response curve also forms an inverted U over a 10,000-fold dose range (33).

Taken together, these findings suggest that effects of fetal exposure to low doses of bisphenol A and ethinylestradiol occur through multiple mechanisms. In addition, the low doses of these estrogenic chemicals to which human fetuses are exposed may

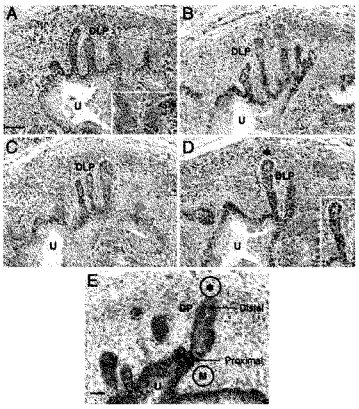

Fig. 3. Immunohistochemical localization of PCNA in developing dorsolateral prostate ducts (DLP) of the UGS of GD-19 fetal mice. (*A–D*) Representative sections of comparable regions are shown for oil-treated control mice (*A*) and animals exposed to the estrogenic compounds DES (0.1 μg/kg per day) (*B*), bisphenol A (10 μg/kg per day) (*C*), and ethinylestradiol (0.1 μg/kg per day) (*D*). In each of the groups studied, the predominant site of PCNA staining was associated with the proximal region of the longest ducts. In *D*, the *Inset* is a digitally enhanced image of developing prostate duct [PCNA-stained cells (brown) vs. nonstained cells (blue)]. These proliferating cells were continuous with the basal layer of urothelial epithelial cells in the urethra (U). PCNA was not detected at the distal tips of the longest ducts that are juxtaposed to a condensation of mesenchyme (*D*; area with *). This region of mesenchyme is associated with induction of postnatal branching morphogenesis, after which formation of a ductal lumen and development of differentiated, secretory epithelial cells occurs. A negative immunohistochemistry control section is shown in *A Inset*. A comparison of cellular proliferation in these experimental groups is presented in Table 1. (Scale bar, 50 μm.) (*E*) Immunohistochemical localization of MK5, a marker for basal epithelial cells, in the developing prostate ducts of an oil-treated control GD-19 mouse fetus. The majority of labeled cells are found in the proximal region of the developing ducts (DP, dorsal prostate) and the basal epithelial cells of the urethra (U). The initial duct formation is associated with periurethral mesenchyme (M). Cells in the distal portion of the duct are infrequently stained. The latter region is juxtaposed to a condensation of mesenchyme (*). The pattern of staining seen with this antibody closely resembles the pattern seen for PCNA staining. (Scale bar, 20 μm.)

have opposite effects on the developing human prostate (and other tissues) relative to the high doses of DES experienced by human males whose mothers took DES during pregnancy (5). The cohort of "DES sons" may thus not be an appropriate reference population for predicting effects of exposure to low doses of estrogenic chemicals. Based on these and other findings, we propose that the use of high, pharmacological doses of estrogenic chemicals in research with experimental animals does not provide information that is relevant to understanding the role of endogenous estradiol in development (20, 32), and that the results are not relevant for predicting the effects of exposure to low levels of man-made estrogenic chemicals present in the environment (6).

An increase in prostate androgen receptors as a result of fetal exposure to very low doses of estrogenic chemicals is likely only one of many potential bases for the altered rate of epithelial proliferation. Extensive research involving experimental recombination of mesenchyme and epithelial tissues has shown that mesenchyme adjacent to specific regions of epithelium "instructs" epithelial differentiation under the control of androgen (34, 35). There have been numerous studies seeking to determine the androgen-mediated factors secreted by adjacent mesenchyme that regulate epithelial growth and differentiation. Results of these studies suggest that multiple paracrine factors, both stimulatory, such as FGF-10 (36), and inhibitory, such as TGF-β, and bone morphogenetic protein 4 (37), are involved (2, 35). A permanent derangement by estrogenic chemicals in the activity of genes that regulate cell proliferation could be a basis for the reawakening of prostate growth (both benign and malignant) during aging in men (38). There is evidence that disruption of the temporal organization of developmental events is associated with permanent functional outcomes in other organs that form ducts, such as the lungs (39), breast (40), and salivary glands (41).

Our findings show a marked increase in PCNA staining in the epithelium of the primary dorsolateral prostate ducts. Basal cells are a subset of epithelial cells found in the undifferentiated UGS that contribute to the proliferative pool of epithelial cells in the developing prostate ducts (35). Based on the known instructive mechanisms that involve mesenchymal induction of epithelial proliferation, our finding that PCNA had a similar distribution to cells that stained for MK5 suggests that estrogenic chemicals influence mesenchymal growth factors, which results in stimulation of the epithelial basal cell population. The recent identification of specific markers, such as p63 (42), which distinguishes progenitor cells in developing epithelial tissues, will permit further elucidation of the subpopulations of these cells that exhibit the greatest proliferative response to estrogenic stimulation.

In assessing whether organs in different species are homologous, functional as well as structural similarities should be assessed. With regard to functional similarities, mesenchyme from the mouse prostate has been shown to produce the appropriate regulatory factors that induce differentiation of human bladder epithelium into epithelium characteristic of the human prostate (43). The dorsolateral prostate in both human and mouse male fetuses also shows structural similarities during early fetal development, namely, the same pattern of epithelial duct formation. Based on these findings, we have proposed that the dorsolateral region of the human prostate is homologous to the dorsolateral region of the mouse prostate (2, 28). In contrast, for the ventral region of the prostate, there are differences, because unlike the mouse prostate, the human adult prostate does not contain ducts that emerge from the ventral region of the urethra. We, and others, also have reported differences between the rodent dorsolateral and ventral prostate in regulatory factors and the response to other chemicals (34, 44). The rodent dorsolateral prostate thus may be a valuable model for predicting the effects of estrogenic chemicals on human prostate development.

The changes in the structure of the urethra induced by estrogenic chemicals may have implications for human disease. Low doses of bisphenol A, ethinylestradiol and DES caused similar morphological changes relative to oil-treated controls in the region of the paired prostatic sulci that form the lateral walls of the colliculus. This region is of particular interest with regard to estrogen-induced malformations, because the primary secretory ducts in the human prostate, which are long ducts that project into the peripheral zone, develop from these urethral ridges, and it is within these ducts that the great majority of malignant prostate tumors form in men as they age (45). The unexpected observation of a malformation of the urethra at the bladder neck (specifically, a narrowing of the urethral lumen by approximately one-third) could affect bladder function and have implications for bladder outlet obstruction disease (46).

There are now >90 published studies showing adverse effects of low doses of bisphenol A in a wide variety of experimental animals (32). For example, during postnatal life very low doses of bisphenol A have been shown to disrupt chromosomes during meiosis in mouse oocytes (47) and decrease sperm production in male rats (48). Fetal exposure to very low doses of bisphenol A accelerates postnatal growth and advances puberty (49) and also stimulates mammary gland epithelium in mice (50). A recent case-control study has shown a correlation between blood levels of bisphenol A and both obesity and polycystic ovarian disease in Japanese women (51).

With regard to ethinylestradiol, the focus of the relatively few studies of exposure by human fetuses during the critical period of reproductive organ development due to continued use of oral contraceptives during an undetected pregnancy has been on externally visible malformations at birth (reviewed in ref. 52). Our findings concerning ethinylestradiol exposure in fetal mice should be viewed from the perspective that the long-term effects of fetal DES exposure in mice and humans have been documented to be highly concordant (3–5), and we show here that the effects of the same low doses of ethinylestradiol and DES on the developing prostate and urethra are virtually identical.

Based on the general absence of grossly observable external malformations at birth, DES was considered safe for administration to millions of women during pregnancy for more than two decades. DES was subsequently found to result in serious harm to offspring that became apparent in adulthood (3–5). Similar conclusions about the safety of ethinylestradiol exposure for human fetuses based on the absence of consistent findings from studies focusing on grossly observable external malformations should be reevaluated based on several lines of evidence. Ethinylestradiol has been shown to readily pass from the maternal to fetal circulation across the primate placenta (52). Fetal exposure to DES caused uterine cancer in humans and mice (3, 4), and similar to our prostate and urethra findings here, DES and ethinylestradiol have virtually identical effects on the developing uterus in female rats (11, 53). The dose of ethinylestradiol in oral contraceptives is typically ≈4- to 8-fold higher than the dose used in our experiment with mice that caused malformations of the urethra and altered differentiation and growth of the prostate. In summary, we propose that the data from this and other published animal studies, and the similarity to effects of low doses of DES, warrant a thorough reevaluation of the risks posed by doses of both ethinylestradiol and bisphenol A below those to which human fetuses are exposed.

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Serum Dioxin and Diabetes Mellitus in Veterans of Operation Ranch Hand

Gary L. Henriksen, Norma S. Ketchum, Joel E. Michalek, and James A. Swaby

We studied diabetes mellitus and glucose and insulin levels in Air Force veterans exposed to Agent Orange and its contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), during the Vietnam War. The index subjects of the Air Force's ongoing 20-year prospective epidemiologic study are veterans of Operation Ranch Hand (N = 989), the unit responsible for aerial herbicide spraying in Vietnam from 1962 to 1971. Other Air Force veterans who served in Southeast Asia during the same period but were not involved with spraying herbicides serve as Comparisons (N = 1,276). The median serum dioxin level in the Ranch Hand group was 12.2 parts per trillion (ppt) (range = 0–617.8 ppt), and the median dioxin level in the Comparison group was 4.0 ppt (range = 0–10 ppt). We found that glucose abnormalities [relative risk = 1.4; 95% confidence limits (CL) = 1.1, 1.8], diabetes prevalence (relative risk = 1.5; 95% CL = 1.2, 2.0), and the use of oral medications to control diabetes (relative risk = 2.3; 95% CL = 1.3, 3.9) increased, whereas time-to-diabetes-onset decreased with dioxin exposure. Serum insulin abnormalities (relative risk = 3.4; 95% CL = 1.9, 6.1) increased with dioxin exposure in nondiabetics. These results indicate an adverse relation between dioxin exposure and diabetes mellitus, glucose metabolism, and insulin production. (Epidemiology 1997;8:252–258)

Keywords: diabetes mellitus, dioxin, glucose metabolism, insulin, cohort study, occupational exposures.

Studies of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) in animals have found a wide range of species-, strain-, age-, and sex-specific effects, including carcinogenicity, immunotoxicity, reproductive and developmental toxicity, hepatoxicity, neurotoxicity, chloracne, and loss of body weight.[1] Endocrine toxicity, however, is not widely acknowledged.[1,2] Many studies of endocrine toxicity focus on either thyroid function[3–7] or reproductive function[8–15] in laboratory animals. Another series of studies has demonstrated reduction in glucose transport activity in adipose and other tissues *in vivo* and *in vitro* in Guinea pigs, mice, and rats.[16–21] The mechanism of dioxin toxicity is not clearly defined, however, and the relevance of these animal studies to endocrine disorders in humans has not been established.

Diabetes mellitus results in a number of adverse effects on the body, including macroangiopathy, neuropathy, and cataracts. Primary risk factors for diabetes are family history, obesity (over 80% of all diabetics are overweight when they are diagnosed), and physical or emotional stress.[22] Little has been reported on diabetes prevalence or glucose and serum insulin levels in Vietnam veterans. Here, we summarize the prevalence of diabetes mellitus, blood glucose and serum insulin levels, and exposure to dioxin in veterans of Operation Ranch Hand, the unit responsible for the aerial spraying of herbicides, including Agent Orange, in Vietnam from 1962 to 1971. These data have been gathered during 10 years of follow-up in the ongoing Air Force Health Study from veterans whose exposure in Vietnam occurred from 24 to 35 years ago.

Methods

The details of study design and subject selection are published elsewhere.[23] The study seeks to determine whether veterans of Operation Ranch Hand (the personnel tasked with spraying operations during the Vietnam conflict) have experienced adverse health and whether those health effects, if they exist, can be attributed to exposure to herbicides or their dioxin contaminant. Ranch Hand veterans were exposed to herbicides during flight operations and maintenance of the aircraft and herbicide spray equipment. The study compares the current health and cumulative mortality experience of Ranch Hand veterans with a comparison group of other Air Force veterans who served in Southeast Asia during the same period (1962–1971) that the Ranch Hand unit was active and who were not involved with spraying herbicides. Comparisons were matched to Ranch Hands on age, race, and military occupation. The study includes periodic analyses of noncombat mortality, in-person interviews, and physical examinations. Physical examinations were conducted in 1982, 1985, 1987, and 1992, and additional examinations are planned for 1997 and 2002.

From the Armstrong Laboratory, Brooks Air Force Base, TX.

Address correspondence to: Joel E. Michalek, 2606 Doolittle Road, Building 807, Brooks Air Force Base, TX 78235-5250.

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In 1987, blood from willing participants was collected and assayed for dioxin.[24] Participation was voluntary, and consent forms were signed at the examination site. Veterans with no quantifiable dioxin result in 1987, those who refused in 1987, and subjects new to the study were also asked to give blood for the assay at the 1992 examination.

Diabetes mellitus cases included for analysis were diagnosed during the post-Vietnam period from the end of the veteran's last tour of duty to June 1995. We report cumulative post-service diabetes, diabetes severity, and time-to-onset of diabetes. Each case was verified from medical records and may represent a diagnosis at any of the four physical examinations. Every veteran who attended at least one examination, regardless of his current vital status, was considered for inclusion in the analysis.

We excluded from all statistical analyses veterans with a history of diabetes mellitus before service in Southeast Asia, those with no dioxin measurement, those with a nonquantifiable dioxin result, and Comparisons with a dioxin result greater than 10 parts per trillion (ppt), the value we regard as the threshold for background dioxin exposure. Table 1 shows study size reductions by group (Ranch Hand, Comparison).

We reviewed medical records and laboratory results to determine diabetic status, diabetes severity, and time-to-diabetes-onset. We classified veterans who attended at least one examination and had a verified history of diabetes mellitus by medical diagnosis or exhibited a 2-hour postprandial glucose laboratory value of 200 mg per dl or greater as diabetic. We defined veterans not meeting these criteria as nondiabetic.

We estimated the initial dioxin dose at the end of the tour of duty in Vietnam in Ranch Hands having current dioxin levels above background using a constant half-life of 8.7 years[25] and assigned each veteran to one of four exposure categories, named "Comparison," "Background," "Low," and "High," according to his group, current dioxin level (D), and initial dioxin level (I), defined in Table 2. The cutpoint separating the Low and High categories (94 ppt) is the median initial dioxin level among all Ranch Hands having current dioxin levels greater than 10 ppt. Table 2 shows sample sizes by dioxin category. For fasting glucose and insulin, we reduced the sample to include only those who had laboratory values from the 1992 physical examination [Comparisons (N = 1,197), Background Ranch Hands (N = 400), Low Ranch Hands (N = 263), and High Ranch Hands (N = 265)]. We included only veterans given Glucola at the 1992 examination in the analysis of 2-hour postprandial glucose [Comparisons (N = 1,123), Background Ranch Hands (N = 381), Low Ranch Hands (N = 241), and High Ranch Hands (N = 234)].

TABLE 1. Study Size Reduction by Group

	Ranch Hand	Comparison	Total
Compliant at any examination	1,108	1,494	2,602
Missing dioxin	(100)	(140)	(240)
Nonquantifiable dioxin	(17)	(50)	(67)
Diabetes before service in Southeast Asia	(2)	(3)	(5)
Current dioxin >10 ppt	(0)	(25)	(25)
Net	989	1,276	2,265

TABLE 2. Exposure Category Definition and Associated Study Sizes

Dioxin Category	Definition*	Study Size
Comparison	D ≤ 10	1,276
Ranch Hand		
Background	D ≤ 10	422
Low	10 < D and I ≤ 94	284
High	10 < D and I > 94	283
Total		2,265

* D = current serum dioxin; I = initial serum dioxin; in parts per trillion.

We defined diabetes severity based on a review of medical records and the latest questionnaire responses. We assigned each veteran with diabetes to one of four categories of control: "Insulin Therapy," "Oral Medications," "Diet Only," or "No Control" and included veterans without diabetes in a category named "No Diabetes." When assessing associations between diabetes severity and dioxin, we considered each severity category separately. When studying insulin therapy, we restricted the analysis to diabetic veterans taking insulin and nondiabetic veterans. We analyzed diabetics on oral medications and diabetics on diet control in a similar fashion. Lastly, we combined "Diet Only," "Oral Medications," and "Insulin Therapy" into a single category named "Any Control" and assessed its association with dioxin category.

In veterans with diabetes, we defined time-to-onset as the number of years between the end of the last tour of duty in Southeast Asia and the date of first diagnosis of diabetes mellitus. In veterans without diabetes, time-to-onset was the number of years between the end of the last tour of duty in Southeast Asia and the last physical examination. We considered time-to-onset for nondiabetic veterans as censored on the right. We used a Weibull model to study the relation between time-to-onset and dioxin category. We defined the dependent variable to be the logarithm of time-to-onset in years, whereas the independent variable was the dioxin exposure category as defined in Table 2. The unit of the coefficient of dioxin category is log(years) per category. A negative coefficient implies that the time-to-diabetes-onset is shorter for Ranch Hands than for Comparisons.

We also report glucose and serum insulin abnormalities determined at the 1992 Air Force Health Study physical examination.[26] Fasting glucose (mg per dl) and 2-hour postprandial glucose (mg per dl) were determined with Paramax equipment. The 100-gm glucose load for the postprandial assay was standardized by the use of Glucola and was not given to known diabetics. Serum insulin (mIU per ml) was measured by radioimmunoassay. We defined a fasting glucose greater than 115 mg per dl as abnormally high. We defined 2-hour postpran-

TABLE 3. Distribution of Dioxin and Demographic Characteristics by Dioxin Exposure Category

Characteristic	Comparison	Ranch Hand		
		Background	Low	High
Dioxin (ppt)*				
Median	4.0	5.7	52.7	197.5
Range	0–10	0–10	27–94	94–3,290
Age (years)				
Mean (SD†)	53.5 (7.6)	54.6 (7.2)	54.9 (7.6)	50.9 (7.4)
Percent body fat				
Mean (SD)	21.8 (5.1)	20.2 (4.5)	22.2 (5.3)	23.4 (5.6)
Current smoker (%)	24.8	27.7	28.5	31.1
Current drinker (%)	20.5	21.8	25.3	17.3
Occupation				
Officer (%)	38.5	61.6	38.0	2.5
Enlisted flyer (%)	16.1	11.6	21.5	20.8
Enlisted ground crew (%)	45.4	26.8	40.5	76.7

* Current serum dioxin levels in the Comparison and Background categories, initial serum dioxin in the Low and High categories, in parts per trillion.
† SD = standard deviation.

dial glucose levels equal to or greater than 140 mg per dl and less than 200 mg per dl as impaired and levels equal to or greater than 200 mg per dl as abnormal. We defined serum insulin abnormalities according to the distribution of observed Comparison values. We computed high and low cutpoints separately for fasting and nonfasting diabetics and nondiabetics. Serum insulin greater than the 97.5th percentile of observed Comparison values was abnormally high, whereas serum insulin less than the 2.5th percentile was abnormally low. In nondiabetics, we classified serum insulin greater than 346 mIU per ml as abnormally high and less than 14 mIU per ml as abnormally low. In fasting diabetics, we defined serum insulin levels greater than 189 mIU per ml as abnormally high and less than 7 mIU per ml as abnormally low. In nonfasting diabetics, we defined serum insulin greater than 528 mIU per ml to be abnormally high, whereas values less than 20 mIU per ml were abnormally low.

We defined percent body fat[27] as 1.26 × body mass index − 13.305, where body mass index is weight (kg) per height squared (m^2), and we adjusted all analyses for birth year (born before 1942, born during or after 1942) and percent body fat at the time of the dioxin blood draw (25% or less, more than 25%) using stratification.[28] We report relative risk, defined as the ratio of the prevalence of the abnormality or disease in the Ranch Hand cohort to the corresponding prevalence in the Comparison cohort, and compute 95% confidence limits (CL) using the method of Rothman.[28]

Because dioxin half-life increases with percent body fat measured in 1982,[25] we conducted additional analyses by calculating a half-life in each of three strata determined by the tertiles of 1982 percent body fat and used these to revise our estimate of the initial dioxin level (I) and the Low and High categories. In a separate series of analyses, we matched Ranch Hands in the Background, Low, and High categories to Comparisons on age to within 1 year, race (black, nonblack), percent body fat to within 3%, and military occupation (officer, enlisted flyer, enlisted ground crew). We computed the point estimates of the relative risk and associated confidence limits for a varying number of Comparisons matched to each Ranch Hand.[28] We also studied the effect of changing our definition of exposure by using the tertiles of the current dioxin distribution in Ranch Hands to define the dioxin categories. These additional analyses are summarized in the text.

Results

Demographic characteristics of all veterans are presented in Table 3. Ranch Hands in the High dioxin category are younger and smoke more than Ranch Hands in the Low and Background categories. Most of the Ranch Hands in the High dioxin category are enlisted ground personnel, and those in the Background category are predominantly officers. The median (and range) of current dioxin levels, in ppt, in the Low and High categories were: Low: 15.0 (10.0–26.6), High: 46.2 (18.0–617.8); the intervals overlap because these categories were defined by initial, not current, dioxin.

The percentages of Ranch Hands in the Low (relative risk = 1.3) and High (relative risk = 1.5) dioxin categories having diabetes are increased relative to the Comparisons (Table 4). The percentage of Ranch Hands in the Background category with diabetes is less than the Comparison percentage (relative risk = 0.7).

The percentages of diabetic Ranch Hands in the Low (relative risk = 1.6) and High (relative risk = 1.5) categories on diet control are increased (Table 5), but the percentage of diabetic Ranch Hands in the Background category on diet control is decreased (relative risk = 0.5). Diabetic Ranch Hands in the High category are more likely than diabetic Comparisons to be controlling their glucose levels with oral medications (relative risk = 2.3), taking insulin (relative risk = 2.4), or to be using any control (relative risk = 1.8). The risks of insulin therapy (relative risk = 2.7) and any control (relative risk = 1.4) are increased in the Low category.

TABLE 4. Risk of Diabetes Mellitus According to Dioxin Exposure Category

Condition	Comparison	Ranch Hand		
		Background	Low	High
Diabetes mellitus				
Number (%)	169 (13.2)	40 (9.5)	49 (17.2)	57 (20.1)
Relative risk	1.0	0.7	1.3	1.5
95% CL	Referent	0.5, 1.0	1.0, 1.7	1.2, 2.0

TABLE 5. Level of Diabetes Mellitus Severity According to Dioxin Exposure Category

		Ranch Hand		
Severity Level	Comparison	Background	Low	High
Diet only				
Number (%)	47 (4.1)	8 (2.0)	16 (6.4)	15 (6.2)
Relative risk	1.0	0.5	1.6	1.5
95% CL	Referent	0.2, 1.0	0.9, 2.7	0.9, 2.7
Oral medications				
Number (%)	39 (3.4)	2 (0.5)	7 (2.9)	19 (7.8)
Relative risk	1.0		0.9	2.3
95% CL	Referent		0.4, 1.9	1.3, 3.9
Insulin therapy				
Number (%)	12 (1.1)	8 (2.0)	7 (2.9)	6 (2.6)
Relative risk	1.0	1.9	2.7	2.4
95% CL	Referent	0.8, 4.6	1.1, 6.8	0.9, 6.4
Any control				
Number (%)	98 (8.1)	18 (4.5)	30 (11.3)	40 (15.0)
Relative risk	1.0	0.5	1.4	1.8
95% CL	Referent	0.3, 0.9	0.9, 2.0	1.3, 2.6

The increased risks of any control are similar to those in Table 4 because the population using any control comprises veterans with diabetes mellitus excluding those using no control, whereas all diabetics are included in the totals in Table 4.

The time-to-diabetes-onset is decreased in the Low (coefficient = −0.125) and High (coefficient = −0.181) categories and increased in the Background category (coefficient = 0.203) relative to Comparisons (Table 6). Figure 1 shows the disease-free percentage of veterans in each exposure category *vs* years since the end of service in Southeast Asia. The Low and High Ranch Hand curves are shifted to the left of the Background and Comparison curves, indicating earlier onset in the Low and High categories. The first decile of time-to-onset in years (and standard errors) for the four exposure categories are: Comparison: 20.3 (0.80), Background: 24.9 (1.8), Low: 17.9 (1.2), and High: 17.0 (1.1).

Table 7 shows time-to-diabetes-onset stratified by percent body fat. The coefficients for Low and High Ranch Hand exposure categories are negative (implying earlier onset relative to Comparisons), regardless of percent body fat category. The negative association between time-to-onset and dioxin is strongest for lean subjects (percent body fat ≤25%) in the High exposure category (coefficient = −0.231 log(years) per category).

TABLE 6. Time-to-Diabetes-Mellitus-Onset and Dioxin Exposure Category

		Ranch Hand		
	Comparison	Background	Low	High
Time-to-onset				
Coefficient*	0.0	0.203	−0.125	−0.181
Standard error*		0.083	0.076	0.072
95% CL		0.040, 0.365	−0.275, 0.024	−0.323, −0.039

* Coefficient and standard error for Ranch Hand *vs* Comparison contrast in a failure-time analysis model, using a censored Weibull distribution. Units of coefficient are log(years)/category.

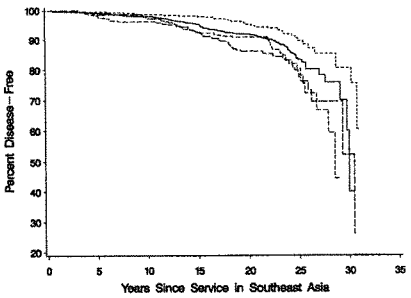

FIGURE 1. Time-to-diabetes mellitus-onset *vs* dioxin category. Percentage disease-free is shown for Comparisons (——), Background Ranch Hands (- - - -), Low Ranch Hands (– – –), and High Ranch Hands (— —).

A study of fasting glucose levels by dioxin category (Table 8) found increased risks of abnormalities in the Low (relative risk = 1.3) and High (relative risk = 1.2) categories and a decreased risk in the Background category (relative risk = 0.7). With regard to 2-hour postprandial glucose, the risk of impaired glucose tolerance was increased in the Low (relative risk = 1.2) and High (relative risk = 1.6) categories and decreased in the Background category (relative risk = 0.9), and the risk of an abnormal response was increased in the Low category (relative risk = 1.2) and decreased in the High (relative risk = 0.9) and Background categories (relative risk = 0.7). The risk of either an impaired or abnormal response at 2 hours was increased in the Low (relative risk = 1.2) and High (relative risk = 1.4) categories and decreased in the Background (relative risk = 0.8) category.

The number of diabetic subjects with abnormally high or abnormally low serum insulin was too small to analyze (Table 9). In nondiabetic veterans, the risk of abnormally high serum insulin is lowest in the Background category (relative risk = 0.8) and highest in the High category (relative risk = 3.4). The risk of abnormally low serum insulin is increased in Ranch Hands in the Background (relative risk = 1.9) and Low (relative risk = 2.1) categories, and the number (N = 2) in the High category was too small to analyze.

We accounted for the dependence of dioxin half-life on 1982 percent body fat by calculating a separate half-life in each of three strata defined by the tertiles of 1982 percent body fat. We used the three half-lives to revise our initial dose (I) and then re-analyzed all data. These results (which do not appear in any table) are briefly summarized as follows. The results for fasting glucose, 2-hour postprandial glucose, and diabetes severity were similar to those

TABLE 7. Time-to-Diabetes-Mellitus-Onset by Dioxin Exposure and Percent Body Fat Category

Percent Body Fat Category	Comparison	Ranch Hand		
		Background	Low	High
>25%				
Number	281	54	65	89
Coefficient*		0.154	−0.061	−0.027
Standard error*		0.115	0.090	0.082
95% CL		−0.070, 0.380	−0.115, 0.112	−0.187, 0.134
≤25%				
Number	994	368	219	192
Coefficient*		0.145	−0.164	−0.231
Standard error*		0.118	0.120	0.119
95% CL		−0.086, 0.376	−0.399, 0.071	−0.465, 0.002

* Coefficient and standard error for Ranch Hand vs Comparison contrast in a failure-time analysis model, using a censored Weibull distribution. Units of coefficient are log(years)/category.

reported here. The revised initial dose produced slightly different results for cumulative diabetes, time-to-diabetes-onset, and nondiabetic serum insulin. Using the revised dose, the risks of diabetes among Ranch Hands in Low and High dioxin categories were still elevated (Low: relative risk = 1.5; High: relative risk = 1.3). The time-to-onset still decreased (Low: coefficient = −0.192; High: coefficient = −0.111), and the risk of abnormally low serum insulin in nondiabetics was increased among Ranch Hands in all three exposure categories (Background: relative risk = 1.9; Low: relative risk = 1.2; High: relative risk = 1.5).

To address the possibility that our method of adjustment may be masking an effect, we matched Ranch Hands in the Background, Low, and High categories to Comparisons, on a one-to-many basis, on race, military occupation, age to within 1 year, and percent body fat to within 3% and re-analyzed again. Matched results for diabetes and diabetes severity were similar to those reported here. Matched analyses for glucose and serum insulin produced slightly different results. The risk of abnormal fasting glucose was not elevated in the Low exposure category (relative risk = 1.0) but was increased in the High category (relative risk = 1.3). The risk of abnormal 2-hour postprandial glucose was not decreased in the Background category (relative risk = 1.0) but was increased in the Low (relative risk = 1.1) and High (relative risk = 1.6) categories. Nondiabetic Ranch Hands in the Background category showed an increased risk (relative risk = 1.4) of abnormally high serum insulin; the risk in the Low category was decreased (relative risk = 0.8); and the risk in the High category was increased (relative risk = 3.0).

Finally, we used tertiles of the current dioxin distribution to define the dioxin exposure categories for Ranch Hands and again re-analyzed. The results for diabetes, diabetes severity, time-to-diabetes-onset, fasting glucose, and 2-hour postprandial glucose were similar to results reported here. Analysis using dioxin tertiles did produce different results for serum insulin in nondiabetic subjects. Among nondiabetics, the risk of abnormally low serum insulin was increased in the Background (relative risk = 2.0), Low (relative risk = 1.6), and High (relative risk = 1.1) categories.

TABLE 8. Glucose Abnormalities and Dioxin Exposure Category in Veterans Who Attended the 1992 Physical Examination

Abnormality	Comparison	Ranch Hand		
		Background	Low	High
Fasting glucose				
Abnormal (%)	155 (12.9)	38 (9.5)	43 (16.3)	42 (15.8)
Relative risk	1.0	0.7	1.3	1.2
95% CL	Referent	0.5, 1.0	0.9, 1.7	0.9, 1.7
2-Hour postprandial glucose*,†				
Impaired (%)	142 (13.4)	43 (11.7)	36 (16.0)	48 (21.6)
Relative risk	1.0	0.9	1.2	1.6
95% CL	Referent	0.6, 1.2	0.9, 1.7	1.2, 2.2
2-Hour postprandial glucose*,‡				
Abnormal (%)	61 (5.4)	14 (3.7)	16 (6.6)	12 (5.1)
Relative risk	1.0	0.7	1.2	0.9
95% CL	Referent	0.4, 1.2	0.7, 2.1	0.5, 1.7
2-Hour postprandial glucose*				
Impaired or diabetic (%)	203 (18.1)	57 (15.0)	52 (21.6)	60 (25.6)
Relative risk	1.0	0.8	1.2	1.4
95% CL	Referent	0.6, 1.1	0.9, 1.6	1.1, 1.8

* Only veterans given Glucola at the 1992 physical examination were included.
† Veterans with glucose ≥200 mg per dl were excluded.
‡ Veterans with impaired glucose were combined with those having normal glucose levels.

Discussion

We found the risk of diabetes mellitus increased, the time-to-onset decreased, and diabetes severity increased with dioxin in Ranch Hands. We found consistent increases in the risk of glucose abnormalities with dioxin and, in nondiabetic Ranch Hands, the risk of abnormally high serum insulin increased with dioxin exposure. Results in other epidemiologic studies are mixed. A follow-up study of German industrial workers exposed to

TABLE 9. Serum Insulin Abnormalities and Dioxin Exposure Category by Diabetic Status

Diabetic Status	Comparison	Ranch Hand		
		Background	Low	High
Diabetic				
Abnormal High (%)	4 (2.4)	2 (4.9)	3 (6.4)	1 (2.0)
Abnormal Low (%)	2 (1.2)	2 (4.9)	0 (0.0)	0 (0.0)
Nondiabetic				
Abnormal High (%)	25 (2.5)	7 (2.0)	5 (2.4)	18 (8.4)
Relative risk	1.0	0.8	1.0	3.4
95% CL	Referent	0.4,1.9	0.4,2.5	1.9,6.1
Abnormal Low (%)	20 (2.0)	13 (3.7)	9 (4.3)	2 (1.0)
Relative risk	1.0	1.9	2.1	
95% CL	Referent	0.9, 3.7	1.0, 4.6	

dioxin found diabetes less often in the exposed group than among referents.[29] In another study of the same cohort, mean fasting glucose levels in the exposed group appeared to increase with current dioxin, but not back-extrapolated initial dioxin.[30] A study of U.S. industrial workers exposed to dioxin found an increased mean dioxin level in diabetic workers as compared with non-diabetic workers and increased mean fasting serum glucose in workers as compared with referents.[31] In the Vietnam Experience Study,[32] diabetes prevalence in the Vietnam veteran cohort was similar to that in the non-Vietnam veteran cohort (relative risk = 1.1).

Studies of glucose transport in animals dosed with dioxin in the range 0.03–1.0 μg per kg have demonstrated reduced glucose transport in adipose, liver, and pancreas tissue in Guinea pigs, mice, and rats.[16–21] These doses are biologically relevant to this study because the median dioxin body burden in Ranch Hands is 0.07 μg per kg, the first and third quartiles are 0.03 μg per kg and 0.14 μg per kg, and the 99th percentile is 1.0 μg per kg. That dioxin may be associated with diabetes and glucose and insulin levels in Ranch Hands therefore appears plausible, although a specific mechanism of dioxin alteration of glucose transport has not been established, and studies of glucose transport in human adipocytes have not been carried out. Other studies of rats given much higher doses of dioxin (more than 100 μg per kg) found glucose[33] and insulin[34] decreased in exposed animals.

The strengths of this study include high participation and low attrition rates, a Comparison population closely matched to the index population, and 10 years of follow-up. Repetitive examinations and active quality control incorporating double-blind entry of data with discordances referred for third-party review and medical review of potential outliers reduced errors that would bias the study toward the null result.

The serum dioxin measurements are accurate[35] and correlate with reported skin exposure to herbicide in Vietnam[36] but were made up to 30 years after exposure. Our initial dose calculation was based on a first-order decay law with an assumed constant half-life. This approach must be considered approximate in light of recent findings that the decay rate may depend on body fat.[25] We accounted for a changing half-life by calculating a separate half-life in each of three strata defined by the tertiles of 1982 percent body fat and using those figures to revise our initial dose. The results using the revised initial dose were similar to those reported here and did not lead us to a different conclusion.

Confounding is another concern. Although we adjusted for all known confounders, there is the possibility that others exist that we have not taken into account. Ranch Hand veterans in the High category were younger and heavier than Ranch Hands in the Background category. These differences are most likely due to the greater percentage of enlisted personnel in the High category (97.5%) than in the Background category (38.4%), because officers are generally older than enlisted personnel, and most officers are college educated whereas most enlisted personnel have only a high school education. Our adjustment for age and percent body fat was motivated by these differences and the strong relation between percent body fat and diabetes mellitus.

We considered the possibility that our method of adjustment may be masking an effect and matched Ranch Hands in the Background, Low, and High categories to Comparisons on a one-to-many basis on age, race, percent body fat, and military occupation. The matched results were similar to those reported here and did not lead us to a different conclusion. We studied the effect of changing our definition of Background, Low, and High dioxin exposure in Ranch Hands by using the tertiles of the current dioxin distribution in Ranch Hands to define the categories. Analysis using the tertiles of dioxin produced negligible changes in the results. We investigated the possibility of selection bias for the dioxin assay and found no evidence that Ranch Hands who volunteered for the assay were different from those who refused with regard to mean 2-hour postprandial glucose, fasting glucose or insulin, or diabetes prevalence.

Our ability to detect associations is limited by the fixed size of the Ranch Hand cohort. Since all Ranch Hands have been identified and invited to participate in the study, their number cannot be increased. Thus, the rarity of some abnormalities led to imprecise measures of association, as indicated by wide confidence limits, and small numbers prevented us from strong inferences on the most heavily exposed Ranch Hands.

Overall, the prevalence and severity of diabetes mellitus and the risk of abnormally high glucose increased and time-to-onset of diabetes decreased with dioxin in Ranch Hand veterans. Among nondiabetics, the risk of abnormally high insulin increased with dioxin. Taken together, these results indicate a possible relation between dioxin exposure and diabetes mellitus, glucose metabolism, and insulin production.

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24 CME REVIEW ARTICLE

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OBSTETRICAL AND GYNECOLOGICAL SURVEY

CHIEF EDITOR'S NOTE: This article is part of a series of continuing education activities in this Journal through which a total of 36 *AMA/PRA Category 1 Credits*™ can be earned in 2007. Instructions for how CME credits can be earned appear on the last page of the Table of Contents.

Antenatal Exposure to DES: Lessons Learned . . . Future Concerns

Mary M. Rubin, RNC, PhD, CRNP

Associate Clinical Professor, School of Nursing; and Research Coordinator, Gynecologic Oncology Clinical Research, University of California, San Francisco, California

The short- and long-term effects of the widespread use of diethylstilbestrol (DES) over 3 decades have become a distant memory for many clinicians. Others are too young to remember the flurry of activity in the early 1970s on the part of many medical centers to identify the offspring of women who were prescribed DES during their pregnancies. This medication was given in an attempt to prevent multiple pregnancy-related problems such as miscarriage, premature birth, and abnormal bleeding. The recognition of the association of DES with an increased incidence of cervical and vaginal cancers in very young women led the Food and Drug Administration to ban its use during pregnancy in 1971. Other pregnancy-related problems for the daughters and genitourinary tract changes in the sons did not become apparent until years later. Ongoing follow-up of these offspring has raised concerns for their future as well as their mothers' future. Clinicians need to be up-to-date with current knowledge regarding risks for cancer and other health-related issues.

Target Audience: Obstetricians & Gynecologists, Family Physicians

Learning Objectives: After completion of this article, the reader should be able to relate that since 1971 there has been an intense follow-up of DES-exposed daughters, explain that certain complications have no time limit, recall that pregnancy complications of these daughters is not insignificant, and state that continued follow-up is necessary.

BACKGROUND

The effects of diethylstilbestrol (DES), a nonsteroidal estrogen used for many years in the United States to improve pregnancy outcome, are nearly forgotten or unknown to many in the medical community (1). The aftermath of the widespread antenatal use of this drug by nearly 5 million women needs to be revisited to bring to consciousness the current and future health care needs of these individuals. It is estimated that 10 million mothers, daughters, and sons were exposed to DES between the years 1938 and 1971 (2). This review is an attempt to assist health care providers to be well informed about this topic.

HISTORICAL REVIEW

DES was first synthesized in 1938. Many in the medical community embraced the use of this potent medication and were relieved that there was finally an estrogen that would be easy to dispense and well tolerated. Much of the initial research to improve pregnancy outcome for diabetic patients was conducted in the United States. It was noted that urinary estrogen and progesterone were decreased in first trimester abortions, leading to testing the hypothesis that exogenous estro-

Dr. Rubin has disclosed that she is a Consultant/Advisor for GlaxoSmithKline, was on the Speakers Bureau for Cytyc and Digene, and is on the Speakers Bureau for Merck.

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Reprint requests to: Mary M. Rubin, RNC, PhD, CRNP, University of California, San Francisco, 1600 Divisadero St., MZ Bldg. A 758, Box 1702, San Francisco, CA 94143-1702. E-mail: mary.rubin@ucsfmedctr.org.

gens could prevent spontaneous abortions by administering DES to any woman who presented for prenatal care in the first half of her pregnancy. This was not a randomized placebo-controlled study as would be required today in testing drug efficacy. In 1948, published reports declaring that DES prevented pregnancy loss led the Food and Drug Administration (FDA) to approve the use of DES to prevent spontaneous abortion. The Smith and Smith regimen of dosing DES, starting with 5 mg at 6 weeks' gestation and gradually increasing the dose throughout the pregnancy, was widely utilized by many clinicians (3).

Over the next 50 years, DES was manufactured by over 267 companies under a variety of names and was administered by several methods. Although the most popular method was by pills, DES was also given by injections, or in suppositories and creams. In addition, it was often combined with progestins, androgens, and vitamins. DesPLEX, for example, also contained vitamin C, B complex, including folic acid and B12, so many women thought they were just taking prenatal vitamins. Doses were not standardized and varied widely by brand name and formulations. See Table 1 for the listing of common names under which DES was dispensed (4).

Some medical journals in the 1950s ran advertisements recommending the use of DES to prevent threatened abortion, habitual abortion, premature labor, and other pregnancy complications. In addition, they went further to recommend the use of DES for "routine prophylaxis" in all pregnancies. The author has personal experience with interviewing and counseling many DES mothers and daughters in one East Coast city in the 1970s. It was standard of care in at least one obstetrical practice to begin giving DES at the first prenatal visit and to increase the dose throughout the pregnancy as described earlier. The ability to obtain the original prenatal records confirmed this practice. The remaining sources for obtaining those records are from 1) the physician who prescribed the drug, 2) the pharmacy that dispensed the drug, and 3) the medical record at the hospital where the child was born.

TABLE 1
DES-related compounds

Benzetrol	Estrobene DP	Pabestrol D
Chlorotrianisene	Estrosyn	Palestrol
Comestrol	Fonatol	Restrol
Cyren A	Gynben	Stil Rol
Cyren B	Gyneben	Stilbal
Divinal	Hexestrol	Stilbestrol
DES	Hexoestrol	Stilbestonate
DESPlex	Hi-Bestrol	Stilbetin
Dibestil	Menocin	Stilbinol
Diestryl	Meprane	Stilboestrol
Dienestrol	Mestilbol	Stilboestrol DP
Dienoestrol	Microest	Stilestrate
Diethylstilbestrol	Mikarol	Stilpalmitate
Dipalmitate	Mikarol forti	Stilphostrol
Diethylstilbestrol	Milestrol	Stilronate
Diphosphate,	Monomestrol	Stilrone
Diethylstilbestrol	Neo-Oestranol I	Stils
Dipropionate	Neo-Oestranol II	Synestrin
Dithylstibenediol	Nulabort	Synestrol
Digestil	Oestrogenine	Synthoestrin
Domestrol	Oestromenin	Tace
Estilben	Oestromon	Vallestril
Estrobene	Orestrol	Wallestrol
Estrogen-androgen combination	Estrogen-progesterone combination	Vaginal cream suppositories
Amperone	Progravidum	AVC Cream w/Dienstrol
Di-Erone		Dienstrol Cream
Estane		
Metystil		
Teserene		
Tylandril		
Tylosterone		

INDICATIONS FOR THE USE OF DES

There were multiple reasons for prescribing the drug (see Table 2). Over the years, studies were conducted regarding the efficacy of DES such as one published in 1953 (5). This randomized double-blind study at the University of Chicago Lying-in-Hospital included 1646 patients and showed no improvement in pregnancy outcome. Despite the results, DES continued to be used. The question is why? Speculation regarding the answer includes the theory that because no known ill effects were readily identifiable, doctors did not believe the studies.

CLEAR CELL CANCER

In 1971 Herbst et al reported a series of young women with a rare type of cervical and vaginal cancer, clear cell adenocarcinoma (CCA) (6) (see Fig. 1). This type of cancer was previously seen in older women only in their sixth to ninth decade of life. Efforts were made to identify a common risk

TABLE 2
Indications for use of DES

Bleeding or threatened miscarriage
Diabetes
Hypertension
Prior miscarriage or spontaneous abortion
RH incompatibilities
Prophylaxis "to make normal pregnancies more normal"

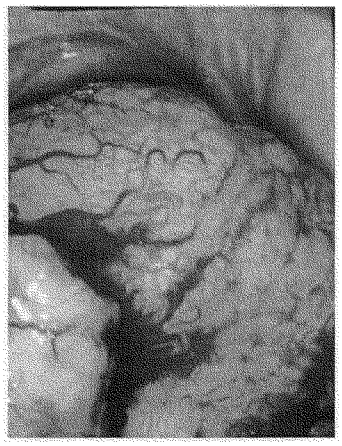

Fig. 1. Clear cell adenocarcinoma of the cervix. Note the atypical vessels on this exophytic lesion.

factor and DES was found to be that drug. The FDA banned DES for use in pregnancy in 1971.

A tumor registry for transplacental carcinogenesis was established in 1971. To date, more than 750 cases have been reported. Sixty percent have confirmed exposure and in the remainder exposure was excluded or unconfirmed. The risk of CCA is approximately 1:1000 to 1:10,000 and the age at diagnosis ranges from 7 to 48. Some unanswered questions regarding this cohort of women exposed in utero to DES include: What is the risk as these women age and is it increased or the same as the general population? Thus far, there is no upper age limit for CCA!

Symptoms for CCA include bleeding or abnormal vaginal discharge, gross lesions on the vagina or cervix, and nodules palpated on the cervix or vagina. Most often diagnosis is not easy. There is little role for effective screening cytology, because there is no known premalignant lesion. When clinical exam reveals a suspicious lesion, biopsy is mandatory to rule out cancer. Therapy must be managed by a gynecologic oncologist, once the diagnosis of CCA is established.

Treatment choices most often include radical hysterectomy with lymph node resection. Partial or full vaginectomy, depending on the primary site, may be required. Preservation of the ovaries and vaginal reconstruction are individualized.

Radiation with or without surgery is another treatment approach. Local excision has been associated with higher recurrence rates and unfortunately should not be considered. Chemotherapy has also not proven to be effective.

There is a 5-year survival rate of 83% and a 10-year rate of 77%. Long-term follow-up is essential. Some recurrences have been diagnosed as long as 20 years later. The most common metastatic site is lung or brain (7).

TABLE 3
Health risks for DES daughters

Malignancy of cervix, vagina, breast
Intraepithelial neoplasia
Congenital anatomic anomalies
Infertility
Pregnancy problems
Immunologic disorders (e.g., lupus erythematosus)
Paraovarian cysts (not leiomyomata)

HEALTH RISKS FOR DES DAUGHTERS

Many health risks have been noted in DES-exposed female offspring (see Table 3).

In 1998 a combined study of 3 research cohorts including 4536 DES-exposed daughters and 1544 controls was published. They found the relative risk (RR) for breast cancer to be 1.18 and no increased risk of any cancer other than CCA (8). One needs to keep in mind that the average age at last follow-up for patients in this study was only 38 years. Therefore, these women need further surveillance to know the true incidence.

More commonly, adenosis, mucin-producing epithelium extending out onto the vagina, is found. It occurred when DES was given between 6 and 18 weeks' gestation and affects 30% of the exposed population (9).

CERVICAL INTRAEPITHELIAL NEOPLASIA

In 1984 the National Cooperative Diethylstilbestrol-Adenosis (DESAD) project data with 3980 DES-exposed daughters and 744 controls were reviewed. A higher incidence of intraepithelial neoplasia in the DES-exposed group with 15.7 versus 7.9 cases of intraepithelial neoplasia per 1000 women-years was found. Most cases were low-grade intraepithelial neoplasia (LSIL) (10).

In a 2001 study, the RR of 2.12 for DES daughters for high-grade squamous neoplasia was found (11).

TABLE 4
Epithelial changes

Adenosis
Ectropion
Increased squamous metaplasia
Wide transformation zone often extending into the vagina

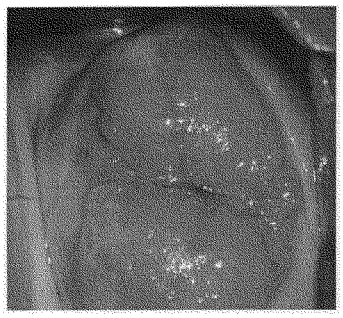

Fig. 2. Ectropion, or an abundance of columnar epithelium covering the entire portio of the cervix.

Fig. 3. Active squamous metaplasia, or columnar epithelium evolving into mature squamous epithelium.

Individualized treatment plans are suggested. Clinicians must exercise caution with treatment because there is a tendency for scarring easily. Cryotherapy has been associated with stenosis. Follow the American Society for Colposcopy and Cervical Pathology Consensus Guidelines for the management of these individuals when they present with abnormal cytology and appropriate treatment for biopsy-proven cervical intraepithelial neoplasia (12,13).

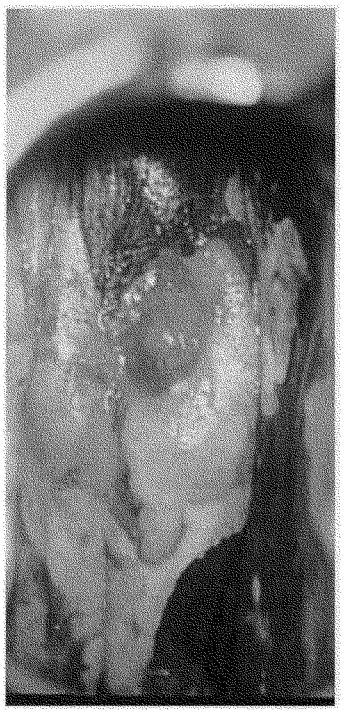

Fig. 4. Wide posterior transformation zone highlighted as nonstaining with Lugol's solution.

TABLE 5
Structural variations in DES daughters

Site	Cervix	Vagina	Uterus
Lower genital tract	Cockscomb Collar (partial or complete) Ridges and folds Hood Pseudopolyp Loss of pars	Ridge or septum Constriction rings	
Upper genital tract			T-Shaped Constrictions (upper, middle, corneal) Irregular uterine margins

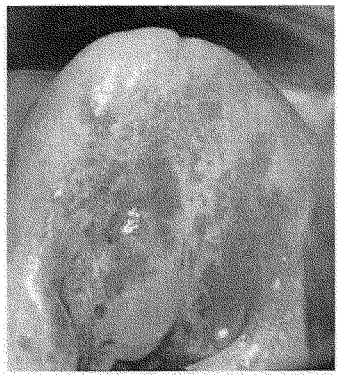

Fig. 5. Cockscomb, an anterior cervical structural variation.

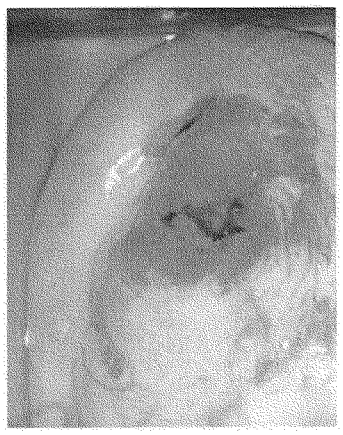

Fig. 6. Partial cervical collar and wide active transformation zone.

EPITHELIAL CHANGES

Unique cellular changes can often be observed in the lower genital tract during routine gynecologic examination, although colposcopic examination clarifies the details of these changes (14) (see Table 4 and Figs. 2–4). As DES daughters mature, the extent of these changes is more difficult to document.

STRUCTURAL VARIATIONS

Since the 1970s, upper and lower genital tract structural changes have been documented in 25% to 33% of the young women exposed to DES (see Table 5 and Figs. 5–8). Many have been found to resolve over time (15).

INFERTILITY

Analysis of the Dieckmann and DESAD cohorts shows significant risk for infertility at 33%. Twenty-four percent of the DES-exposed nulligravidas versus 18% of the nonexposed (RR 1.3) never achieved pregnancy. Twenty-eight percent of DES-exposed women attempted pregnancy for 12 months without success versus 16 nonexposed controls (RR 1.8). DES exposure is associated with primary infertility (RR 2.0) and secondary infertility (RR 2.0) (16).

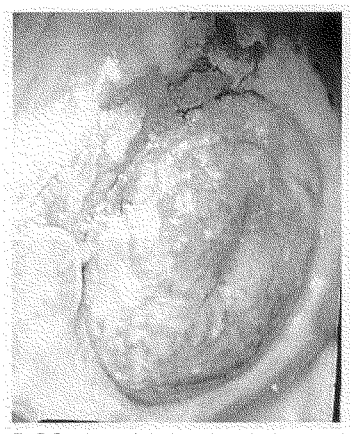

Fig. 7. Complete cervical collar and pseudopolyp cervix with adenosis.

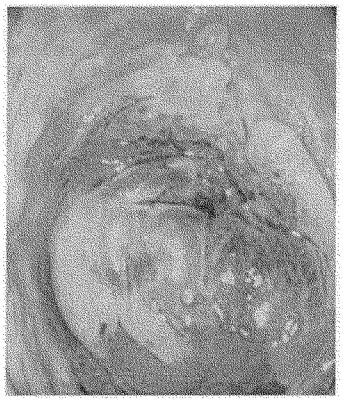

Fig. 8. Cervical ridges and folds undergoing active squamous metaplasia.

PREGNANCY OUTCOME

Overall outcome is significantly poorer. There is an increased incidence of spontaneous first trimester loss (RR 1.31), spontaneous second trimester loss (RR 4.25), premature birth (RR 2.93), and ectopic pregnancy (RR 3.84). Only 64.1% of pregnancies resulted in full-term live births versus 84.5% in unexposed women. However, 85% resulted in live births (17).

HEALTH CARE GUIDELINES FOR WOMEN

DES-exposed daughters should have annual examination. Cytology sampling should include collection of endocervical and ectocervical cells as well as those from the vaginal fornices. Four-quadrant vaginal wall biopsies are no longer routinely performed.

The examination should include thorough vaginal/cervical inspection, taking time to view underneath the speculum. This can be done by slowly withdrawing the speculum, allowing the anterior and posterior vaginal walls to come into view. This is best accomplished while looking through the colposcope. Careful palpation of the cervix and entire length of the vagina (include fornices) should be done. Ridges or structural changes should be noted and a bimanual rectal-vaginal examination is most effective in assessing the vagina for nodularity. A biopsy of any areas of thickening or induration, palpable nodularity, or atypical colposcopic finding is mandatory. Colposcopy is not always routinely done, but most DES experts around the country still follow patients with colposcopy at least periodically. It should always be the next step in management after abnormal cytology. The frequency of Pap smears is best individualized, although yearly is usually sufficient. With each visit, focus on changes from the initial examination and ask about interval bleeding or abnormal vaginal discharge (18).

DES SONS

Recent studies show no increase in infertility even among those with genital malformation. It is difficult to assess DES-related effects associated with testicular cancer due to its low prevalence. Several studies showed no increase in testicular cancer (19,20). Studies in mouse model continue to raise concern for the future for increased rates of testicular cancer. Follow-up of DES-exposed men as they age is critical, because the true risk in humans is unknown. The level

TABLE 6
DES effect in men

Decreased sperm count
Epididymal cysts (20.8% vs. 4.9%)
Hypospadius
Microphallus, undescended testicles
Testicular hypoplasia

of concern for prostate cancer among DES-exposed men in the future is as yet unknown. To date, no other associated cancers have been reported (21). See Table 6 for other DES-related effects in male offspring.

THIRD-GENERATION EFFECTS

Animal studies have shown tumor growth in older third-generation mice (human equivalent to age 70) (22). Multigenerational studies in humans are currently underway. Several small studies of teenage third-generation females have not shown the same type of changes as in their mothers. Sons of DES daughters are at increased risk for hypospadias (23).

DES MOTHERS

DES mothers are now aged 50 to 90. Breast cancer has an RR of 1.3. No study has shown an RR of 2 or greater. However, if there is a family history of breast cancer, the RR is 2.1.

For those using hormone replacement therapy (HRT), the risk of breast cancer is 1.35. The mothers should be followed up and evaluated as age-appropriate with mammography, clinical evaluation, and self breast examination. To date, no other cancers have been associated with their exposure to DES (24).

FUTURE CONCERNS

Autoimmune disorders are currently being studied since the DESAD project has noted an increased incidence in their cohort of DES offspring.

Environmental and dietary chemical exposure as well as estrogen modulators used in chemoprevention are being studied in animal models and raise concern for humans with antenatal exposure to DES (25).

Many unanswered questions remain for these 10 million individuals exposed to DES years ago. With more than 40 years of data gathering, some of the early effects of antenatal exposure are now better understood. However, health care providers must remain vigilant to the possible unknown long-term effects of exposure to this synthetic estrogen. Identifying these individuals remains a challenge, as the prenatal records become less accessible.

RESOURCES

CDC DES Update

- CDC Web site for health care providers (www.cdc.gov/DES)
- 1-888-232-6789 NCI 1-800-4CANCER

Patient Support

- DES Action USA (www.desaction.org)
- DES Cancer Network (www.descancer.org)

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Perinatal Exposure to Bisphenol-A Alters Peripubertal Mammary Gland Development in Mice

Monica Muñoz-de-Toro, Caroline M. Markey, Perinaaz R. Wadia, Enrique H. Luque, Beverly S. Rubin, Carlos Sonnenschein, and Ana M. Soto

Department of Anatomy and Cellular Biology (C.M.M., P.R.W., B.S.R., C.S., A.M.S.), Tufts University School of Medicine, Boston, Massachusetts 02111; and Laboratorio de Endocrinologia y Tumores Hormonodependientes (M.M.-d.-T., E.H.L.), Faculty of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina

Developmental exposure to estrogenic chemicals induces morphological, functional, and behavioral anomalies associated with reproduction. Humans are exposed to bisphenol-A (BPA), an estrogenic compound that leaches from dental materials and plastic food and beverage containers. The aim of the present study was to determine the effects of perinatal exposure to low, environmentally relevant doses of BPA [25 and 250 ng BPA/kg body weight (bw)·d] on the peripubertal development of the mammary gland. BPA exposure enhanced the mammary glands' sensitivity to estradiol in ovariectomized CD-1 mice. In their intact 30-d-old littermates, the area and numbers of terminal end buds relative to the gland ductal area increased whereas their apoptotic activity decreased. There was a positive correlation between ductal length and the age at first proestrus; that was reduced as the BPA dose increased, suggesting that BPA exposure slows down ductal invasion of the stroma. There was also a significant increase of progesterone receptor-positive ductal epithelial cells that were localized in clusters, suggesting future branching points. Indeed, lateral branching was significantly enhanced at 4 months of age in mice exposed to 25 ng BPA /kg bw·d. In conclusion, perinatal exposure to environmentally relevant BPA doses results in persistent alterations in mammary gland morphogenesis. Of special concern is the increased terminal end bud density at puberty as well as the increased number of terminal ends reported previously in adult animals, as these two structures are the sites at which cancer arises in humans and rodents. (*Endocrinology* 146: 4138–4147, 2005)

PERTURBATIONS IN THE fetal environment may predispose individuals to disease and/or dysfunction, such as hypertension and coronary heart disease that become apparent in adulthood (1). Epidemiological studies also suggest that the intrauterine milieu may have an influential role in predisposing an individual to carcinogenesis. For example, dizygotic twin births correlate with an increased incidence of breast cancer in female siblings (2); on the other hand, females born to mothers who had eclampsia or preeclampsia, have a decreased breast cancer risk. These outcomes have been attributed to changes in the fetal hormonal milieu, in particular estrogens (3). The mechanisms underlying this observation are presently unknown.

During the last decade, observations made in the estrogen receptor (ER)-α and -β knockout models suggested that the mammary gland develops in an estrogen-independent manner until puberty (4, 5). The corollary of this particular view of development was that perinatal exposure to exogenous estrogens would have no effect on the development of the mammary gland. This concept was reinforced by the fact that the mouse mammary gland does not express a proliferative response to estrogens before the third week of age (6). However, administration of supraphysiological doses of estradiol (E_2) (*i.e.* doses of 35 μg/mouse·d or higher) to mice at postnatal d (PNDs) 1–5 resulted in changes in the mammary gland, such as increased ductal branching, which did not become obvious until the fifth week of life (7). Similarly, prenatal exposure to pharmacological levels of the estrogen diethylstilbestrol (DES) enhanced the sensitivity of the gland to hormones and carcinogens administered during adulthood, thus increasing mammary cancer incidence in rodent models (8, 9). These studies required addressing the notoriously pathological effects of *in utero* exposure to high doses of DES in humans (10, 11). Presently the concern about effects of estrogen exposure is focused on a far more subtle exposure, represented by environmental estrogens, which may affect mammary gland development and/or enhance the risk of breast cancer later in life.

Over the last 60 yr, humans have been exposed to a plethora of synthetic hormonally active chemicals overtly because of their deliberate use in agriculture and medicine; inadvertently as byproducts of industrial use; or as waste released into rivers, lakes, and the atmosphere. Environmental exposure to these chemicals has coincided with an increase in endocrine-related diseases of the male reproductive system (12) and increases in testicular (13) and breast cancers (14).

Among the endocrine disruptors, bisphenol-A (BPA) is receiving increased attention due to its high potential for human exposure. Used in the manufacture of polycarbonate plastics and epoxy resins, BPA leaches from food containers (15), beverage containers (16), and dental sealants and composites (17) under normal conditions of use (18). BPA is also

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Abbreviations: BPA, Bisphenol-A; BrdU, 5-bromo-2′-deoxyuridine; bw, body weight; DAB, diaminobenzidine; DES, diethylstilbestrol; E_2, estradiol; ER, estrogen receptor; PND, postnatal day; PR, progesterone receptor; RT, room temperature; TEB, terminal end bud; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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used in the manufacture of many products in addition to those stated above (19), which would further increase levels of human exposure to this compound. These reports suggest that humans routinely ingest BPA. Indeed, a recently published study, the first involving a reference human population (394 samples analyzed), reported that BPA was found in 95% of the urine samples (20). In a smaller study (36 samples analyzed), Arakawa *et al.* (21) reported a median daily urinary excretion of BPA of 1.2 μg/d and a maximum daily intake of BPA per body weight to be 0.23 μg/kg·d.

Before these studies, research on the effects of low-dose BPA exposure in animal models have used measurements of the amount of BPA leached from food cans, containers, and dental sealants as guidance for estimating a plausible exposure range of 2–20 μg BPA/kg body weight (bw)·d administered orally (22). BPA has recently been measured in human sera (adult men: 1.49 ± 0.11 ng/ml; adult women: 0.64 ± 0.10 ng/ml) (23), the maternal and fetal plasma, and placental tissue at birth in humans (24, 25). The concentration of BPA in amniotic fluid was approximately 5-fold higher than that measured in maternal plasma. The range of BPA concentrations assessed in the placenta was 1–100 ng/g with a median level of 12 ng/g, whereas in fetal plasma, levels ranged from 0.2 to 9.2 ng/ml. Recognizing that it is not feasible to delineate definitive exposure levels from the existing data, we have chosen to administer 25 and 250 ng BPA/kg bw·d sc by means of osmotic pumps to female mice on d 9 of pregnancy until PND4. Based on the data reported by Arakawa *et al.* (21), we estimate that this level of BPA exposure should fall within the range of human exposures reported to date.

Intrauterine exposure to BPA has been shown to advance puberty and disrupt estrous cyclicity in mice (26–28). Although the mechanism by which BPA is able to induce developmental abnormalities in estrogen-target tissues is unknown, it is plausible that ERs may mediate BPA-induced effects because this chemical binds both ERα and ERβ (29–31). Interaction between BPA and ERs may take place during fetal development because ERα and ERβ are first detected in the mouse mammary gland primordium at d 12.5 of gestation (32).

The purpose of the present study was to test whether *in utero* and neonatal exposure to environmentally relevant levels of BPA alters the peripubertal development of the mammary gland.

Materials and Methods

Animals

Sexually mature female CD-1 mice (8 wk of age; Charles River Laboratories, Wilmington, MA) were maintained in temperature-controlled and light-controlled (14-h light, 10-h dark cycle) conditions in the Tufts University School of Medicine animal facility. All experimental procedures were approved by the Tufts University–New England Medical Center Animal Research Committee in accordance with the Guide for Care and Use of Laboratory Animals. Food, cages, and bedding all tested negligible for estrogenicity by the E-SCREEN assay (33); water was supplied from glass bottles only. Female mice were mated with CD-1 males of known reproductive competence, and the morning on which a vaginal plug was observed was designated pregnancy d 1. On d 9 of pregnancy, mice were weighed and implanted with Alzet osmotic pumps (Alza Corp., Mountain View, CA) designed to deliver either dimethyl sulfoxide (vehicle control), 25 or 250 ng BPA/kg bw·d dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) for 14 d, which encompassed the remainder of the pregnancy through PND4 (n = 6–10/treatment). Offspring born on d 20 of gestation were culled to 10 pups per mother on PND7. The actual dose decreased as pregnancy progressed because the weight of the mother increased over this period. Female offspring were killed by CO_2 inhalation on PND20 and 30 and at 4 months of age (one pup/litter, n = 6–10/treatment). For mice killed at first proestrus, vaginal smears were taken daily after vaginal opening until a proestrus smear was detected. For animals killed at 4 months of age, vaginal smears were assessed daily for 2–3 wk to assess the pattern of estrous cyclicity; these mice were killed on the afternoon of proestrus. Proestrus was confirmed by the presence of uterine ballooning.

To assess whether perinatal BPA treatment altered the response to E_2, animals (one pup/litter) from each treatment group were ovariectomized at 25 d of age and immediately implanted with one Alzet pump delivering vehicle or 0.5 μg E_2/kg bw·d (n = 10/group). Animals were killed 10 d after ovariectomy.

Tissue collection. One and a half hours before kill, the animals were weighed and injected ip with 5-bromo-2′-deoxyuridine (BrdU) (1.5 mg/100 g bw; Roche, Indianapolis, IN). Trunk blood was collected in heparinized tubes and plasma was separated by centrifugation and stored at −80 C until assayed for E_2. The fourth and fifth mammary gland pairs were dissected out; the right mammary glands were whole mounted, and the left mammary glands were embedded in paraffin for light microscopy and immunohistochemistry. Mammary glands from an additional series of mice killed at first proestrus were collected under RNase-free conditions and immediately frozen for subsequent RNA isolation and RT-PCR.

Whole mounts and paraffin sections

To prepare the whole mounts, the mammary glands were spread onto glass slides, fixed with 4% paraformaldehyde in 0.1 M PBS overnight, stained with Carmine Alum (Sigma), dehydrated, and whole mounted with Permount. To prepare paraffin sections, mammary glands were fixed with 4% paraformaldehyde in 0.1 M PBS for 18–20 h at room temperature (RT), washed in 0.1 M PBS, dehydrated through a series of alcohol and xylene, and embedded with Paraplast paraffin (Fisher, Pittsburgh, PA) under vacuum. Five-micrometer sections were cut on an RM2155 rotary microtome (Leica, Nusslock, Germany), mounted on Superfrost slides (Fisher Scientific International Inc., Hampton, NH), and stained with hematoxylin and eosin or processed for immunohistochemistry to assess DNA synthesis, apoptosis, or expression of ERα and progesterone receptor (PR).

Immunohistochemistry

BrdU was immunolocalized within paraffin sections of mammary glands from 30-d-old mice using the streptavidin-biotin labeling system as described previously (34). Sections were hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, subjected to acid hydrolysis for DNA denaturation, and treated for both endogenous peroxidase and nonspecific binding with methanol/H_2O_2 and 2% normal goat serum in 0.01 M PBS, respectively. Sections were incubated with monoclonal BrdU antibody IgG (1:400; clone 85-2C8; Novocastra, Newcastle upon Tyne, UK) overnight in a humid chamber at 4 C. Biotinylated goat antimouse IgG (Sigma) was applied and reaction visualized by streptavidin-peroxidase-diaminobenzidine (DAB) (Sigma) to assess cellular incorporation of BrdU. Sections were lightly counterstained with Harris' hematoxylin and mounted with a glass coverslip for light microscopy.

The expression of ERα and PR was evaluated by immunohistochemistry in paraffin sections of mammary tissue obtained from 30-d-old mice, as previously described (35). Using the biotin-streptavidin-peroxidase method, tissue was incubated with primary antibodies for ERα (1:60; mouse 6F-11 clone; Novocastra Laboratories) and PR (1:100; mouse PR-AT 4.14 clone; Affinity BioReagents Inc., Golden, CO) at 4 C for 14–16 h, and DAB was used as the chromogen to visualize reactions. Sections were counterstained with Mayer's hematoxylin. Each immunohistochemical run included positive and negative controls. In the negative control slides, the primary antibody was replaced with nonimmune mouse serum (Sigma). To minimize spurious variation among experi-

ments, each immunostaining procedure was performed in sets containing tissues from an equal number of subjects from all the experimental groups (0, 25, and 250 ng BPA/kg bw·d).

In situ detection of apoptosis

Apoptosis was evaluated in mammary glands from 30-d-old mice. Sections were analyzed for *in situ* detection of cells with DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (ApopTag; Intergen Co., Purchase, NY) as previously described (36). Briefly, after deparaffinization and rehydration, sections were incubated with proteinase K (5 μg/ml; Intergen) for 10 min at 37 C and then treated with hydrogen peroxide PBS for 10 min at RT to quench endogenous peroxidase activity. Sections were then incubated with a mixture containing digoxigenin deoxynucleotide triphosphate, unlabeled deoxynucleotide triphosphate, and terminal dideoxy transferase enzyme in a humidified chamber at 37 C for 1 h. Subsequently slides were rinsed with PBS and incubated with antidigoxigenin-peroxidase for 30 min at RT and substrate-chromogen mixture (DAB; Sigma) for 4 min. Samples were counterstained with Mayer's hematoxylin, dehydrated, and mounted with permanent mounting medium. Negative control slides were run using exactly the same procedures, except distilled water was added instead of terminal dideoxy transferase enzyme. An involuting mouse mammary gland collected 3 d after weaning was processed in an identical manner and used as positive control.

Quantitative analysis of the expression of BrdU, ERα, PR, and apoptosis

The incorporation of BrdU in mammary glands from 30-d-old mice was quantified using a Zeiss microscope (Carl Zeiss Inc., Göttinger, Germany) and a ×100 objective oil immersion lens. A graticule was placed in the eyepiece to quantitate the percentage of BrdU-stained cells within the epithelium per total number of cells in approximately 50 arbitrarily chosen fields per mammary gland.

Quantitative analysis of ERα and PR expression was performed in both epithelial and stromal compartments of the mammary gland. Two different stromal areas were defined: first, a periepithelial stroma (stroma immediately surrounding epithelial structures, composed of fibroblastic-like cells and a dense extracellular matrix) and second, adipose stroma (fatty stroma not adjacent to epithelial structures) (37). Receptors associated with the capsule of the mammary gland were not included in the analysis.

For analysis of cells expressing ERα and PR, 50 representative fields (at least 1000 epithelial cells/section) in each section were analyzed using a Dplan ×100 objective lens. All immunostained epithelial and stromal nuclei in the defined regions, regardless of intensity, were defined as positive. Positive cells were expressed as the percent ratio of total number of epithelial or stromal cells evaluated. In tissue from 30-d-old mice, the percentage of apoptotic cells was quantified in the body cells of the terminal end buds (TEBs) at the peripheral leading edge of the advancing ductal mass, according to the methodology described by Humphreys *et al.* (38).

Morphometric analysis of whole mounts

Digital images of whole mounted mammary glands were captured with a SPOT-real time digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a dissecting microscope (Carl Zeiss). Quantitative analyses of mammary glands were performed using the Optimus 6.5 program (Media Cybernetics, Silver Spring, MD). In 30-d-old mice, the length of the ductal tree along its longitudinal axis, the total area of the mammary gland occupied by the epithelial ducts, and the total number and size of all TEBs were analyzed. The ductal area was determined by measuring the region defined by the outermost edges of the epithelial tree relative to the area of the entire fat pad (or the total area of the mammary gland epithelium was determined on digital images of mammary glands (for d 20: objective, ×1, magnified, ×400; for d 30: objective, ×0.8, magnified, ×400) by using the threshholding tool within Optimus, taking advantage of the greater staining intensity of the mammary gland epithelium relative to the remainder of the gland). The total number and size of TEBs were measured on digital images of the entire mammary gland (×0.8 objective, magnified ×400); the size of the TEBs was determined by placing each structure into a size category, ranging from smallest to largest, based on its area and perimeter length.

Morphometric analysis of ductal side branching was performed on whole mounted mammary glands of 4-month-old mice. Digital images were captured using a ×4 objective. Side-branching evaluation was carried out using Image Pro-Plus 4.1.0.1 system (Media Cybernetics) by counting the total number of branch points along the ductal network. Results were expressed as the number of branch points per 500 μm of duct length.

RNA isolation

Total RNA was isolated from whole mammary glands using TRIzol (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions and stored at −80 C until analyzed. RNA concentration was determined using the ratio of UV absorbance read at 260/280 nm.

Analysis of Wnt4 mRNA expression using RT-PCR

To remove genomic DNA, RNA isolated from mammary glands of 30-d-old mice was treated with DNase (Invitrogen) before performing reverse transcription with random primers and Superscript II RNase H$^-$ reverse transcriptase (Invitrogen). All of the above procedures were carried out according to the manufacturer's instructions. An aliquot of cDNA (2.9 μl) from control and treated mammary glands was added to each PCR tube along with 10 μl master mix (Quantitect SYBR Green PCR kit, QIAGEN Inc., Valencia, CA) containing SYBR green as the detection dye and 6-carboxy-X-rhodamine as the normalizing dye, and 1 U/tube uracil-DNA-glycosylase, heat labile (Roche Diagnostics Corp., Indianapolis, IN) to prevent DNA contamination from previous PCRs, according to the instructions of the manufacturer. Forward and reverse primers for Wnt4, GATGTGCAAACGGAACCTTGA, and GTCACCACCTTCCCAAAGACAG (PCR product = 144 bp), respectively, were also added to the tubes. The internal control, ribosomal protein L19, was run in separate tubes using the primers: forward, ATCGCCAATGCCAACTCC and reverse, TCATCCTTCTCATCCAGGTCA (PCR product = 205 bp). Real-time PCR was performed in the MX4000 thermocycler (Stratagene, La Jolla, CA) under the following conditions: 50 C for 2 min and 95 C for 15 min, followed by 45 cycles of 95 C for 15 sec, 63 C for 30 sec, and 72 C for 30 sec.

The SYBR green fluorescence data for each sample were analyzed on the MX4000 software (Stratagene); after normalization with the 6-carboxy-X-rhodamine signal followed by baseline correction, an amplification curve was obtained to calculate the threshold cycle for each sample. Absolute standards (200×10^3 to 390 copies/tube) for both Wnt4 and L19, prepared from purified cDNA identical with real-time PCR products, were included on each plate to ensure equal efficiency of amplification between standards and PCR products generated in sample wells. The efficiency of the PCR was more than 95% for both Wnt4 and L19 as calculated from the standard curves with $R^2 = 0.9861$ and $R^2 = 0.9714$, respectively. The expression of Wnt4 was then determined by interpolating the threshold cycle values of each sample from the Wnt4 standard curve and was normalized with the expression of L19 of that sample, which was calculated in the same way. An average of values obtained from three runs was used as the final result to compare Wnt4 expression between control and treated groups. Contamination by genomic DNA was assessed in each sample by running the reaction in the absence of reverse transcriptase. The purity and specificity of the PCR products was confirmed by the SYBR Green dissociation curve set up at the end of each PCR run.

RIA for plasma E_2

Steroids were extracted from 200 μl of plasma using 6 ml diethyl ether (>99% pure; Sigma). Percent recovery of extraction was calculated by the addition of a fixed amount of tracer. Plasma E_2 levels were determined by RIA (DSL-39100; Diagnostic Systems Laboratories Inc., Webster, TX) following the manufacturer's instructions.

Statistics

The statistical program SPSS (SPSS, Inc., Chicago, IL) was used. Statistical analysis was performed by one-way ANOVA and significance

TABLE 1. Morphometric analyses of mammary gland structures and plasma estradiol levels in animals exposed perinatally to vehicle or BPA

Parameter	0 ng BPA/kg bw·d	25 ng BPA/kg bw·d	250 ng BPA/kg bw·d
No. of TEBs at PND 20	2.0 ± 1.1	3.0 ± 1.38	2.1 ± 0.7
No. of TEBs at PND30	12.0 ± 1.75	15.67 ± 1.52	15.83 ± 1.16
No. of TEBs/ductal area at PND 30	0.184 ± 0.029	0.271 ± 0.029[a]	0.290 ± 0.016[b]
Total area (mm^2) TEBs at PND30	0.580 ± 0.091	0.715 ± 0.076	0.802 ± 0.073
Area TEBs/ductal area at PND30	0.0091 ± 0.0017	0.012 ± 0.0014	0.153 ± 0.0012[c]
Ductal area (mm^2) at PND30	67.55 ± 6.60	65.22 ± 7	59.47 ± 5.08
Ductal tree length (mm) at PND30	5.79 ± 0.64	6.11 ± 0.62	5.73 ± 0.33
% ER-positive epithelial cells at PND 30	52.03 ± 1.62	53.4 ± 1.56	58.17 ± 2.83
E_2 levels at 1st proestrus pg/ml	18.11 ± 0.99	18.42 ± 0.812	15.92 ± 0.875

Data are represented as the mean ± SEM.
[a] $P = 0.054$.
[b] $P = 0.007$.
[c] $P = 0.005$ when compared with the respective vehicle-treated control.

between groups was determined by Dunn's *post hoc* test. In specific cases in which the data were not distributed normally, a Kruskal-Wallis test was employed and differences between the control and BPA treatment groups were assessed by Mann-Whitney *U* tests (39). Results were considered significant at $P < 0.05$.

Results

Number, size, and area of TEBs

The period around puberty, *i.e.* between 20 and 30 d of age, is characterized by the reinitiation of ductal growth in the mammary glands. Estrogens play a major role in this process (40). Bulbous TEBs (area > 0.03 mm^2) form at the tips of the ducts. These structures invade the stroma and mediate the longitudinal growth of the subtending ducts. When the ductal tree reaches the edge of the fat pad, the TEBs mature into a resting structure, the terminal ends, and ductal growth ceases. The number of bulbous TEBs was similar in the mammary glands of all groups at 20 d of age (Table 1). At 30 d of age, there was a significant increase in the number of TEBs relative to the area occupied by the ductal tree in the animals exposed to 250 ng BPA/kg bw·d, compared with that in the vehicle-treated controls ($P = 0.008$), whereas the increase in the 25 ng BPA/kg bw·d approached significance ($P = 0.054$) when compared with control (Fig. 1A and Table 1). Similarly, when these data were expressed as TEB area relative to ductal tree area, a significant increase was observed at 250 ng BPA/kg bw·d with respect to the vehicle-treated control ($P < 0.05$) (Fig. 1B and Table 1). The absolute number of TEBs and the total area occupied by TEBs were increased in the mammary glands of BPA-treated animals when compared with the control group (Table 1); however, this increase was not statistically significant. The increased number and area of TEBs relative to the ductal area in the BPA-exposed animals suggested that ductal growth might be impaired.

Analysis of epithelial ducts in whole mounts in 30-d-old animals

To assess whether ductal growth was affected at 30 d of age, two parameters were measured: the length of the ductal tree from the center of the lymph node to the leading edge and the total area covered by the ductal tree. There were no significant differences among the experimental groups regarding the area covered by the epithelial ducts of the mammary gland; however, a trend toward reduction of the ductal area was observed in the BPA-treated animals (Table 1). A measure of the length of the ductal tree from the center of the lymph node to the leading edge also did not show a significant difference between the control and treated groups.

The length of the ductal tree was also measured at the first proestrus. There was a positive correlation between ductal length from the center of the lymph node to the leading edge and the age at first proestrus (Fig. 2). The slope was steepest in the controls (m = 0.8822) and became reduced as the BPA dose was increased (25 ng BPA/kg bw·d: m = 0.501; 250 ng BPA/kg bw·d: m = 0.1249), suggesting that BPA exposure

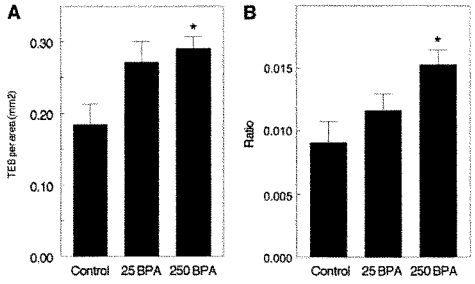

FIG. 1. Effect of perinatal exposure to BPA (25 and 250 ng/kg bw·d) on the number of terminal end buds (A) and the terminal end bud area (B) relative to the total ductal area in the fourth mammary gland at 30 d of age (*bars* indicate mean ± SEM). The *asterisk* indicates statistical significance relative to the control (A, $P = 0.008$; B, $P = 0.005$).

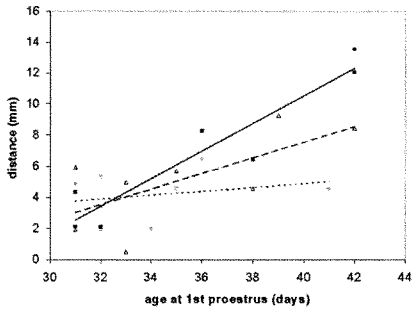

FIG. 2. Length of the ductal tree from the center of the lymph node to the leading edge measured in mammary glands of control (■, *solid line*) and BPA [25 ng (△, *dashed line*) and 250 ng (◆, *dotted line*) per kg bw·d] treated mice killed at the first proestrus.

slows down ductal invasion of the stroma, a phenomenon that is regulated by estrogens. Indeed, there was a statistically significant difference between the slopes of the control and 250 ng BPA/kg bw·d-exposed groups ($P = 0.005$). Moreover, the length of the ductal tree was significantly reduced in the subset of animals exposed to 250 ng BPA/kg bw·d, which had their first proestrus at 34 d of age or later (4.43 ± 0.71 mm), when compared with the controls (10.05 ± 1.63; $P = 0.027$). Two possible underlying causes are that perinatal exposure to BPA alters either the plasma E_2 levels or the sensitivity of the mammary gland to these hormones. Measuring the plasma E_2 levels did not provide a clear-cut answer. Whereas there were no significant differences among the treatments regarding the plasma level of E_2 at proestrus (Table 1), E_2 levels were slightly, but not significantly, lower in the 250 ng BPA/kg bw·d group than the vehicle or 25 ng BPA/kg bw·d.

Perinatal BPA treatment alters the sensitivity of the mammary gland to E_2

To assess whether the increase in the number of TEBs/ductal area observed at 30 d of age in animals exposed perinatally to BPA was due to altered sensitivity to E_2, a set of animals exposed perinatally to vehicle or to 250 ng BPA/kg bw·d was ovariectomized at 25 d of age and treated for 10 d with pumps delivering 0 or 0.5 μg E_2/kg bw·d. Perinatal exposure to 250 ng BPA/kg bw·d significantly increased the response to 0.5 μg E_2/kg bw·d regarding the following parameters: total number of TEBs, total TEB area, mean TEB size, TEB number/ductal area, and TEB area/ductal area (Table 2 and Fig. 3). These findings suggest that perinatal exposure to BPA enhances the sensitivity of the mammary gland to estrogens.

Apoptotic and proliferative activity in the mammary gland

At puberty, as the TEBs become bulbous, they show both high proliferative and high apoptotic activity. Death of the body cells is essential for the formation of the lumen on the proximal side of the TEBs (38) and to the growth of the subtending duct. A large number of apoptotic cells were identified by the TUNEL method in TEBs of control mice at 30 d of age as shown in Fig. 4A. *In utero* exposure to BPA resulted in a significant decline in the number of apoptotic cells in TEBs of both treated groups (25 ng BPA/kg bw·d, $P < 0.001$; 250 ng BPA/kg bw·d, $P < 0.05$) relative to the controls (Fig. 4B). This decreased apoptotic activity suggests impaired ductal growth and may explain the increased number of TEBs/ductal area.

In contrast to the strong inhibitory effect on apoptosis, BPA exposure did not affect BrdU incorporation, a marker of proliferative activity, in the epithelial compartment. On the other hand, the percentage of stromal cells that incorporated BrdU decreased by 50% ($P < 0.05$) in the 250 ng/kg BPA group at 30 d of age (Table 3). Because the development of ductal structures involves remodeling of the stroma, these

TABLE 2. Perinatal exposure to 250 ng/kg·d alters the sensitivity to estradiol in the mammary glands of ovariectomized animals

	A.0BPA/0E2 (mean ± SEM)	B.0BPA/0.5E2 (mean ± SEM)	*P* (A and B)	C.250BPA/0E2 (mean ± SEM)	D.250BPA/0.5E2 (mean ± SEM)	*P* (C and D)	*P* (B and D)
No. of TEBs	1.4 ± 0.58119	5.5 ± 1.16667	0.004	2.09091 ± 0.77991	10 ± 0.64979	0.000	0.007
TEB area	0.048 ± 0.0201	0.219 ± 0.05374	0.007	0.07818 ± 0.02885	0.428 ± 0.0396	0.000	0.005
Ductal extension	2.6155 ± 0.62599	4.41091 ± 0.74227	ns	2.80273 ± 0.7631	4.686364 ± 0.5776	0.016	ns
Ductal area	79.081 ± 8.65917	126.135 ± 9.20329	0.002	82.2555 ± 6.96275	118.5518 ± 8.21476	0.005	ns
TEB size	0.0137 ± 0.00559	0.03384 ± 0.0043	0.023	0.02021 ± 0.00599	0.04228 ± 0.00164	0.000	ns
No. of TEBs/area	0.0146 ± 0.00622	0.04195 ± 0.00896	0.029	0.02289 ± 0.00599	0.084893 ± 0.00621	0.000	0.003
TEB area/area	0.0005 ± 0.00021	0.00167 ± 0.00041	0.019	0.00086 ± 0.0003	0.003576 ± 0.00029	0.000	0.004

Animals exposed perinatally to 250 ng BPA/kg bw·d or to vehicle were ovariectomized at 25 d of age and implanted with osmotic pumps delivering 0 or 0.5 μg estradiol/kg bw·d from 25–35 d of age. A, Perinatal treatment with vehicle and implanted with pumps delivering vehicle at 25–35 d old; B, perinatal treatment with vehicle and implanted with pumps delivering 0.5 μg estradiol/kg bw·d at 25–35 d old; C, perinatal treatment with 250 ng BPA/kg bw·d and implanted with pumps delivering vehicle at 25–35 d old; D, perinatal treatment with 250 ng BPA/kg bw·d and implanted with pumps delivering 0.5 μg E_2/kg bw·d at 25–35 d old. *P* (A and B) denotes the *P* value of the comparison between groups A and B; *P* (C and D) denotes the *P* value of the comparison between groups C and D; and *P* (B and D) denotes the *P* value of the comparison between groups B and D. ns, Lack of statistically significant differences. No. of TEBs denotes number of TEBs at the leading edge of the 4th mammary gland; ductal area is expressed in mm^2. Ductal extension denotes the distance from the center of the lymph node to the leading edge in mm; TEB size is expressed in mm^2. TEBs/area denotes the number of TEBs relative to the ductal area; it is expressed as number/mm^2, and TEB area/area denotes the area (mm^2) occupied by the TEBs relative to the total area (mm^2) of the ductal tree. There were no statistically significant differences between the mice treated perinatally with 0 and 250 ng BPA/kg bw·d after ovariectomy for any of the parameters measured (groups A and C).

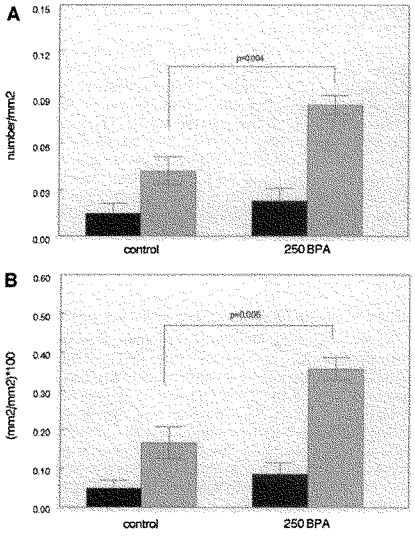

FIG. 3. Effect of perinatal exposure to 250 ng BPA/kg bw·d on the response to 0.5 μg E_2/kg bw·d administered from 25 to 35 d of age in animals ovariectomized at 25 d of age. A, Comparison of the number of TEBs per ductal area. B, Comparison of the area occupied by TEBs relative to the ductal area of the fourth mammary gland. The *gray bars* represent E_2 treatment; *black bars* represent controls (*bars* indicate mean ± SEM).

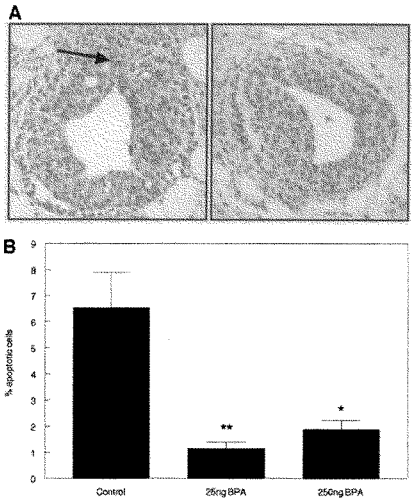

FIG. 4. Detection of apoptotic cells by the TUNEL method in mammary glands of mice treated perinatally with BPA or vehicle. A, Photomicrographs showing cells stained positive for apoptosis in terminal end buds of 30-d-old mice treated perinatally with vehicle (*left panel*) and BPA (*right panel*). The *arrow* indicates a cluster of apoptotic bodies. B, Graph summarizing percentage of apoptotic cells in TEBs of control and treated mice (*bars* indicate mean ± SEM). *, $P < 0.05$; **, $P < 0.01$.

results suggest that perinatal exposure to BPA also alters stroma-epithelium interactions.

Expression of ERα and PR in the mammary glands

Because ER and PR are clearly involved in the pathways regulating ductal development (41, 42), their expression was assessed at 30 d of age. Expression of ERα was observed in both the epithelial and stromal compartments of the mammary glands, whereas expression of PR was observed only in the epithelium. Treatment with BPA had no effect on the expression of ERα in the stroma or the epithelial ducts of mammary glands at 1 month of age (Table 1). The number of cells expressing PR in the epithelial compartment of both treated groups was found to be significantly higher (25 ng BPA/kg bw·d, $P < 0.001$; 250 ng BPA/kg bw·d, $P < 0.05$) than in controls (Fig. 5A). In addition, a characteristic cluster of PR-positive cells was frequently seen in the ductal epithelium of BPA-treated mice (Fig. 5B). These clusters are believed to be indications of future branching points (43).

Expression of Wnt4 in the mammary glands

The PR expression results described above suggested that BPA might increase lateral branching. Wnt4 expression is an important mediator of lateral branching downstream of PR (44). Compared with the basal levels of Wnt4 seen in controls, the mean expression of Wnt4 was increased in the 250 ng BPA/kg bw·d group, whereas the levels in the 25 ng BPA/kg bw·d group remained similar to the control levels (Fig. 6). However, this increasing trend of Wnt4 levels did not reach statistical significance at the single time point chosen for this study.

Analysis of ductal branching

As suggested by the clustering of PR positive cells at 30 d of age, an analysis of mammary gland whole mounts at 4 months of age revealed a significant increase in the number of lateral branches in mammary glands of mice treated with

TABLE 3. Percent of BrdU-positive cells in the epithelial and stromal compartment of the 4th mammary gland of 30-d-old animals

BPA dose	0 ng/kg bw·d	25 ng/kg bw·d	250 ng/kg bw·d
Epithelium	8.18 ± 2.51	3.31 ± 1.02	5.51 ± 1.15
Stroma	1.78 ± 0.29	1.27 ± 0.18	0.87 ± 0.10[a]

Data are represented as the mean ± SEM.
[a] A significant difference with the control ($P < 0.05$).

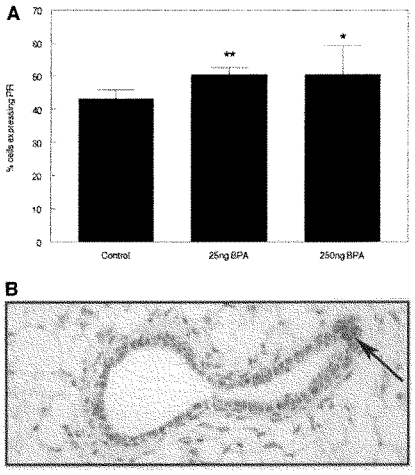

FIG. 5. A, Histogram representing the percentage of cells stained positive for PR in the epithelial compartment of mammary glands of 30-d-old mice treated perinatally with vehicle, 25 and 250 ng BPA/kg bw·d (**, $P < 0.01$; *, $P < 0.05$) (*bars* indicate mean ± SEM). B, Photomicrograph showing clusters (as indicated by the *arrow*) of PR-expressing cells in the mammary gland ducts of BPA-treated mice.

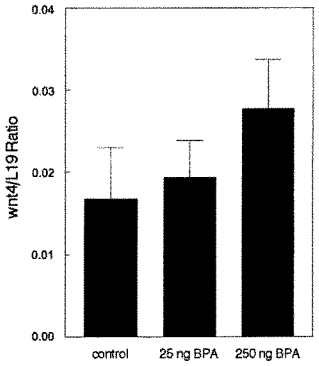

FIG. 6. Expression of Wnt4 mRNA in mammary glands of control and BPA-treated mice (25 and 250 ng BPA/kg bw·d) at 30 d of age estimated by quantitative RT-PCR. Wnt4 mRNA levels were measured relative to L-19 expression (internal control). *Bars* indicate mean ± SEM.

25 ng BPA/kg bw·d ($P < 0.05$), whereas an increase observed in the 250 ng BPA/kg bw·d group was not statistically significant ($P = 0.09$) (Fig. 7, A and B). Interestingly, in the latter group, the data points form two clusters, one control-like and the other similar to the 25 ng BPA/kg bw·d dose. This plot also reveals that there is less variance among the controls than among the individual animals in the treated group ($P = 0.007$) (Fig. 7A).

Discussion

Prior data obtained from siblings of the animals used in the present study revealed that prenatal exposure to environmentally relevant levels of BPA resulted in alterations of the reproductive tract and mammary gland that were manifested long after exposure ended. These alterations encompassed functional changes such as alteration of estrous cyclicity observed at 3 months of age (27); morphological changes in the ovary, uterus, and vagina, also observed at 3 months of age (27); and cellular changes, such as an increase in the ER- and PR-positive cells observed in the lining of the endometrium at 3 months of age (45). In the mammary gland, an increase in the total area of the ductal tree and increases in the number of terminal ducts, terminal ends, and alveolar buds were observed at 6 months of age (34).

The objective of the present study was to assess whether perinatal BPA exposure also affected the development of the mammary gland at puberty, a phenomenon initiated by the rise in estrogen plasma levels. During this period, the ductal tree undergoes growth by invasion of the stroma, accrual of new cells, and lumen formation; these processes are mediated by a highly dynamic structure, the TEB. We observed a positive correlation between ductal length from the center of the lymph node to the leading edge and the age at first proestrus; the slope of this curve was significantly reduced as the BPA dose increased. At 30 d of age, the number of TEBs relative to the ductal area increased significantly in animals exposed perinatally to BPA. Whereas the distal aspect of the TEB is the invasive organ, the solid primordium of body cells near the neck is involved in the formation of the subtending duct and its lumen, a process that is manifested by a high apoptotic rate (38); the ductal lumen is formed as a consequence of this apoptotic activity. A large and significant decrease in the number of apoptotic cells was observed within the TEBs in animals of both BPA-treated groups. Hence, decreased cell death may be the factor underlying the impaired ductal elongation and the increased number and area of TEBs per ductal area in the BPA-treated animals. Cell proliferation, instead, seemed to be unaffected because no significant changes in the BrdU labeling index of epithelial cells were observed. However, a significant decrease of the labeling index of stromal cells was found in animals exposed to 250 ng BPA/kg bw·d. Because mammary gland morphogenesis is mediated by complex stromal-epithelial interactions, this result suggests that BPA also affects the stromal compartment and may disrupt important stromal-epithelial interactions.

The morphological changes found in 30-d-old animals exposed perinatally to BPA could be attributed, at least in part, to an increased sensitivity to estrogens. Indeed, the magnitude of the response to E_2 was significantly enhanced in their siblings that were ovariectomized and exposed to E_2 for 10 d. In particular, a significant increase in the number and area

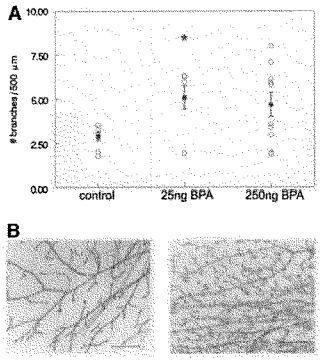

FIG. 7. A, Number of side branches per 500 μm of ductal length in mammary glands of 4-month-old mice treated perinatally with vehicle, 25 and 250 ng BPA/kg bw·d (*, $P < 0.05$) (n = 6, 6, 10 for controls, 25 and 250 ng BPA/kg bw·d, respectively). *Error bars* indicate SEM. B, Photomicrographs of whole mounts of mammary glands of animals treated with vehicle (*left panel*) and 25 ng BPA/kg bw·d (*right panel*). The *bar* represents 1 mm.

of TEBs relative to the ductal area was observed in these ovariectomized E_2-treated animals as well as in their intact siblings that were exposed perinatally to BPA. Although it seems counterintuitive that E_2 would decrease ductal growth, we observed that the response of the normal mammary gland to E_2 is biphasic, with low doses producing a larger effect than higher doses (Vandenberg L., personal communication). This nonmonotonic behavior was also observed regarding the percent area of the mammary gland occupied by the ductal tree (46). Alternatively, BPA may affect ductal growth by acting on other yet-unknown end points in addition to increasing the sensitivity to estrogens. Indeed, recent evidence indicates a complex interaction among E_2, IGF-I, and progesterone on ductal morphogenesis (47).

Progesterone is the main mediator of lateral branching and alveolar growth, a fact clearly illustrated by the PR null mutant mice (48). Perinatal BPA exposure resulted in a significant increase in the number of epithelial cells expressing PR. This finding may result from the observed increased estrogen sensitivity of the mammary gland in BPA-exposed animals. In these animals, the ductal PR-positive cells often formed clusters. Lateral branching points and alveoli originate in these clusters (49). In the normal mammary gland, the transition from uniform PR expression to PR clustering takes place between 8 and 12 wk of age (49). Wnt 4 expression is a crucial event downstream from PR signaling in the lateral branching process (44). Perinatal BPA exposure resulted in increased wnt4 mRNA levels at puberty, the only time point assayed; however, this increase did not reach significance. Nevertheless, morphometric analysis of the mammary glands at 4 months of age clearly revealed an increase in the number of lateral branches in the animals exposed to BPA.

These observations suggest that perinatal exposure to BPA significantly enhances the response to estrogens and increases the expression of PR. Furthermore, it suggests that the increased expression of PR may be the mechanism underlying the enhanced side branching observed at 4 months of age and the significant increase in the percentage of alveolar buds observed at 6 months of age (34).

The mechanisms by which BPA affects the morphology of the mammary gland long after the period of exposure are largely unknown. One pathway may involve the direct action of BPA on the fetal mammary gland by altering the expression of genes that regulate mesenchymal-epithelial interactions and ductal morphogenesis (50). Misexpression of these genes has been associated with mammary gland dysgenesis and carcinogenesis (51). E_2 regulates certain homeobox genes (51–53); thus, it is plausible that BPA may also have this effect.

Prenatal exposure to DES down-regulates the expression of Wnt7a in the uterus (54) and AbdB Hoxa in the müllerian duct (55) and induces a posterior shift in Hox gene expression and homeotic anterior transformations of the reproductive tract (56), and this correlates with structural abnormalities of these organs. The observation that the response to E_2 was enhanced in ovariectomized animals exposed perinatally to BPA is compatible with this scenario. An additional pathway by which BPA may indirectly affect mammary gland development is by disrupting the hypothalamic-pituitary-ovarian axis. This would alter the secretory patterns of pituitary and ovarian hormones, which are important in postnatal mammary gland morphogenesis. The observations that perinatal exposure of rats to low-dose BPA reduces serum LH levels after ovariectomy in adulthood (57), disrupts estrous cyclicity, and results in morphological changes in the ovary (27) support the potential for alterations in the hypothalamus-pituitary-ovarian axis of BPA-exposed offspring.

What are the implications of these findings regarding human health? Surrogate animal models provide an understanding of human diseases. Although the relationship between the two is not always direct, surrogate models are most useful when used to develop hypotheses linking exposures and health outcomes. They also increase our understanding of the mechanisms underlying these pathologies. For instance, the mouse model has proven to be a generally outstanding model of human DES exposure, thus providing a means to understand the mechanisms underlying the DES syndrome. This excellent performance strengthens the human relevance of the current findings in mice. Within this context, it is useful to speculate how the findings described here may also apply to humans. On the one hand, exposure to estrogens is a main risk factor for the development of breast cancer in humans and also increases the development of mammary cancer induced by chemical carcinogens in rodent models (58). Thus, the increased sensitivity to E_2 suggests that prenatal exposure to BPA may increase the likelihood of neoplastic development. On the other hand, TEBs are the structures in which mammary cancer originates in both rodents and humans (59, 60). The increase in the number of TEBs/ductal area is also consistent with an increased risk of breast cancer. Another well-established risk factor for breast cancer is increased mammographic density.

Mammographic density is attributed to an increased epithelial compartment and increased nonadipose stroma. The mammary glands of BPA-exposed animals contained significantly more ducts due to lateral branching at 4 months of age. Previous data obtained from sisters of animals in this study revealed that at 6 months of age, the number of all the epithelial structures were significantly increased (34), including terminal ducts, *i.e.* the structures in which neoplasia originates in adult animals (59). These correlations suggest that perinatal exposure to BPA in particular, and to estrogens in general, may increase susceptibility to breast cancer. This hypothesis is being further tested in our laboratories.

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Address all correspondence and requests for reprints to: Ana M. Soto, Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111. E-mail: ana.soto@tufts.edu.

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Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses

Tyrone B. Hayes*, Atif Collins, Melissa Lee, Magdelena Mendoza, Nigel Noriega, A. Ali Stuart, and Aaron Vonk

Laboratory for Integrative Studies in Amphibian Biology, Group in Endocrinology, Museum of Vertebrate Zoology, Department of Integrative Biology, University of California, Berkeley, CA 94720-3140

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Atrazine is the most commonly used herbicide in the U.S. and probably the world. It can be present at several parts per million in agricultural runoff and can reach 40 parts per billion (ppb) in precipitation. We examined the effects of atrazine on sexual development in African clawed frogs (*Xenopus laevis*). Larvae were exposed to atrazine (0.01–200 ppb) by immersion throughout larval development, and we examined gonadal histology and laryngeal size at metamorphosis. Atrazine (≥0.1 ppb) induced hermaphroditism and demasculinized the larynges of exposed males (≥1.0 ppb). In addition, we examined plasma testosterone levels in sexually mature males. Male *X. laevis* suffered a 10-fold decrease in testosterone levels when exposed to 25 ppb atrazine. We hypothesize that atrazine induces aromatase and promotes the conversion of testosterone to estrogen. This disruption in steroidogenesis likely explains the demasculinization of the male larynx and the production of hermaphrodites. The effective levels reported in the current study are realistic exposures that suggest that other amphibian species exposed to atrazine in the wild could be at risk of impaired sexual development. This widespread compound and other environmental endocrine disruptors may be a factor in global amphibian declines.

In the last 10 years, a great deal of attention has focused on the global presence of endocrine-disrupting contaminants in the environment (1, 2). Similarly, a great deal of attention has focused on global amphibian declines (3, 4). In the case of amphibian declines, efforts focus on identifying causes (5), whereas for endocrine disruptors, the "causes" have been identified and studies focus on identifying effects of endocrine disruptors in the environment (6–11).

Atrazine (2-chloro-4-ethytlamino-6-isopropylamine-1,3,5-triazine) is the most commonly used herbicide in the U.S. and probably the world. The U.S. Department of Agriculture reports that more than 30,000 tons (60 million pounds) are used annually in the U.S. alone (12). Atrazine has been used for over 40 years and currently it is used in more than 80 countries. Despite its widespread intensive use, atrazine is considered safe because of its short half-life and negligible bioaccumulation and biomagnification (13). Also, atrazine seems to have very few effects on adults and reportedly induces abnormalities and deformities only at very high doses. As a result of the high doses required to produce deformities, it has been suggested that "direct toxicity of atrazine is probably not a significant factor in recent amphibian declines" (14). Here, we test the hypothesis that atrazine may interfere with metamorphosis and sex differentiation at ecologically relevant low doses via endocrine-disrupting mechanisms.

Materials and Methods

Animal Breeding and Larval Care. We report results from two experiments that used frogs from two separate sources. Adults from Exp. 1 were from a long-term captive colony maintained at the University of California, Berkeley, whereas adults from Exp. 2 were obtained from Nasco (Fort Atkinson, WI). In both experiments, three females and three males were injected with human choriogonadotropin (1,000 international units) 6 h before harvesting gametes. Eggs were manually stripped from the female and fertilized *in vitro* in 0.3 × modified mammalian Ringer's solution by using the sperm obtained from the dissected testes of the three males. The embryos were allowed to hatch. After 4 days, the larvae were all mixed and netted into tanks 5 at a time repeatedly, until all tanks contained 30 larvae. Larvae were reared in 4 liters of aerated 10% Holtfreter's solution (15) and fed a solution of ground Purina rabbit chow daily. Food levels were adjusted as the animals grew to maximize growth.

Dosing. In Exp. 1, we exposed larvae to atrazine at nominal concentrations of 0.01, 0.1, 1.0, 10.0, and 25 parts per billion (ppb), whereas the second experiment used 0.1, 0.4, 0.8, 1.0, 25, and 200 ppb atrazine. Concentrations were confirmed by two independent laboratories (PTRL West, Richmond, CA, and the Iowa Hygienic Laboratory, Univ. of Iowa, Iowa City, IO). All stock solutions were made in ethanol (10 ml), mixed in 15-gallon containers, and dispensed into treatment tanks. Controls were treated with ethanol such that all tanks contained 0.004% ethanol. Water was changed and treatments were renewed once every 72 h. Each treatment was replicated 3 times with 30 animals per replicate (total of 90 animals per treatment) in both experiments. All treatments were systematically rotated around the shelf every 3 days to ensure that no one treatment or no one tank experienced position effects. Experiments were carried out at 22°C with animals under a 12-h/12-h light/dark cycle (lights on at 6 a.m.). Animals were exposed throughout the entire larval period, from hatching [Niewkwoop–Faber (NF) Stage 48 (16)] until complete tail reabsorption (NF Stage 66). In all experiments, all treatments and analyses were conducted blindly with color-coded tanks and treatments and number-coded specimens.

Gross Measurements. At metamorphosis (complete tail reabsorption—Niewkwoop–Faber Stage 66), the date was recorded for each animal. Each animal was weighed to the nearest 0.002 g on a Mettler AT 261 Delta Range balance and its total length was measured to the nearest 0.5 mm. Animals were anesthetized in 0.2% benzocaine (Sigma), assigned a unique identification number, fixed in Bouins' fixative, and preserved in 70% ethanol until further analysis.

Gonadal Analysis. Initially, the sex of all individuals was determined based on gross gonadal morphology (Fig. 1). Sex identification was confirmed by histology for 10 animals per tank. Further, histological analysis was conducted on all animals for which the sex was ambiguous when determined by gross mor-

Abbreviation: ppb, parts per billion.

*To whom reprint requests should be addressed. E-mail: tyrone@socrates.berkeley.edu.

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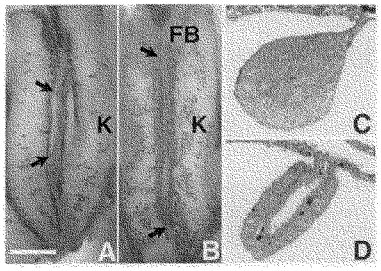

Fig. 1. Gonads of a control postmetamorphic male (*A* and *C*) and female (*B* and *D*) *X. laevis*. *A* and *B* show the entire dissected kidney–adrenal–gonadal complex preserved in Bouins' fixative. *C* and *D* show 8 μm of transverse cross-sections through the animals' right gonad stained with Mallory's trichrome stain. [Bar = 0.1 mm (*A* and *B*) and 10 μm (*C* and *D*)]. FB, fatbody; K, kidney. Arrows (in *A* and *B*) show the anterior and posterior ends of the animals' right gonads. The yellow color in *A* and *B* is a result of fixation in Bouins' fixative. Without fixation, the gonad is transparent. The ovary is distinguished by its greater length, lobed structure, and melanin granules. Although some specimens' ovaries lack pigment (especially atrazine-treated animals), testes never have melanin in this species. Histologically, the ovary is distinguished by the ovarian vesicle (hole in the center) along its entire length and the internal ring of connective tissue (in blue). Note the melanin granules (black) in the connective tissue in *D*.

phology. All histology was conducted according to Hayes (17). In brief, tissues of interest were dissected and dehydrated in graded alcohols, followed by infiltration with histoclear and paraffin. Sections were cut at 8 μm and stained in Mallory's trichrome stain.

Laryngeal Size. Serial transverse histological sectioning was conducted on the larynges of 10 males and 10 females from each replicate from all treatments in both experiments. Histology was conducted as described above. To estimate the size of the larynx, the *M. dilator laryngis* was measured. We used the largest cross-sectional area (transverse section) as a measure of muscle size. Initially, 10 sections were taken from 100 animals (distributed over all treatments from Exp. 1) until a region approximately one-third through the larynx was repeatedly determined to be the largest section. For the final analysis this region was identified by shape. Thus, similar sections were measured for each individual. Images of this section from each animal were recorded with a Sony DKC-5000 and analyzed with METAMORPH software (version 2.75, Universal Imaging, Media, PA).

Adult Treatments. Newly metamorphosed animals were too small to obtain enough plasma to measure hormone levels. Thus, studies of effects of atrazine on hormone levels focused on adults. For adult studies, males and females were obtained from a long-term captive colony at University of California, Berkeley. Adults were maintained under the same light and temperature cycles as described for larvae. Animals were acclimated in 10% Holtfretter's solution for 5 days and then exposed to 25 ppb atrazine. Water was not aerated, animals were fed Purina trout chow daily, and water was changed and treatment renewed every 72 h. Animals were treated for 46 days. At the end of the exposure, animals were killed by decapitation, and the blood was collected. Plasma was collected and stored frozen until analysis.

RIA. For testosterone analysis, plasma was extracted with diethyl ether and dried under nitrogen. All samples were reconstituted in PBS with gelatin (PBS-g). Hormone assays were conducted as described in Hayes and Licht (18). Testosterone antisera were obtained from Endocrine Sciences (Calabasas, CA) and were validated for several species including *Xenopus laevis*. Plasma from controls and treated animals was assayed in the same assay at 3 doses and the assay was repeated 3 times. Intraassay variation was 1.0%, and interassay variation was 1.3%.

Statistical Analysis. Statistical analysis was conducted with the aid of SYSTAT software (SPSS, Chicago). Sex ratios were analyzed by using the G test with Wilkin's g- adjustment as described in Hayes and Menendez (19). Similarly, mortality was analyzed by using the G test. Time to metamorphosis and size (length and weight) at metamorphosis were analyzed by using ANOVA with treatment, tank, and sex (sex nested within tank and tank nested within treatment) as independent variables. In addition, we conducted correlational analyses to determine whether laryngeal size correlated with time to metamorphosis, size, or atrazine dose. Also, we scored all animals as to whether they were greater or less than the mean laryngeal size for controls and then conducted a G test to determine whether the number of affected animals in the treatment group changed with atrazine treatment. Finally, we used Kendall's ranked coefficient to determine whether the percentage of below-average animals varied with the dose of atrazine.

Results

Mortality, Development, and Growth. At the doses tested, atrazine exposure had no effects ($P > 0.05$) on mortality, time to metamorphosis, length, or weight at metamorphosis (not shown).

Effects on Primary and Secondary Sex Differentiation. Males and females were sexually differentiated at metamorphosis based on gonadal morphology and histology (Fig. 1). At all doses tested (except 0.01 ppb), atrazine produced gonadal abnormalities. Up to 20% of the animals (16–20%) had multiple gonads (up to 6 in a single animal) or were hermaphrodites (with multiple testes and ovaries; Fig. 2). These abnormalities were never observed in control animals in the current experiments or in over 10,000 observations of control animals in our laboratory over the last 6 years.

Control males had larger larynges than females at metamorphosis, but males exposed to atrazine ($\geq$1 ppb) had reduced larynges (both studies; Fig. 3 *A* and *B*). When we examined the proportion of "below-average" animals against dose, we found a threshold effect at 1 ppb (both studies; Fig. 3*C*), but Kendall's rank coefficient suggested a dose effect with increasing proportions of affected males associated with increasing atrazine doses ($P < 0.01$; Fig. 3*D*).

We hypothesized that the effects of atrazine were caused by a disruption of steroidogenesis (20–27). Further, we showed that sexually mature males suffered a 10-fold decrease in plasma testosterone (Fig. 4).

Discussion

Although data from two experiments are reported here, these studies have been repeated four times, including an unpublished report and a study submitted to the U.S. Environmental Protection Agency (28). In total, atrazine exposure at these levels has been replicated 51 times by our laboratory with similar results. We chose *X. laevis* for these studies, because it is a well studied laboratory model for which the effects of sex steroids are well known. Exposure to exogenous estrogen in this species results in 100% females (29, 30), whereas androgens increase laryngeal growth but do not affect gonadal differentiation (30,

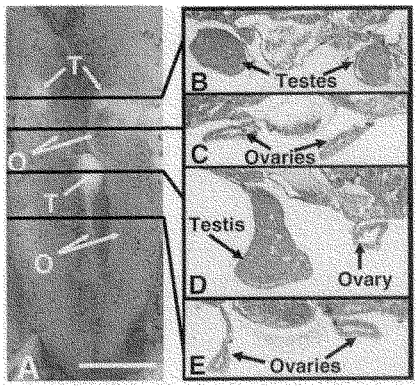

Fig. 2. An atrazine-treated hermaphrodite. The specimen shown was treated with 1 ppb atrazine. *A* shows the entire dissected kidney–adrenal–gonadal complex. *B–E* show 8 μm of transverse cross-sections (stained with Mallory's trichrome stain) through the areas indicated by the lines in *A*. [Bar = 0.1 mm (*A*) and 25 μm (*B–E*)]. FB, fatbody; K, kidney; O, ovary(ies); T, testis(es). Note the absence of pigment in the ovaries, which was typical of hermaphrodites.

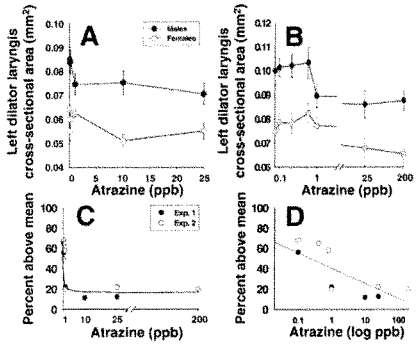

Fig. 3. Results of measurements of the left laryngeal muscle (*M. dilator laryngis*) in control males and females compared with atrazine-treated animals. In Exp. 1 (*A*), atrazine (≥1 ppb) reduced laryngeal size in males but did not affect females. Doses of 0.01 and 0.1 ppb did not have a significant effect. In Exp. 2 (*B*), 0.1–0.8 ppb atrazine did not have a statistically significant effect on laryngeal size but again, exposure to ≥1 ppb atrazine significantly reduced laryngeal size in males ($P < 0.05$). Laryngeal size was greater in animals from Exp. 2 compared with Exp. 1, suggesting a population difference in the absolute size of the larynges, but the relative sizes (male to female and atrazine-treated compared with controls) were similar within each experiment. *C* and *D* show two interpretations of the data by using analysis of the proportion of above-average males for both experiments. Atrazine exposure (≥1 ppb) significantly decreased the proportion of males that were at or above the mean for control males (G test; $P < 0.05$) and suggested a threshold effect at 1.0 ppb in which 80% of the exposed males were below average (*C*). Kendall's rank coefficient analysis ($P < 0.01$), however, suggested a relationship between dose and the proportion of affected males with a decrease in the proportion of normal males with increased dose (*D*). Note that control males were normally distributed with exactly 50% of the individuals above the mean in both experiments.

31). Thus, endpoints for detecting sex steroid-like or antagonistic effects are well defined for this species. The current findings suggest that atrazine inhibits testosterone and induces estrogen secretion.

Previous studies have suggested that atrazine is an endocrine disruptor, but these effects have been observed in a single strain of rat or were produced only at high doses (32–38). In fact, no published studies have addressed effects of atrazine at concentrations considered safe in drinking water or safe for limited human exposure—3 and 200 parts ppb, respectively (39). Also, until now, the potential endocrine-disrupting effects of atrazine have not been examined in amphibians, although teratogenesis, mortality, and growth effects have been examined at high doses (14, 40–45). In the cited amphibian studies, deformities, acute toxicity, or physiological impairments were not detected below atrazine doses of 47.6 ppm.

Disruption of steroidogenesis by atrazine has been reported in mammals (20–26) and reptiles (27), however. Several of these studies reported the induction of aromatase and an increase in estrogen. Here, we suggest that the same mechanism may explain the effects observed in *X. laevis*. An induction of aromatase may result in the decrease in androgens (as androgens are the substrate for aromatase). The loss of masculine features, such as the decreased laryngeal size, may be a result of the decreased androgens, whereas the induction of ovaries may be a result of increased estrogen synthesis and secretion. The possible common mechanism underlying the abnormal sexual development in the current study and reproductive abnormalities in reptiles and mammals has significant implications for environmental and public health. The effects observed in mammals were dismissed as a concern for public health because the exposure levels were very high (20–26, 32–38). The effective doses in the current study, however, demonstrate the sensitivity of amphibians relative to other taxa, validate the use of amphibians as sensitive environmental monitors/sentinels, and raise real concern for amphibians in the wild. The effects on the gonads in the current study were produced at 0.1 ppb, which was more than 600 times lower than the dose required to induce aromatase in human adrenocortical carcinoma (25) and placental choriocarcinoma studies (25–26) and 30,000,000 times lower than the dose required to produce reproductive effects in rats (24).

Furthermore, the current data demonstrate the importance of considering endocrine-regulated endpoints in assessing the potential impact of pesticides on amphibians. Reported teratogenesis, growth inhibition, and mortality in amphibians in response to atrazine were not considered environmental concerns because of the high doses required to produce these effects (40). Effects in the current study, however, occurred at levels 10,000 times lower than the dose required to produce effects in amphibians in these previous studies (40–45). Allran and Karasov (14) reached the conclusion that atrazine was probably not a significant factor in amphibian declines based on their studies of toxicity, deformities, and effects on feeding and ventilation in leopard frogs that did not produce noticeable effects below 3 ppm. The current data show that negative effects on sex differentiation occur at doses 30,000 times lower than effective doses reported by Allran and Karasov. The Allran and Karasov study, however, examined a different species and different endpoints.

The current data raise new concerns for amphibians with regards to atrazine. Effective doses (0.1 ppb for the production of hermaphrodites and 1 ppb for reduction in laryngeal size) are ecologically relevant. The recommended application level of

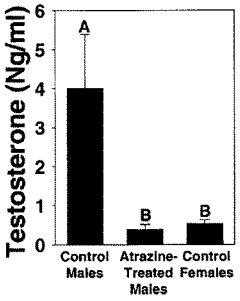

Fig. 4. Effect of 46-day exposure to atrazine on plasma testosterone levels in sexually mature male *X. laevis*. Sexually mature males were housed individually. Experimental animals were treated every 3 days with 25 ppb atrazine, and controls were treated identically except without atrazine exposure. Control females are shown for comparative purposes. Letters above bars show statistical groupings (ANOVA, $P < 0.05$).

atrazine ranges from 2,500,000–29,300,000 ppb (46), the allowable contaminant level for atrazine in drinking water is 3 ppb (39), and short-term exposures of 200 ppb are not considered a health risk. Atrazine can be as high as 21 ppb in ground water, 42 ppb in surface waters, 102 ppb in river basins in agricultural areas, up to 224 ppb in Midwestern streams, and up to 2,300 ppb in tailwater pits in Midwestern agricultural areas (47, 48). Atrazine can be found in excess of 1 ppb in precipitation in localities where it is not used and up to 40 ppb in rainfall in Midwestern agricultural areas (49–51). Further, Davidson *et al.* (52) recently reported that at least one species (*Rana aurora*) may be affected by aerial transport of agrichemicals. They showed that declines and extirpations of *R. aurora* populations were strongly correlated with areas that were downwind of agricultural activity. Furthermore, Cory *et al.* (53) showed that agrichemicals can be transported aerially and accumulated in amphibians' tissues. Thus, the likelihood that wild amphibians are exposed to 0.1 ppb or even 1 ppb atrazine is extremely high.

Furthermore, atrazine is typically applied when the soil is tilled, such that levels are highest during spring rainfall (13). This pattern of use puts amphibians at great risk, because the highest atrazine levels coincide with the breeding season for amphibians. Throughout areas where atrazine is used, atrazine levels peak while larval amphibians are at critical developmental stages. Also, depending on the species, amphibians breed in every possible freshwater microhabitat—from temporary pools, irrigation ditches, and flooded fields, to streams, rivers, lakes, and other permanent sources of water. The current data raise the question of the threat of atrazine, in particular, and of pesticides, in general, to amphibians in the wild. Low-dose endocrine-disrupting effects, which have not been addressed extensively in amphibians, are of special concern in this regard. If such effects do occur in the wild in other species, exposed animals could suffer impaired reproductive function. The described effects are all internal and may go unnoticed by researchers—unlike mortality and external malformations. Thus, exposed populations could decline and even go extinct without any recognition of the developmental effects on individuals. Already, it has been suggested that pesticides may play a role in amphibian declines (3, 52, 54, 55). Also, Reeder *et al.* (56) found that atrazine exposure may be associated with intersexual cricket frogs in the wild in the Illinois. Because the *P* value in the Reeder *et al.* study was 0.07 and because no laboratory data were available, they concluded that "[w]hether atrazine accounts for findings of intersexuality is less clear" (ref. 56, p. 265). We believe that the current data strongly suggest a connection between atrazine exposure and intersexuality. Combined with the decreases in dissolved oxygen, pH, and available food sources (phytoplankton, periphyton, and macrophytes) caused by atrazine (45), this common contaminant could be a contributing factor in amphibian declines. Ongoing investigations of the effects of atrazine on other species and amphibians in the wild will assess the realized role of this widespread compound in amphibian declines.

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REVIEW ARTICLE

Thyroid disrupting chemicals: mechanisms and mixtures

Kevin M. Crofton

Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC, USA

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Correspondence:
Kevin M. Crofton, PhD, Neurotoxicology Division, MD-B105-05, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA.
E-mail: crofton.kevin@epa.gov

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Summary

Environmental contaminants are known to act as thyroid disrupting chemicals (TDCs). Broadly defined, TDCs are xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated with thyroid hormone (TH) homeostasis or change circulating or tissue concentrations of THs. For THs, homeostasis is defined as the normal range of THs and TSH in circulation and tissues. TDCs include a wide range chemical structures that act through a variety of mechanisms. Concern about TDCs has increased because of the critical role that thyroid hormones play in brain development. A major uncertainty regarding the endocrine disrupting potential of environmental xenobiotics is the potential for additive, antagonistic or synergistic effects following exposure to mixtures. In addition, there are a number of uncertainties in both interpretation and extrapolation of results from studies of TDC mixtures. Extrapolation of data from laboratory animals to humans is tempered by uncertainty in how the mechanism(s)-of-action of the TDCs may differ between species. The variety of mechanisms by which TDCs alter thyroid homeostasis also yields a difficulty in determining at what level of biological organization to cumulate effects. Should it be at the molecular level, which could be chemical class specific or at the level of a downstream consequence (e.g. circulating hormone levels, brain biochemistry and behaviour) which would be mechanism-independent? To date, the limited data from TDC mixture studies suggest that dose addition is reasonably accurate in predicting the effects on serum T4 concentrations. Assessing the health risks of thyroid disruption by environmental xenobiotics will need to include an improved understanding of how divergent mechanisms alter THs and consequent adverse impacts on nervous system development.

Introduction

A variety of data suggest an increased need for characterizing the potential risks of xenobiotics as endocrine disruptors (IPCS, 2002; Daston *et al.*, 2003; Guillette, 2006). Environmental chemicals are known to adversely impact a number of hormonal systems, including oestrogen, androgens and thyroid hormones. Concern about thyroid hormone disrupting chemicals (TDCs) has increased because of the critical role that thyroid hormones play during development, especially in the nervous system (Porterfield & Stein, 1994; Brucker-Davis, 1998; Zoeller & Crofton, 2000; Morreale de Escobar *et al.*, 2004; Zoeller & Tan, 2007). Broadly defined, TDCs are xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated with thyroid hormone (TH) homeostasis, or change circulating or tissue concentrations of THs (Crofton *et al.*, 2005). TDCs include a wide range of chemical structures that act through a variety of mechanisms. A large number of environmental chemicals and stressors are known to influence measures of thyroid function in experimental animals (Capen, 1994; Brouwer *et al.*, 1998; Brucker-Davis, 1998; DeVito *et al.*, 1999). There is also evidence that xenobiotics disrupt thyroid hormone homeostasis in humans (Gaitan, 1990; Longnecker *et al.*, 2003; Delange, 2005; Blount *et al.*, 2006; Steinmaus *et al.*, 2007).

Uncertainties hamper predictions of the health risks of TDCs. One uncertainty is the potential for additive, antagonistic or synergistic effects following exposure to

endocrine disrupting mixtures (Wade *et al.*, 2002; Daston *et al.*, 2003; De Rosa *et al.*, 2006). In addition, there are a number of uncertainties in the interpretation of results from animal studies, as well as extrapolation of findings to humans. Extrapolation of data from laboratory animals to humans is tempered by the uncertainty in how the mechanism(s)-of-action of the TDCs may differ between species [cf. (McClain, 1995; Capen, 1997; Tabb *et al.*, 2004)]. The variety of mechanisms by which TDCs alter thyroid homeostasis yields yet another difficulty in determining at what level of organization to cumulate effects. Should it be at the molecular level, which could be chemical class specific or at the level of a downstream consequence (e.g. circulating hormone levels, brain biochemistry and behaviour) which would be mechanism independent? Assessing the health risks of thyroid disruption by environmental xenobiotics will need to include an improved understanding of how divergent mechanisms alter THs and consequent adverse impacts on nervous system development.

Impact of xenobiotics on thyroid hormones

Thyroid hormone homeostasis involves a complex interplay of homeostatic regulatory processes (Hill *et al.*, 1989; Atterwill *et al.*, 1992; Thomas & Williams, 1992). Regulation of THs includes control of iodine uptake, synthesis and storage of THs in the thyroid gland, release into and transport of THs within an out of circulation, tissue-specific deiodination and degradation by catabolic hepatic enzymes (Fig. 1). The hypothalamic-pituitary axis controls the synthesis and secretion of THs in the thyroid gland via production of thryotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH). Once released into circulation, THs are bound to plasma transport proteins. Circulating levels of T3 and T4 provide a negative feedback to the hypothalamus and pituitary; conversely, lowered levels of T3 and T4 active the feedback mechanism, resulting in increased TRH and TSH, which then increases TH production in the thyroid gland. T3 and T4 levels are also controlled by extra-thyroidal mechanisms. Peripheral tissue deiodinases are responsible for conversion of T4 to T3 (the major source of circulating T3) and also metabolites of T3 and T4 (e., reverse T3, T2) (Kohrle, 2002). There are also two other important regulatory processes. The first are cellular thyroid hormone transporters (e.g. OATP1C1, MCT8), responsible for moving T4, T3 and metabolites into and out of cells (Friesema *et al.*, 2005; Kohrle, 2007). The other major regulatory process is hepatic catabolism of THs.

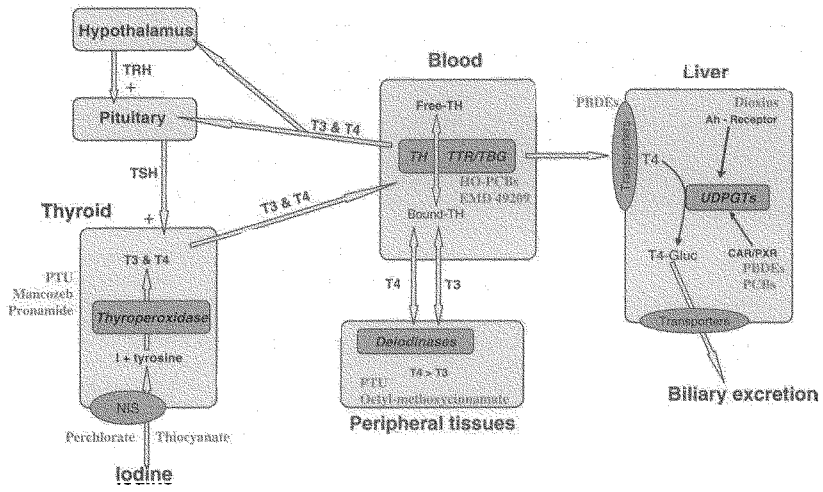

Figure 1 Thyroid hormone control pathways and sites of disruption by xenobiotic chemicals. Sites or processes where xenobiotics are known or hypothesized to act as TDCs are indicated in the boxes and ovals. Xenobiotics that block, inhibit or upregulate these processes are shown in red text.

A number of hepatic uridine diphospho-glucuronosyltransferase (UGT) or sulfotransferase (SULT) isozymes are responsible for Phase II metabolism of THs, resulting in biliary excretion of glucuronide or sulphate conjugates of T4 or T3 (Kester *et al.*, 2003; Mackenzie *et al.*, 2005).

A wide range of structurally diverse xenobiotics act through a variety of mechanisms to alter thyroid hormone homeostasis (Fig. 1, Table 1) (Atterwill *et al.*, 1992; Brouwer *et al.*, 1998; Brucker-Davis, 1998; Hurley, 1998; DeVito *et al.*, 1999). Table 1 lists chemicals with known or suspected mechanisms-of-action and the effects on thyroid hormones. Some chemicals such as perchlorate or thiocyanate, inhibit the uptake of iodide into the thyroid and subsequently decrease TH synthesis (Wolff, 1998). Other chemicals decrease TH synthesis by inhibition of thyroid peroxidase thus blocking incorporation of iodide into thyroglobulin, the stored form of T4. This occurs with several classes of drugs and chemicals such as thionamides, aniline derivatives, substituted phenols, the herbicide amitrole and the fungicide metabolite ethylenethiourea (Atterwill *et al.*, 1992; McClain, 1995; Capen, 1997, 1998; Brucker-Davis, 1998; Hill *et al.*, 1998; Hurley, 1998).

A well-characterized effect of TDCs is increased biliary elimination of THs via induction of UGTs (Oppenheimer *et al.*, 1968; McClain *et al.*, 1989; Visser *et al.*, 1993; Hood & Klaassen, 2000). Xenobiotics also appear to upregulate hepatic SULTs (Liu & Klaassen, 1996), but the impact of this on circulating or tissue levels of THs is currently unknown. In addition, there is evidence that some xenobiotics, such as pentachlorophenol or triclosan, inhibit SULTs (Schuur *et al.*, 1998; Wang *et al.*, 2004; Wang & James, 2006).

Xenobiotics also impact the transport of THs, both in the blood, and into and out of cells. *In vitro* data support the hypothesis that xenobiotics displace T4 from transthyretin (TTR), the major serum transport protein for T4 in rat (Kohrle *et al.*, 1989; Lueprasitsakul *et al.*, 1990; Kohrle, 1992; Lans *et al.*, 1993; Cheek *et al.*, 1999; Chauhan *et al.*, 2000; Ishihara *et al.*, 2003). However, the *in vivo* consequences of such displacement are harder to determine for two reasons. First, TTR knockout mice appear to exhibit no adverse phenotype (Palha *et al.*, 2000; Schreiber, 2002), suggesting no effects during development. Second, most chemicals screened in T4-TTR displacement assays are not also screened for other thyroid hormone disrupting activities, making it difficult to ascribe circulating or tissue changes in THs to one specific mechanism. One exception is the flavinoid EMD 49209, which displaces T4 from TTR, but does not inhibit deiodinases *in vivo* (Schroder-van der Elst *et al.*, 1997). This suggests that tissue levels of T3 are reduced because of shortage of T4 as substrate for deiodinases and not to a direct effect on type I or II 5′-deiodinase (Schroder-van der Elst *et al.*, 1998).

Recent evidence supports the conclusion that xenobiotics alter the expression of cellular proteins important for hormone transport into and out of cells. Xenobiotic-induced changes in mRNA and proteins levels of a number of multi-drug resistant proteins and organic anion-transporting polypeptides (OATP) have been demonstrated with both *in vivo* and *in vitro* preparations (Staudinger *et al.*, 2001; Guo *et al.*, 2002; Jigorel *et al.*, 2006; Petrick & Klaassen, 2007). Some of these transporters control thyroid hormone influx into brain and liver cells (Friesema *et al.*, 2005). Changes in the expression of these transporter proteins may interact with other changes induced by TDCs and exacerbate alterations in hormone homeostasis.

A number of xenobiotics may alter thyroid hormone homeostasis via interference with deiodinases. Red dye #3 exposure in rats led to decreases in serum T3 and increases in serum reverse T3 (rT3), T4 and TSH, with inhibition of 5′-deiodinase as a hypothesized mechanism (Capen, 1997). Propylthiouracil, in addition to inhibiting thyroperoxidase (TPO), also inhibits 5′deiodination (Cavalieri & Pitt-Rivers, 1981). Recent work by Korhle *et al.* (Klammer *et al.*, 2007) reports short-term exposure to octyl-methoxycinnamate, a UV inhibitor, resulted in a decreased serum T3, T4 and TSH, along with a decrease in hepatic type I 5′-deiodinase, a pattern of effects quite different than Red dye #3 (Capen, 1997). Developmental exposure to Aroclor 1254, a commercial polychlorinated biphenyl (PCB) mixture, increased whole brain type II 5′-deiodinase in brain homogenates from both dams and offspring (Morse *et al.*, 1993). The upregulation of whole brain type II 5'-deiodinase was likely a compensatory response to maintain tissue T3 concentrations in response to decreased circulating and brain concentrations of T4 (Morse *et al.*, 1993).

The biological actions of TH are driven by the binding of T3 to nuclear thyroid receptors (TRs) (Yen, 2001; Bernal *et al.*, 2003). These ligand bound receptors act as signal transducers and transcription factors by binding to nuclear response elements. Whether xenobiotics bind directly to thyroid receptors is currently a controversial issue: there are data that both support and refute this hypothesis. While there is convincing evidence that xenobiotics alter thyroid hormone-responsive genes (Moriyama *et al.*, 2002; Gauger *et al.*, 2004; Bansal *et al.*, 2005; Kitamura *et al.*, 2005), there is also data indicating a lack of binding to the TR by these chemicals (Cheek *et al.*, 1999; Ishihara *et al.*, 2003; Gauger *et al.*, 2004). Thus, although it appears unlikely that PCB congeners or their metabolites competitively bind to the TR, it may well be that they produce allosteric effects on TRs that alter their

Table 1 Classes, mechanisms-of-action and effects of TDCs on thyroid hormone homostasis

Class	Mechanism	Effects on THs	Chemicals	References
Iodine transport	Competition/block of NIS	Decreased thyroidal synthesis of T3 and T4	Perchlorate, chlorate, bromate	(Wolff, 1998; Van Sande *et al.*, 2003)
Synthesis inhibitors	Inhibition of TPO	Decreased thyroidal synthesis of T3 and T4	Methimazole, propylthiourea, amitrole, mancozeb, soy isoflavones, benzophenone 2, 1-methyl-3-propyl-imidazole-2-thione	(Biegel *et al.*, 1995; Capen, 1997; Hurley, 1998; Doerge & Sheehan, 2002; Schmutzler *et al.*, 2007)
Transport disruption	Altered binding to serum transport proteins	unknown	Hydroxyl-PCBs, EMD 49209; pentachlorophenol	(van den Berg, 1990; Lans *et al.*, 1993; Schroder-van der Elst *et al.*, 1997)
Enhanced hepatic catabolism	Upregulation of glucuronylsyltransferases or sulfotransferases (via CAR/PRX or AhR)	Increased biliary elimination of T3 and T4	Acetochlor, phenobarbital, 3-methylcolanthrene, PCBs, 1-methyl-3-propyl-imidazole-2-thione	(Biegel *et al.*, 1995; Liu & Klaassen, 1996; Brucker-Davis, 1998; Hurley, 1998; Hood & Klaassen, 2000)
Enhanced cellular transport	Upregulation of OATPs or MCT transporters via CAR/PRX or AhR	Increased biliary elimination of T3 and T4	TCPOBOP, pregnenolone-16α–carbonitrile, TCDD, rifampicin, phenobarbital, oltipraz	(Staudinger *et al.*, 2001; Guo *et al.*, 2002; Jigorel *et al.*, 2006; Petrick & Klaassen, 2007)
Sulfotransferases	Inhibition of sulfotransferases (SULTs)	Decrease sulfation of THs	Hydroxlyated PCBs, triclosan, pentachlorophenol	(Schuur *et al.*, 1998; Wang *et al.*, 2004; Wang & James, 2006)
Deiodinases	Inhibition or upregulation of deiodinases	Decreased peripheral synthesis of T3	FD&C Red dye #3, propylthiouracil, PCBs, octyl-methoxycinnamate	(Visser *et al.*, 1979; Morse *et al.*, 1993; Capen, 1998; Klammer *et al.*, 2007)
TR agonists and antagonists	Direct or indirect alterations in TR-TRE binding	Altered activation of TH dependent gene transcription	Tetrabromobisphenol A, bisphenol A, hydroxyPCBs	(Moriyama *et al.*, 2002; Gauger *et al.*, 2004; Kitamura *et al.*, 2005)

ability to mediate thyroid hormone action (Yamauchi & Ishihara, 2006; Zoeller & Tan, 2007).

Not only do structurally diverse chemicals act through different mechanisms to alter serum TH concentrations, they may also cause different patterns of effects on circulating concentrations of THs. For example, synthesis inhibitors (e.g. propylthiouracil) decrease T3 and T4, and increase TSH (Cooper *et al.*, 1983; Hurley, 1998). Decreased T3 and increased T4, rT3 and TSH may be indicative of deiodinase inhibition (Capen, 1997). In contrast, inducers of T4 glucuronidation (i.e. induction of hepatic UGTs) tend to decrease predominately T4 with lesser effects on serum T3 or TSH (Hurley, 1998; Hood & Klaassen, 2000). Upregulation of both T4 and T3 glucuronidation yield decreases in both T4 and increases in TSH (Hood & Klaassen, 2000). Once more is known about the mechanism(s) by which of TDCs alter thyroid hormone homeostasis, patterns of effects on circulating hormone concentrations may play a greater predictive role in risk assessment.

Biological impacts of thyroid hormone disruption

Thyroid hormone insufficiency has two major consequences depending on the stage of development when the exposure occurs. In adult humans, hypothyroidism results in a number of non-specific signs such as constipation and fatigue, dry skin, muscle cramps and menorrhagis (Ladenson *et al.*, 2000). An additional effect of long-term hypothyroidism in adult rodents is thyroid tumour formation. These tumours are the result of long-term overstimulation of the thyroid gland via upregulation of TSH (McClain, 1995; Capen, 1997; Hill *et al.*, 1998). Amelioration of the TSH upregulation, by removal of the disrupting agent or hormone supplementation, will lead to a normal clinical state in both humans and rodents. It is widely recognized that this rodent mode-of-action that results in thyroid tumours is not relevant to humans because of a decreased sensitivity of the human thyroid pituitary axis (Atterwill *et al.*, 1992; Capen, 1997; Hill *et al.*, 1998; Meek *et al.*, 2003). In contrast, a large body of research in both animals and humans documents the permanent and definitely adverse consequences of developmental disruption of thyroid function. Even transient disruption of normal thyroid homeostasis will lead to disastrous outcomes, especially in the developing nervous system [cf. (Boyages & Halpern, 1993; Morreale de Escobar *et al.*, 2000; Delange, 2005)]. Very important to note is that the effects of TDCs in the developing organism are independent of TSH, and instead result from decreases in

tissue levels of T4 and T3 (Porterfield, 2000; Howdeshell, 2002), and may occur via modes-of-action that are likely to be relevant to humans (Crofton, 2004; Crofton & Zoeller, 2005; Zoeller & Crofton, 2005).

The diverse array of xenobiotics that disrupt TH levels (Atterwill *et al.*, 1992; Brucker-Davis, 1998) and the potential for concurrent exposure to many of these compounds (CDC, 2005), make it imperative to develop an understanding of the potential impact of exposures to mixtures of TDCs.

Mixtures models

One issue that has handicapped mixtures toxicology has been the lack of a standard nomenclature to describe the outcomes of mixtures studies. The lack of a common nomenclature has been recognized for at least 50 years. In 1953, Loewe stated 'Indeed, the quantitative problems of combined drug effect still persist unchanged, revolving around the two terms synergism and antagonism – which are in need of clarification as ever before.' (Loewe, 1953). Loewe described 10 terms to describe drug interaction, based on whether the outcome was homodynamic or ahomodynamic (de Jong, 1961). Earlier, Bliss introduced the concept of independent joint action (Bliss, 1939). Since that time a plethora of terms, with overlapping definitions, have been used to describe the outcome of mixtures effects testing. Wessinger listed over 20 terms commonly used to describe outcomes of drug–drug interaction studies (Wessinger, 1986). Clearly, a common nomenclature will facilitate the development of a framework for assessing the risk of combined exposures.

Most models used to predict the effects of chemical mixtures use the concepts of dose and effect addition. The following definitions are used in the text that follows. *Effect addition* predicts the effectiveness of a mixture of chemicals that affect the same end point but act through dissimilar modes-of-action (USEPA, 2000). Effect addition involves simple summation of the effectiveness of each component of the mixture. *Dose addition* predicts the effectiveness of mixtures containing chemicals that have similar modes-of-action (USEPA, 2000). Dose addition assumes that each chemical behaves as a dilution of every other chemical in the mixture, and that the response to the mixture is the same as that expected from an equivalent dose of an index chemical. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical (USEPA, 2000).

Figure 2 provides hypothetical examples of effect addition and dose addition for a mixture of three chemicals A, B and C. The upper panels illustrate the dose-response function for each chemical alone. Chemical A is more potent compared with chemical B and chemical B is more potent than chemical C. Effect addition and dose addition predict very different outcomes from a mixture of the three chemicals. The mixture contains the dose of each chemical illustrated by the arrows in the upper panel. Effect addition sums the effectiveness (y-axis) of each chemical alone: 0 + 0 + 0 = 0 and predicts that the mixture should have no efficacy (middle panel). Dose addition predicts the effect of the mixture by summing the relative potency of each chemical (middle panel, in this case the potency of each chemical is expressed as a percentage of an equivalent effectiveness, the ED_{50}): 30 + 30 + 40 = 100% (of the ED_{50}). The lower panels illustrate an experiment where each chemical is administered alone (groups A, B and C) and in combination in a mixture (Group Mix). Effect addition predicts no effect of the mixture on this hypothetical outcome. Dose addition predicts a 50% decrease in the response, *even though each chemical alone produces no measurable effect on the response* (note the lack of effect of chemicals A, B and C in the lower panels of Fig. 2).

TDC mixtures

The number of studies investigating the impact of mixtures on endocrine systems has increased in the past decade. While there are a number of quality studies examining the effects of androgenic or oestrogenic mixtures (Rajapakse *et al.*, 2002; Tinwell & Ashby, 2004; Howdeshell *et al.*, 2007; Metzdorff *et al.*, 2007), there are a limited number of studies of mixtures of TDCs. Desaulniers *et al.* (2003) demonstrated that toxic equivalents (Van den Berg *et al.*, 1998) predicted the additive effects of a mixture of coplanar-PCBs, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) on circulating thyroxine (T4) concentrations in neonatal rats. Wade *et al.* (2002) exposed male rats subchronically to a mixture of 16 organochlorines, lead and cadmium, and reported that thyroid histopathology and hormone effects were under predicted based on effect additivity of published health advisories. More recently, enhanced toxicity was reported for a binary mixture of perchorate and sodium chlorate on thyroid hormone and pathology indicators (Khan *et al.*, 2005). A binary mixture of PCB126 and perchlorate produced a less than additive effects on the hypothalamic-pituitary-thyroid (HPT) axis when exposure included a high dose of PCB126 (McLanahan *et al.*, 2007). These previous efforts investigated the effects of mixtures without concurrent experimental characterization of the effects of the individual chemicals and/or lacked either by study design or statistical approach, the ability to test for additivity. The use of rigorous statistical models is critical for testing hypotheses of effect or dose addition, and determining whether antagonism or synergism exists (Feron & Groten, 2002;

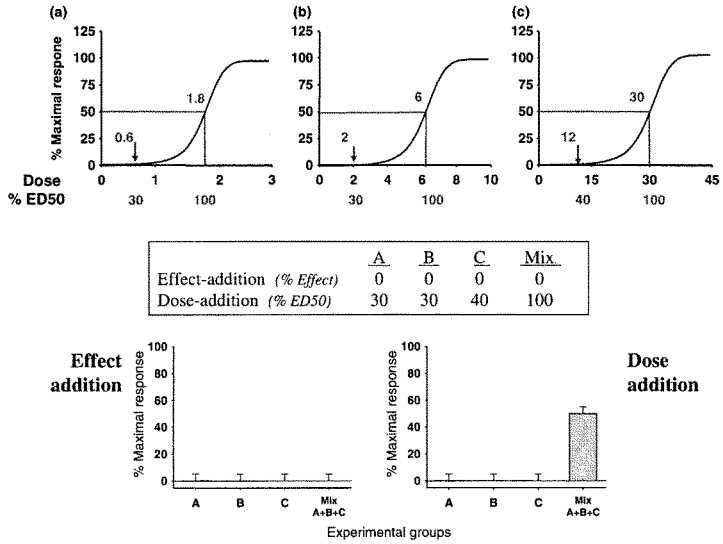

Figure 2 A hypothetical example of three chemicals, A, B and C, and outcomes based on effect-addition or dose-addition (see text for details).

Hertzberg & Teuschler, 2002; Gennings *et al.*, 2004; LeBlanc & Olmstead, 2004; Teuschler, 2007).

Dose-addition theory has been tested with a mixture of 18 polyhalogenated hydrocarbons that contained dioxins, dibenzofurans and PCBs using an up-to-date statistical model (Crofton *et al.*, 2005). One mechanism by which these chemicals alter THs is thought to be via upregulation of hepatic catabolic enzymes (e.g. UGTs). TCDD, dibenzofurans and dioxin-like PCBs activates a network of phase II and III proteins via binding the the aryl-hydrocarbon receptor (Schrenk, 1998). The non-dioxin like PCBs active a slightly different set of enzymes (and possibly transporters) via binding to pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) (Schuetz *et al.*, 1998; Kretschmer & Baldwin, 2005). These chemicals were all shown to decrease circulating concentrations of T4 (Saeed & Hansen, 1997; Craft *et al.*, 2002; Khan *et al.*, 2002; Crofton *et al.*, 2005; McLanahan *et al.*, 2007). Theoretically, dose addition would not predict the effects of a mixture of these chemicals because of differences in mechanisms of action (i.e. AhR agonists and CAR/PXR agonists). This hypothesis was directly tested using a mixture of TDCs (Table 2) that were known to decrease serum total thyroxine (T4) concentrations in a dose-additive manner (Crofton *et al.*, 2005). Young female Long-Evans rats were dosed via gavage with 18 different polyhalogenated aromatic hydrocarbons (PHAHs) (two dioxins, four dibenzofurans and 12 PCBs, including dioxin-like and non-dioxin-like PCBs) for four consecutive days. Serum total T4 was measured in serum samples collected 24 h after the last dose. Extensive (7–9 doses/chemical) dose–response functions were determined for individual chemicals. A mixture was custom synthesized with the ratio of chemical components based on environmental concentrations and serial dilutions of the mixture were tested. Individual chemical potencies and the predicted outcomes from the mixture were calculated using a 'flexible single chemical required' method applicable to chemicals with differing dose thresholds and maximum-effect asymptotes (Gennings *et al.*, 2004). There was no deviation from dose additivity at the lowest doses of the mixture, whereas there was a greater than additive effect at the highest mixtures doses (Fig. 3). At high doses, the dose-additivity model under predicted the empirical effects by two- to threefold. These are the first results to suggest dose-dependent additivity and synergism in TDCs that may act via different mechanisms in a complex mixture. The results imply that cumulative risk approaches

Table 2 The chemical used in the PHAH mixture, the concentration in the highest dose of the mixture tested, the ratio of each chemical in the mixture relative to the total mixture mass and the ratio relative to TCDD (from Crofton *et al.*, 2005)

Chemical	Concentration[a] (μg/mL)	Ratio (TCDD)	Ratio (total mass)
TCDD	0.013	1.0	0.000007
PCDD	0.013	1.0	0.000007
TCDF	0.019	1.4	0.000010
1-PCDF	0.006	0.4	0.000003
4-PCDF	0.026	1.9	0.000013
OCDF	0.065	4.6	0.000032
PCB28	78.600	5605.3	0.039237
PCB52	155.200	11074.7	0.077523
PCB77	2.000	141.1	0.000988
PCB101	153.800	10973.4	0.076814
PCB105	76.700	5468.9	0.038282
PCB118	381.100	27186.0	0.190302
PCB126	0.610	43.1	0.000302
PCB138	380.900	27168.7	0.190181
PCB153	382.200	27265.9	0.190861
PCB156	13.100	934.4	0.006541
PCB169	0.400	28.1	0.000197
PCB180	377.900	26957.1	0.188700

[a]Chemical concentration in the highest dose of the mixture administered.

be considered when assessing the risk of exposure to chemical mixtures that contain TDCs with different mechanisms-of-action.

There are three conclusions apparent from these data. The first is that exposure to the 18 chemical mixture resulted in effects that were greater than those predicted by effect addition, i.e. the mixture actually caused decreases in T4 concentrations even though the individual chemical concentrations in the mixture were below effective doses. This demonstrates that effect addition is not a tenable hypothesis for PHAH TDCs. The second conclusion is that while the greater than dose additive effects are statistically significant, the magnitude of underestimation of the experimental data (Fig. 3, solid line) by the additivity model (Fig. 3, dotted line) is small. On a dose basis, the underestimation is about 2.5-fold for the three highest doses of the mixture. This suggests that, even in the high mixture-dose region, the effects of this mixture are predicted by additivity with a fair degree of accuracy. The third conclusion is that departure from additivity was not detected in the low dose region. While this suggests that dose additivity predicts effects on T4 at low exposures, it is tempered by a presumed low statistical power to detect differences in this area of the dose response.

The significance of these findings for environmental exposures is restrained by some uncertainty (Crofton *et al.*, 2005). The current work used a weanling animal model with a short (i.e. 4 days) exposure duration that may confound extrapolation of long half-life chemicals like PHAHs. Results from these short-term exposures may not accurately predict the effects of real-life chronic low-level exposures. In addition, extrapolation of the current work in rats to humans is hampered by the uncertainty in how the mode(s)-of-action of the TDCs may differ between species (Sonich-Mullin *et al.*, 2001; Seed *et al.*, 2005). Relevancy analysis for PCBs and propylthiouracil suggest that concordance between rodent and

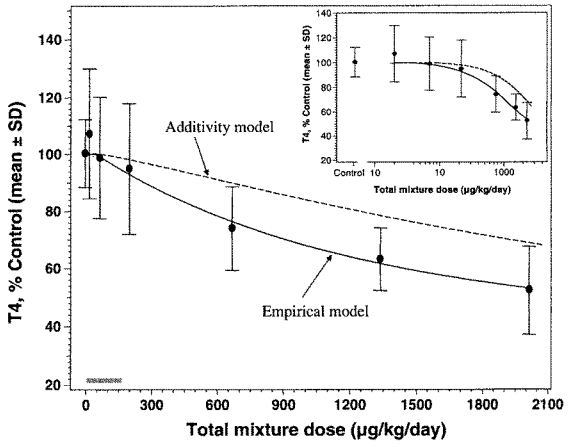

Figure 3 The empirical and predicted effects of the PHAH mixture on serum total thyroxine (T4). The predicted effects were generated using a single-chemicals required additivity model (Gennings *et al.*, 2004). The results demonstrated a significant deviation from dose additivity at the three highest mixture doses. The effects of the lower mixture doses were not significantly different than that predicted by additivity. The grey bar on the x-axis indicates the approximate range of background human exposure. The inset illustrates the same data plotted as log dose. (modified from Crofton *et al.*, 2005).

human modes-of-action depends on the life stage of exposure. There is a good degree of concordance for the developmental neurotoxicity using altered TH concentrations as a key event (Crofton & Zoeller, 2005; Zoeller & Crofton, 2005) but no concordance for the tumourigenic effects that require TSH upregulation as a key event (Axelrad *et al.*, 2005). Data gaps in key events have been identified that should be researched to increase confidence in the relevancy of this mode-of-action (Crofton & Zoeller, 2005; Zoeller & Crofton, 2005) that include: a better characterization of the inducibility of and sensitivity of UGTs during human and rodent foetal development, comparative data on the relationship between serum and brain tissue levels of THs, and comparative studies on the effects of moderate and mild hypothyroxinemia on nervous system development.

Research challenges

A number of research challenges need to be addressed in order to more efficiently and accurately predict the adverse health outcomes of TDC mixtures. These include, choice of the biomarker of effect, developing predictive models that can account for compensatory mechanisms, exposure to mixtures with diverse mechanisms and extrapolation of data from animal models to humans.

A major question for modelling TDC mixtures is the choice of which biomarker to use. Biomarkers of effect range from adverse outcomes (i.e. clinical measures) to molecular interactions at key target sites (Fig. 4). Recent data from Rider *et al.* (2008) suggest that the tissue concentration of androgenic ligands available for interaction with the receptor is critical, and not measures of the initial target mechanisms. These data suggest that chemicals that do not act via a common cellular mechanism of action may result in dose-additive effects on common downstream endpoints. The TDC mixture results discussed above support this to a limited extent. The effect on circulating T4 that result from at least two initial targets of thyroid disruption (i.e. activation of hepatic Ah receptors or CAR/PXR) is reasonably well predicted using dose addition (Crofton *et al.*, 2005). Thus, for TDCs effects on neurodevelopment, the critical mediator of adverse outcome may be a combination of tissue levels of T3 (driven in part by circulating levels of T3 and T4) and tissue concentrations of chemicals that act to augment or antagonize binding of T3 to TRs (Fig. 4).

The complex array of intra-thyroidal and extra-thyroid feedback mechanisms, and our lack of understanding the responses to multiple perturbations (different sites), increase the difficulty in predicting the effects of complex TDC mixtures. Thyroid hormone homeostasis, and more

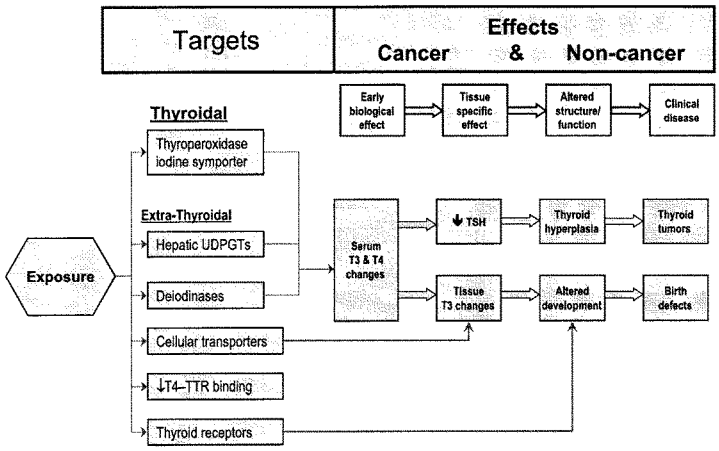

Figure 4 A combined mode-of-action model for the effects of TDCs on cancer and non-cancer outcomes. Mixtures models are needed to better predict effects of mixtures containing xenobiotics that affect multiple targets with common downstream effects (modified from USEPA, 2000; Crofton & Zoeller, 2005).

importantly, tissue concentrations of THs, are regulated by a number of complex compensatory processes that strive to maintain euthyroid status (Fig. 1). For example, increased brain deiodinase activity can compensate for xenobiotic-induced decreases in circulating and tissue concentrations of THs (Morse *et al.*, 1993). Lowered iodine concentration can result in increased expression of the sodium-iodine transporter (Spitzweg *et al.*, 1999; Opitz *et al.*, 2006). Cellular transporter expression changes because of thyroid status and influences T3 concentrations in brain, which is also regulated by both glial production of T3 from T4 which is then transported into neurons, as well as direct uptake in neurons of T3 and T4 by MCT8 transporters (Galton *et al.*, 2007). Once inside the cell, an array of intracellular TH/TR mediated signalling processes are potential targets for disruption by xenobiotics (Yamauchi & Ishihara, 2006; Zoeller & Tan, 2007). These include ligand induced conformational changes of DNA-bound TRs and alterations in the levels of corepressors and coactivators. Disruption of these targets/pathways may alter regulation of intracellular T3, as well as changes in cell sensitivity to T3 (Flamant *et al.*, 2007). Models are needed that can accurately predict the impact xenobiotic mixtures that act at multiple targets and active numerous compensatory processes.

A major uncertainty that hampers the risk assessment of TDCs is the lack of a clear characterization of the dose–response relationship between the degree of change in hormone concentrations and adverse consequences. If accurate predictions of the effects of TDC mixtures circulating or tissue TH are possible, there is still a concern as to what degree of change leads to adverse outcomes, and whether the mechanism(s) responsible for the changes found in animal models are similar in humans. Published literature from animal models consists almost entirety data derived from experimental conditions where THs are severely depleted by high doses of thryotoxicants [e.g. (Uziel *et al.*, 1981; Timiras & Nzekwe, 1989; Morreale de Escobar *et al.*, 1993; Hendrich & Porterfield, 1996; Lavado-Autric *et al.*, 2003]. While these studies provide knowledge about the role of THs in nervous system development, it does not prove as useful in estimating risks from environmental TDCs. Only recently have data been generated that characterize the relationships between low and moderate thyroid hormone perturbations and adverse outcomes (Crofton, 2004; Pedraza *et al.*, 2006; Goodman & Gilbert, 2007). These types of experiments are crucial for correctly modelling outcomes in humans, where xenobiotics are likely to only modestly perturb TH homeostasis. Indeed, data from CDC surveys suggest only mild T4 disruptions from combined mild iodine deficiency and low perchlorate exposures (Blount *et al.*, 2006).

Conclusions

There are a variety of environmental contaminants that disrupt thyroid hormone homeostasis. The critical role of THs for brain development has increased concern over possible additive or synergistic effects of exposure to TDC mixtures. Advances in understanding the mechanisms by which TDCs act, improved models for predicting the effects of mixtures, and use of mode-of-action models is beginning to reduce the uncertainties in both interpretation and extrapolation of results from studies of TDC mixtures. The limited data available to date from mixtures models for TDCs suggest that dose addition is reasonably accurate in predicting the effects on serum T4 concentrations. Assessing the health risks of thyroid disruption by environmental xenobiotics will need to include an improved understanding of how divergent mechanisms alter THs and consequent adverse impacts on nervous system development.

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Research | Review

Large Effects from Small Exposures. I. Mechanisms for Endocrine-Disrupting Chemicals with Estrogenic Activity

Wade V. Welshons,[1] Kristina A. Thayer,[2] Barbara M. Judy,[1] Julia A. Taylor,[1] Edward M. Curran,[1] and Frederick S. vom Saal[2]

[1]Department of Veterinary Biomedical Sciences and [2]Division of Biological Sciences, University of Missouri-Columbia, Columbia, Missouri, USA

Information concerning the fundamental mechanisms of action of both natural and environmental hormones, combined with information concerning endogenous hormone concentrations, reveals how endocrine-disrupting chemicals with estrogenic activity (EEDCs) can be active at concentrations far below those currently being tested in toxicological studies. Using only very high doses in toxicological studies of EEDCs thus can dramatically underestimate bioactivity. Specifically: *a*) The hormonal action mechanisms and the physiology of delivery of EEDCs predict with accuracy the low-dose ranges of biological activity, which have been missed by traditional toxicological testing. *b*) Toxicology assumes that it is valid to extrapolate linearly from high doses over a very wide dose range to predict responses at doses within the physiological range of receptor occupancy for an EEDC; however, because receptor-mediated responses saturate, this assumption is invalid. *c*) Furthermore, receptor-mediated responses can first increase and then decrease as dose increases, contradicting the assumption that dose–response relationships are monotonic. *d*) Exogenous estrogens modulate a system that is physiologically active and thus is already above threshold, contradicting the traditional toxicological assumption of thresholds for endocrine responses to EEDCs. These four fundamental issues are problematic for risk assessment methods used by regulatory agencies, because they challenge the traditional use of extrapolation from high-dose testing to predict responses at the much lower environmentally relevant doses. These doses are within the range of current exposures to numerous chemicals in wildlife and humans. These problems are exacerbated by the fact that the type of positive and negative controls appropriate to the study of endocrine responses are not part of traditional toxicological testing and are frequently omitted, or when present, have been misinterpreted. *Key words:* dose response, endocrine disruptors, estrogen action, estrogen receptors, fetal development, inverted U, MCF-7 cells. *Environ Health Perspect* 111:994–1006 (2003). doi:10.1289/ehp.5494 available via *http://dx.doi.org/* [Online 2 February 2003]

During the past decade a number of pesticides, industrial by-products, manufactured products such as plastics, and natural chemicals have been shown to disrupt the endocrine system. These chemicals are referred to as endocrine-disrupting chemicals (EDCs). These chemicals have received considerable attention, in part because endocrine disruption is a relatively unstudied area in toxicology and is only recently being taken into account in risk assessment. The focus here is on EDCs with estrogenic activity (EEDCs), which are chemicals that act as hormone mimics via estrogen receptor mechanisms; this is currently the largest group of known endocrine disruptors. The main purpose of this article is to present an overview of the mechanisms of hormone action that provide the basis for understanding how EEDCs have the potential to be biologically active at low, environmentally relevant doses. Our strategy is to discuss the receptor mechanisms mediating responses to a natural hormone, 17β-estradiol (E_2), and then to use this information as the basis for describing the low-dose effects of chemicals that disrupt the normal functioning of this hormonal system, either by mimicking, modulating, or antagonizing the activity of the hormone. We have chosen to use estrogen as our example because there is more known about the biology of estrogens and xenoestrogens than other components of the endocrine system for which there is evidence for disruption by environmental chemicals; however, the information presented here is applicable to endocrine disruptors that interfere with other hormonal systems.

We will begin by briefly reviewing information concerning the relationship between dose, receptor occupancy, and responses (such as cell proliferation) after binding of E_2 to estrogen receptors (ER-α) in cultured human MCF-7 breast cancer cells. A number of specific factors influence the dose of an EEDC that reaches the target cells to produce a response. These factors include route of administration, absorption, distribution, metabolism, rate of clearance, plasma transport, cell uptake, affinity for estrogen receptor subtype in the cell, and the interaction of the ligand–receptor complex with tissue-specific factors comprising the transcriptional apparatus. This mechanistic information provides the basis for establishing the dose at the target site in cells (nuclear receptors associated with DNA or more recently identified receptors associated with the cell membrane) for an EEDC required to elicit a biological response similar to that produced by a dose of E_2 with equal estrogenic activity. Modeling that takes into account each of these factors would encompass physiologically based pharmacokinetic information (*1*), as well as quantitative structure-activity relationships (QSAR) (*2,3*). We have previously discussed the factors that influence access of E_2 and EEDCs from blood to estrogen receptors in cells elsewhere (*4–6*). Our primary focus in this review is on the latter part of the overall process that occurs once an estrogenic chemical has reached the nuclear estrogen receptor.

Dose ranges. We have separated dose-specific effects into three general categories: the physiological dose range for estrogenic activity, the toxicological dose range for acute toxicity, and the environmentally relevant dose range related to current exposures. The physiological dose range (of estrogenic activity, whatever the source) is defined by the normal concentration range of an endogenous hormone. More specifically, with regard to steroid hormones, the physiological concentration refers to the amount of free (unbound to plasma proteins and unconjugated) endogenous hormone that the EEDC is mimicking or antagonizing. The free hormone concentration is generally considered to be the biologically active portion of total hormone concentration in blood (*7,8*) and most accurately predicts biological activity (for example, free triiodothyronine and free thyroxine, as opposed to total hormone concentration, are routinely used for clinical diagnosis). The toxicological dose range is identified by some measure of toxicity, such

Address correspondence to W.V. Welshons, Dept. of Veterinary Biomedical Sciences, E102 Veterinary Medicine, University of Missouri-Columbia, Columbia, MO 65211 USA. Telephone: (573) 882-3347. Fax: (573) 884-6890. E-mail: welshonsw@missouri.edu

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as death in the extreme case, a decrease in body weight, or malformations in a developmental study. The environmentally relevant dose range can be established for chemicals where there is information concerning levels monitored in air, food, or water or, less commonly, if there is information based on monitoring of biological tissues in wildlife or human populations.

It is important to note that during fetal and early postnatal life, the pharmacokinetics of chemicals and drugs are markedly different relative to adulthood, and pregnant and nonpregnant females also differ in this regard. Therefore, dose ranges in pregnant females and fetuses cannot be assumed to be the same as in adults and should be evaluated separately.

Low-dose range. The physiological and the environmentally relevant dose ranges typically fall well below the toxicological dose range based on using established protocols for examining acute toxic effects of chemicals. Exceptions would be instances of industrial accidents or workplace exposure, such as the Yu-Cheng incident in Taiwan involving accidental exposure to acutely toxic doses of polychlorinated biphenyls (PCBs) (*9*) or exposure to synthetic estrogens by workers in pharmaceutical plants (*10*).

At a meeting hosted by the National Institutes of Health (NIH) at the request of the U.S. Environmental Protection Agency (U.S. EPA), devoted to the low-dose issue (*11*), low dose was defined as doses below the range typically used in toxicological studies, where the dose range seldom extends more than 50-fold below the maximum tolerated dose (MTD) in an animal (*12,13*). The physiological and the environmentally relevant ranges we describe here fall within this low-dose range defined at the NIH meeting. For example, the MTD for the plastic monomer bisphenol A is 1,000 mg/kg/day (*14*). The U.S. EPA calculated a reference dose (RfD) based on a LOEL (lowest-observed-effect level) of 50 mg/kg/day; this was because a no-observed-adverse-effect level had not been determined, and adverse responses occurred at the lowest dose tested. The RfD of bisphenol A based on application of a safety factor of 1,000 was calculated to be 50 μg/kg/day (*15*).

The environmentally relevant amount of bisphenol A, however, has recently been determined on the basis of direct measurement in the blood of human fetuses at term. Parent (unconjugated, aglycone) bisphenol A concentrations ranged from 0.2 to 9.2 ng/mL, with a mean ± SD of 2.9 ± 2.5 ng/mL (*16*).

Developmental exposures. Although the issues discussed in this review apply to exposure to endocrine disruptors at any time in life, it is generally accepted that EDCs have the greatest impact when exposure occurs during development (*17,18*). In describing the *in vivo* effects of EDCs, we will emphasize effects of endocrine disruptors on fetal development. During fetal life, endogenous hormones regulate the differentiation and growth of cells, and developmental processes appear to have evolved to be exquisitely sensitive to changes in hormone concentrations. A consequence of this evolved strategy of development being epigenetic (that is, based on signals that cells are exposed to rather than due to a fixed genetic program) is that even in animals that are genetically identical, small fluctuations in endogenous hormonal signals during development provide the basis for significant variability in phenotype (*19*). This provides the mechanism via which even slight alterations in hormonal activity due to exposure to EDCs during very brief critical developmental periods in fetal life can potentially lead to irreversible changes in the course of differentiation of cells. These cellular changes are associated with permanent alterations in gene activity and organ function (*20,21*).

Implications. We will review mechanistic information showing that failure to apply fundamental principles of hormone receptor biology to dose selection in toxicological studies can potentially lead to a huge error in estimating risk associated with exposure to doses below the NOEL (no-observed-effect level) determined in traditional toxicological studies. These issues are problematic for toxicology, because they challenge the traditional use of extrapolation from high-dose testing to predict responses at much lower environmentally relevant doses. Additionally, these data also provide evidence that some traditional assumptions used in risk assessment for systemic (noncarcinogenic) toxicants, such as the assumption of a threshold (*22*) and a monotonic dose–response relationship (*23*), cannot be uniformly applied to EDCs (*24,25*). We will relate our findings regarding effects of very low doses (within the range of human exposure) of bisphenol A (the monomer used to manufacture resins and polycarbonate plastic and used as an additive in many other products) and methoxychlor (a currently used insecticide) to current methods of risk assessment for systemic toxicants. The classification of EDCs as systemic toxicants is due to an absence of data and is not based on findings of no genotoxic effects, particularly for estrogenic EDCs (*26*). Because estrogen is implicated in a number of cancers, both as an initiator and promoter, environmental chemicals that mimic estrogen cannot be ruled out as carcinogens. In particular, research is needed to determine whether exposure to EDCs during early life is related to the development of cancer later in life (*26,27*). A recent example of a relevant finding is that at very low doses (0.1–10 nM, 0.023–2.3 ng/mL), bisphenol A induces proliferation of human prostate cancer cells via binding to a mutant form of the androgen receptor found in some prostate tumors (*28*).

It has been known for decades that some environmental chemicals mimic the activity of endogenous hormones. However, the mechanistic information we provide here concerning the functioning of the hormonal systems being disrupted by these chemicals was, in general, not considered in designing toxicological studies conducted to assess safety. This is especially true with regard to doses administered, long-term consequences of exposure during sensitive periods in development, and types of end points examined. With regard to dose, if the mechanistic information concerning hormone action that we review here had been considered, the currently accepted practice of only testing very high doses to predict effects of doses thousands or even millions of times lower would have been recognized as inappropriate. The result would have been that doses of EDCs such as methoxychlor and bisphenol A far below those currently being described as safe would, in fact, have been predicted to produce biological responses, and much lower doses would have been tested. A recent dose range–finding study of the dietary estrogen genistein (*29*) has used a wide range of multiple doses including a low-dose range, and these studies illustrate the importance of this approach (*29,30*). On the basis of the information provided here, we propose that toxicological testing procedures incorporate a much wider dose range, take into account the heightened sensitivity and unique effects (some of which may not be apparent until adulthood) that can occur as a result of endocrine disruption in the fetus, and shift to measuring functional changes in organs (focusing on continuous variables), rather than low-frequency dichotomous variables such as malformations associated with acute toxicity.

Mechanisms of Estrogen Action Predict Low-Dose Effects of EEDCs

Although the mechanism of action of most toxicants is unknown, the mechanism of action for estrogens, including EEDCs, is already known in substantial detail; however, much remains to be learned. For an EEDC to exert a direct estrogenic effect in a cell, the cell must have estrogen receptors (whether the receptors are located in the nucleus, cytoplasm, or cell membrane). With regard to nuclear receptors, the most critical piece of information regarding the mechanism of action of an EEDC is defined by its binding affinity for the subtype of estrogen receptor (alpha or beta) present in the cell. Once affinity for the receptor is estimated, one can

immediately apply information from a vast literature concerning the interaction of estrogenic chemicals with receptors to understand a considerable amount about the mechanisms of action of the chemical. Understanding the mechanism of action for a toxicant allows the incorporation of this information into predicting appropriate doses to use in toxicological studies (*11*). In this section we will describe the relationship between dose, receptor occupancy, and responses, such as cell proliferation, after binding of E_2 to estrogen receptors (specifically, ER-α) in cultured human MCF-7 breast cancer cells. In a subsequent article (*31*), we will relate this information to the results of *in vivo* experiments showing that the bioactive concentration of E_2 in serum during development in mice and rats is very similar to the bioactive concentration that stimulates cell proliferation in human MCF-7 cells. This information will provide the basis for determining doses of EEDCs that produce effects similar to those caused by an increase in E_2 during development in mice, as well as effects caused by low doses of EEDCs administered at other times in life.

Lipophilic and hydrophilic hormones. Hormones do not act directly, but rather indirectly, through binding to specific receptor proteins. When these receptor proteins are occupied by hormone, they become the signal transduction system for inducing the hormonal response. Two basic transduction systems for hormones have been identified. Hydrophilic hormones, such as the hypothalamic and pituitary hormones, do not easily cross cell membranes, but instead bind to the extracellular domain of transmembrane receptors; binding of the hydrophilic hormone to the membrane-bound receptor results in activation of complex intracellular signaling pathways that can lead to rapid changes (in seconds) in cell function (*32*). The second transduction system is used by lipophilic hormones, including the sex steroids such as E_2, which are small (molecular weight of a few hundred daltons) lipophilic molecules that can diffuse into cells. These hormones bind to intracellular receptors and induce transcription of specific genes (a much slower process). These intracellular receptors act as ligand-dependent transcription factors and belong to the nuclear receptor superfamily that, in addition to estrogen receptors, includes receptors for triiodothyronine, retinoic acid, vitamin D_3, cortisol, androgens, progesterone, and aldosterone (*33–35*). In addition to acting via binding to nuclear receptors, there is now considerable evidence that estradiol interacts with transmembrane receptors to stimulate rapid responses in some cells (*36–39*).

Although hydrophilic and lipophilic hormones act through different receptor systems, both require receptor occupancy as a precursor to produce a response in target cells. There is a critical aspect of this issue with regard to the potential for species differences in the response to EEDCs. It is well known that the gene structure and ligand-binding properties of the classical estrogen receptor (ER-α) have been highly conserved (that is, have experienced relatively little change) among vertebrates separated for up to 300 million years of evolution. Thus, the binding of an estrogenic chemical to ER-α in fish, amphibians, reptiles, birds, and mammals (including humans) shows relatively little difference (*40–42*). Binding to the receptor is the initiating step in endocrine disruption by estrogenic chemicals. It is during events prior to and subsequent to receptor binding that species and tissue differences emerge in terms of differences in absorption and metabolism, as well as specific genes regulated by estrogen. There are also tissue-specific components of the transcriptional apparatus (receptor coregulators) involved in determining which genes are regulated by ligand-activated receptors (*43,44*).

Even within a specific tissue in a single organism, there are developmental changes in the genes regulated by specific hormones (*45*). In addition, with regard to unique developmental effects of EEDCs, there is evidence that the functioning of enzyme systems involved in metabolizing endogenous steroids, drugs, and EDCs differs during fetal life and in adulthood (*46,47*). Regardless of these species, tissue, and life stage differences, if a chemical can bind to estrogen receptors in fish, the evidence is that it will also bind to estrogen receptors in humans and other vertebrates. Until there are data to the contrary, one would expect that the possibility of endocrine disruption occurring in humans can be predicted by assessing binding of an estrogenic chemical to estrogen receptors in any vertebrate. With regard to estrogenic EDCs and their potential for disrupting embryonic development, the similarity between vertebrates with regard to the mechanism of action of estrogenic chemicals that act via binding to estrogen receptors argues strongly for the continued use of animal models to assess human risk (*40–42*). Within the field of comparative endocrinology, the finding of highly conserved molecules such as estradiol and the estrogen-receptor complex has led to the general assumption that it is the specific uses to which hormones and their receptors have been put that has changed throughout the evolution of multicellular organisms, not the hormones and receptors themselves (*48*).

Relationship between hormone concentration and receptor occupancy. There are four properties of receptors that predict responses to estrogen and other hormones. The first property is affinity of the ligand for the receptor, which must be high enough for a sufficient number of receptors to be occupied at the concentrations at which the natural or manmade estrogen is present. The second property is saturability. As binding of the hormone to its receptor shows the property of saturation, there is no further increase in number of occupied receptors as a function of increase in dose once all receptors are occupied. Likewise, biological responses to hormones saturate; interestingly, saturation of response frequently occurs considerably below 100% receptor occupancy in what has been traditionally termed "spare receptor" observations (we cover this in more detail below). The third property is ligand specificity, as all compounds that show hormonal activity (or receptor-mediated antihormonal activity) must bind to the hormone receptor, whereas compounds that at a given concentration do not have hormonal activity (or antihormonal activity) do not bind to the receptor. The fourth property is tissue specificity of receptor distribution. Tissues that respond to the presence of a hormone must have receptors for the hormone. If a given cell does not have receptors for the hormone, that hormone is "invisible" to that cell, and the cell can show no primary response to the hormone, although indirect (secondary) effects may be observed. At concentrations above those within a normal physiological range, hormones may bind to receptors for other hormones. For example, E_2 binds to androgen receptors at concentrations approximately 100 times higher than the concentrations required to occupy estrogen receptors and induce responses (*49*). The biological consequences of "cross-talk" with other receptors at high doses of a ligand have not been well characterized for most systems, but this likely contributes to qualitatively different effects at low (physiological) and high (toxicological) doses. We discuss dose–response issues in more detail below.

Receptor occupancy is directly linked to responses, and responses to either a natural estrogen or an EEDC are brought about in relation to the number of occupied receptors. Above 10% receptor occupancy, and particularly above 50% receptor occupancy, which mathematically defines the K_d (the dissociation constant from the law of mass action applied to receptor–ligand binding kinetics) of the binding of hormone and receptor, receptor occupancy is never determined to be linear in relation to hormone concentration. Using a less stringent definition of linearity, proportionality between receptor occupancy and hormone concentration is observed below 10% receptor occupancy, and the relationship

between receptor occupancy and response (such as cell proliferation) is also only proportional below 10% receptor occupancy. We will thus consider that the relationship between receptor occupancy and hormone concentration, as well as between receptor occupancy and response, are approximately linear up to 10% receptor occupancy. At concentrations above the K_d, saturation of response occurs first, and then at higher concentrations, saturation of receptors is observed.

An example based on administration of E_2 to MCF-7 cells of the relationship between hormone concentration, receptor occupancy, and a response (cell proliferation) is presented in Table 1. The data in Table 1 show that as hormone concentration increases by factors of 10, receptor occupancy typically increases by the following relationship: *a*) If the hormone concentration is 1% of its K_d (% K_d: Table 1, middle column), the number of receptors occupied is also approximately 1% of total receptors. *b*) With a 10-fold increase in hormone concentration to 10% of the K_d, receptor occupancy increases to approximately 9%. *c*) The next 10-fold increase in hormone concentration is to the K_d and leads to 50% receptor occupancy. *d*) With another 10-fold increase in hormone concentration, 91% of receptors are occupied. *e*) Finally, another 10-fold increase in hormone concentration only leads to a small increase, from 91 to 99% receptor occupancy.

The importance of the data in Table 1 is that while at the lowest concentration referenced, a 10-fold increase in hormone leads to a 9-fold increase in receptor occupancy (from 1 to 9%), between the highest doses, a 10-fold increase in hormone concentration only leads to less than a 1.1-fold increase in receptor occupancy (from 91 to 99%). The practical result is that while at hormone concentrations below 10% receptor occupancy (10-fold below the K_d) receptor occupancy is close to proportional to hormone concentration, this is not the case above this concentration. The view of the previously mentioned "spare receptor" hypothesis from this perspective is that a system such as this, which we assume evolved to be responsive to small changes in ligand concentration, could only operate in a portion of the binding range that was nearly linear (below 10% receptor occupancy), thus leading to the observation that there appeared to be receptors that were in surplus over those needed for responses, hence spare receptors. Surplus hormone receptors over the number of occupied receptors required for response (*50,51*) was recognized early in the study of the steroid receptors and steroid receptor-mediated action (*52*).

At the dose ranges of EEDCs used in current toxicity testing, chemicals are likely to be present within target cells at concentrations many orders of magnitude above their K_d for estrogen receptors. Within this dose range, changes in hormone concentration cannot have a detectable effect on receptor occupancy, because all receptors are saturated at 100% and no additional binding, which is required to result in an increase in response, can be observed. No primary hormonal effects can be observed in response to changes within this high-dose range, but only secondary effects not mediated by estrogen receptors.

Relationship between receptor occupancy and response. It is sometimes erroneously assumed that hormones act *in vivo* at their K_d (50% receptor occupancy). With a few exceptions, the physiological ranges for natural hormones (more specifically, the free, bioactive fraction (*7,8*) of the total circulating) are typically below the K_d. A biological basis for this observation may be that if natural hormone concentrations were at or above the K_d and thus near receptor saturation, even quite large changes in hormone concentrations would result in only a small change in occupied receptors. This type of system would be relatively insensitive to changes in hormone concentrations and would require dramatic changes in hormone concentrations to elicit changes in response. Because very small changes in hormone concentrations, for example, a 50% increase, were associated with changes in responses in animal studies, it appears that the working range for hormones must be well below the K_d, and indeed the animal data support this hypothesis (*19,23,53,54*).

In many biological systems, saturation of response is observed well below saturation of receptors, and saturation of specific responses may even occur below the K_d. As indicated above, the spare receptor hypothesis is the term applied to this kind of observation (*55–58*) and has been described in detail, particularly on the basis of observations with transmembrane receptors. Specifically, transmembrane receptors show a much greater percent inhibition as the dose of ligand increases (~ 90%) than do nuclear receptors that are members of the nuclear receptor superfamily (~ 50%) (*59,60*). The potential contribution to nonmonotonic dose–response curves of the loss of receptors as dose of ligand increases is covered below.

There is only near-linearity of dose and occupancy up to a dose that results in 10% of receptors being occupied (below 0.01 nM for E_2), and the near-linear range between dose and response is even more restricted (shifted to the left). For example, although the K_d for E_2 binding to ER-α is approximately 0.1 nM, a significant increase in proliferation of MCF-7 estrogen-responsive breast cancer cells is seen with addition of 0.0004 nM E_2 to estrogen-free medium. Half-maximal proliferation is seen at 0.001 nM E_2, and near-maximum proliferation is seen between 0.01 and 0.1 nM. Thus, almost 91% of maximal cell proliferation is observed at a concentration 10-fold below the K_d, at a ligand concentration approximately 100-fold lower than 91% of receptor saturation (Table 1). The relationship between hormone response and receptor occupancy is not limited to permanent cell lines and has also been described for a number of estrogenic chemicals in primary rat uterine cells, where, as above, saturation of response occurs before saturation of receptor occupancy (*61*).

Interestingly, for E_2, the dose required to induce different responses in the same cell is not the same. For example, in GH_3 rat pituitary cells *in vitro*, proliferation of cells is half maximal at an E_2 concentration between 0.001 and 0.01 nM, whereas synthesis of prolactin is half-maximally induced at 0.1 nM (*62*). Progesterone receptors in MCF-7 cells require roughly 10 times more E_2 for induction relative to proliferation (*63*),

Table 1. Mathematical calculations of receptor occupancy versus hormone concentration for an example where the K_d = 0.1 nM.[a]

	Estradiol concentration (nM)	Estradiol concentration (ng/mL)[b]	Percent of K_d	Receptors occupied (%)[c]	Cell proliferation, (% of maximum response)[d]
	10	2.72	10,000	99	100
	1	0.272	1,000	91	100
K_d	0.1	0.0272	100	50	99
	0.01	0.00272	10	9	91
PR[e]	0.001	0.000272	1	1	50
	0.0001	0.0000272	0.1	0.1	9

[a]This K_d was chosen because it represents a midrange value commonly measured for the binding of estradiol to the estrogen receptor. [b]ng/mL = ng/g = μg/kg = ppb. [c]The mathematical relationship described here between ligand concentration and receptor occupancy applies to receptor–ligand interactions for all hormones, although each ligand will have a unique K_d associated with 50% receptor occupancy. [d]This column in the table represents a physiological response, in this example, the estrogen-dependent proliferation of MCF-7 human breast cancer cells. [e]The physiological range (PR), occurring at an EC_{50} of 1 pM for cell proliferation, was determined from both *in vitro* stimulation of cell proliferation at 1 pM = 0.27 pg/mL (Figure 1) and free E_2 at EC_{50} = 0.2 pg/mL (*54*), and *in vivo* studies where free E_2 = 0.21 to 0.54 pg/mL (*23,53*) and is within the range of 1% receptor occupancy. Note that here, as in many systems, response saturates (e.g., 99% response at 0.1 nM and 50% receptor occupancy) well before receptor occupancy saturates (e.g., 10 nM and 99% receptor occupancy).

similar to induction of prolactin in GH_3 cells. This relationship demonstrates that the activation of different genes requires different numbers of receptors to be occupied. Importantly, both of these responses saturate at a percent receptor occupancy far below receptor saturation, that is, spare receptor kinetics still apply.

Nonmonotonic Dose Response to Estrogens

Nonmonotonic (inverted-U) dose–response relationships: in vitro effects of low and high doses of estrogens. Responses to hormones, including estrogens, saturate as does receptor occupancy, and therefore cannot be linear as a function of an increase in dose within the high-dose range. Further, for many responses to a wide range of concentrations, across many powers of 10-fold, the dose–response relationship is nonmonotonic as well, with response decreasing at doses above those that initially reach a level of saturation. There are a number of published examples of this *in vivo* and *in vitro*. In male mouse fetuses, a very small increase in E_2 or a physiologically equivalent increase in estrogenic activity by an estrogenic chemical such as diethylstilbestrol (DES) resulted in prostate enlargement detected later in life (*23,64–66*). In marked contrast to these findings, consistent with numerous prior studies, administration of much higher doses of either natural or man-made estrogens during the prenatal or neonatal period of prostate development caused a reduction in prostate size relative to untreated males (*23,64,66–69*).

The lower doses of DES that resulted in an increase in prostate size (*23,64,65*) were predicted to increase total serum estrogenic activity within a physiological range, based on studies of the free concentration of DES in serum (*5*) and transplacental transport of radiolabeled DES in pregnant mice (*47*). Specifically, a low dose of DES of 0.02 μg/kg/day administered to pregnant mice was predicted to lead to an increase in free, bioavailable DES in the fetus that falls within the physiological dose range of free, bioavailable estrogenic activity during normal fetal development (*54*); this exposure led to the prostate enlargement response (*23*). This dose of DES, in the physiological range of estrogenic activity, falls within the low-dose range of exposure. In contrast, in the same studies, a 10,000-times higher dose of DES (200 μg/kg/day) resulted in gross abnormalities in the reproductive organs, including a marked reduction in prostate size (*23,64*). This dose of DES therefore falls within the toxicological dose range and represents a high-dose range of exposure.

There are many additional examples of nonmonotonic dose–response relationships. For example, it has been known for some time that there are adverse effects at low and high doses, on either side of an optimum physiological range for normal development, for other ligands that bind to receptors in the steroid receptor superfamily, such as vitamin A and thyroid hormone. It is difficult to compile a literature focusing on inverted-U dose–response curves, as these types of dose–response functions are common in endocrine studies and are often not identified in titles or abstracts as a noteworthy finding. Among those that have been reported, nonmonotonic dose–response curves can occur at several levels of organization, ranging from the biochemical based on *in vitro* studies (*28,54,62,70–75*) to the organ or system level based on *in vivo* studies (*23,60,66,76–82*).

MCF-7 cell in vitro model for inverted-U endocrine dose responses. MCF-7 human breast cancer cells (*83*) are a permanent cell line that contains estrogen receptors. These cells have retained estrogen responsiveness for a sustained period of continuous cell culture and show estrogen-dependent stimulation of cell proliferation by natural and xenobiotic estrogens (*84–86*). In addition, the same chemicals that stimulate growth at lower concentrations can slow MCF-7 cell growth at higher concentrations (*72,73,* for example) and inhibit growth by acute cytotoxicity at high concentrations in the micromolar (ppm) range (Figure 1A). The dose–response range required to observe these dual effects by natural and xenobiotic estrogens can be very wide, spanning 1,000- to 100,000-fold for bisphenol A and octylphenol up to and exceeding 100 million-fold for DES and E_2 (Figure 1A) (*54*). These cell responses in tissue culture to very wide concentration ranges create a type of inverted-U dose response that can be used as an *in vitro* model.

Low-dose stimulation of cell proliferation followed by high-dose cytotoxicity is illustrated in Figure 1A in estrogen-responsive MCF-7 cells. Growth was stimulated by E_2 in the concentration range from 0.1 pM to 100 pM. This low part-per-trillion (ppt) range is the physiological range for E_2 determined in studies of free estradiol in rats and mice from fetal life through adulthood (*23,53*); this is the low-dose range indicated in the figure. The cell growth response was saturated and did not increase with increased hormone concentration from 100 pM through to 1 μM. Above 1 μM (the high-dose range indicated in Figure 1A), however, cytotoxicity reduced the cell growth response to E_2, with inhibition of response to below the control level at 100 μM. The physiological dose range for E_2 action was approximately 100 million times lower (0.1–1.0 pg/mL culture medium; 0.1–1.0 ppt; the low-dose range) than the toxicological dose range that results in acute toxicity (which occurred at 10–100 μg/mL culture medium, or 10–100 parts per million (ppm); the high-dose range).

The acute cytotoxicity of E_2 in cultured MCF-7 cells did not depend on the presence of estrogen receptors. We have derived clonal cell lines from MCF-7, including cell line C4-12-5, which no longer express estrogen receptors and are completely estrogen nonresponsive and proliferate in the absence or presence of estrogen (*87*); re-expression of estrogen receptors in these clonal cell lines can lead to recovery of estrogen-dependent cell proliferation (*88*). As stated above, without receptors, these C4-12-5 cells are "blind" to the presence of the hormone. Cytotoxicity occurred within the same high-dose range of E_2 in the clonal C4-12-5 cells (derived from MCF-7 cells) that do not express estrogen receptors (Figure 1B) as in the parental MCF-7 cell (Figure 1A); however, the low-dose range effects to stimulate cell proliferation could not be demonstrated in the estrogen-nonresponsive cells (Figure 1B). These estrogen receptor–negative variants proliferate in the absence of estrogen, and in the absence of estrogen receptors, low doses of estrogen are the incapable of eliciting effects in these cells.

Importantly, stimulatory effects of estradiol in the low-dose range could also be obliterated in estrogen-responsive MCF-7 cells by the presence of a background or contaminating level of another estrogen such as DES (Figure 1C). Background estrogenic activity due to contamination by addition of DES at only 3 ppt (10 pM DES) completely obscured the low-dose range effects of E_2 on cell proliferation, but did not impair detection of the high-dose range, toxic effects observed above 1 μM E_2 (above 0.3 ppm; Figure 1C). Although this background contamination was created experimentally with 3 ppt DES, the presence of contaminating estrogens in the phenol red pH indicator dye included in most tissue culture media limited the recognition of and acceptance of estrogen-dependent cell proliferation by MCF-7 cells until 1985 (*63,89,90*). Unrecognized estrogenic contamination may interfere with any study, *in vitro* or *in vivo*, unless this possibility is excluded by the performance of appropriate controls.

Overall, both low-dose and high-dose effects by E_2 were observed in MCF-7 cells (Figure 1A). Demonstration *in vitro* of the low-dose effects of E_2, but not the high-dose effects, was obscured by testing in the absence of estrogen receptors (Figure 1B) or by testing in the presence of a low level of a contaminating estrogen (Figure 1C). The objective of appropriate control procedures discussed below is to allow one to distinguish whether negative results are due to an actual lack of activity of a compound, or rather due to

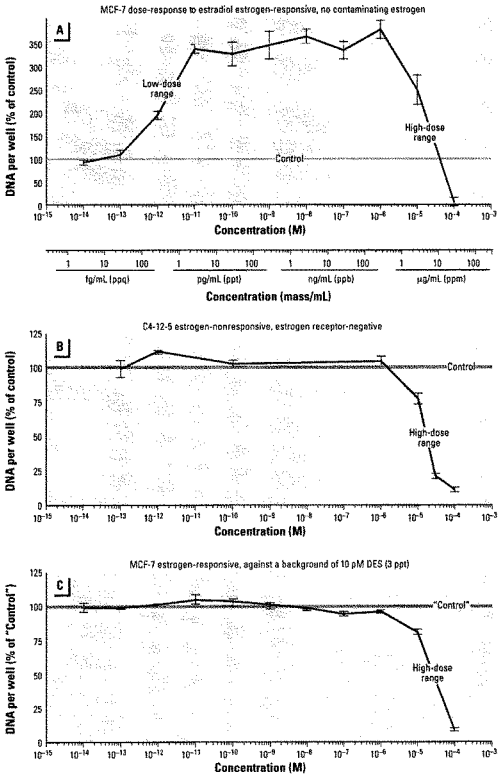

Figure 1. MCF-7 human breast cancer cell proliferation at low through high doses. (*A*) Stimulation of MCF-7 cell proliferation in estrogen-free medium by E_2 up to a dose at which E_2 is cytotoxic. Control line indicates estrogen-free medium. (*B*) Lack of response to E_2 by estrogen receptor–negative, estrogen-non-responsive C4-12-5 cells derived from MCF-7 cells, in estrogen-free medium. Proliferation is independent of dose up to a dose that is cytotoxic. Control line indicates estrogen-free medium. (*C*) Lack of response to E_2 by estrogen-responsive MCF-7 cells to E_2 due to the presence of a background of 10 pM DES (3 ppt) added to the estrogen-free medium to mimic contamination and present in all dose groups. Proliferation is independent of dose up to a dose that is cytotoxic. "Control" line indicates estrogen-free medium plus the 3 ppt DES background. High-dose effects of E_2 are seen in *A*, *B*, and *C*, whereas low-dose effects are visible only in *A*, the dose response performed in estrogen-responsive MCF-7 cells examined in the absence of detectable background estrogen. In *A* the concentration range is shown simultaneously as molarity (M), as mass per milliliter, and as mass ratio (ppq: parts per quadrillion). Half-maximal stimulation of proliferative response occurred at approximately 1 pM E_2 in medium (0.272 ppt) in the low-dose range, whereas inhibition was induced at micromolar concentrations in the high-dose range. Estrogen-dependent cell proliferation and cytotoxicity were determined exactly as described in prior publications (*72,138,139*). Briefly, the very wide dose responses (*54*) were performed for E_2 by incubating the indicated cells in 24-well plates for 4 days in culture medium (phenol red-free medium, charcoal-stripped serum) plus E_2 at concentrations from 0.01 or 0.1 pM through 100 µM, with daily medium changes. Proliferation was determined by DNA assay at the end of the incubation, and results were expressed as percent of the control; control 100% values were 1.0, 3.7, and 5.5 µg DNA/well for *A*, *B*, and *C*, respectively. Values are the mean and standard error of measurements in replicate wells; $n = 3$.

unresponsiveness of a tissue, or contamination that is obscuring all responses.

Importance of Valid Positive and Negative Controls for Endocrine Responses

Although E_2 was clearly capable of exerting effects in the physiological, low-dose range (Figure 1A), demonstration of the low-dose effects was system dependent. Importantly, the inability to detect the low-dose effects of E_2 in Figure 1B and C was due to the experimental conditions and was not due to the absence of estrogenic activity by E_2 itself or due to an absence of the potential to show estrogen responses in uncontaminated MCF-7 cells with estrogen receptors. This conclusion will only be realized if specific positive and negative controls are included to allow for the correct interpretation of results. Without evaluation of the appropriate negative and positive controls, it is not valid to conclude that a chemical lacks low-dose estrogenic activity simply because it fails in assays that may be represented by the conditions in Figure 1B, where the test system is unresponsive, or in Figure 1C, where the test system is responsive but contaminated. In these examples, if the controls were omitted (or ignored), E_2 itself in its own physiological concentration range (as well as any other estrogenic chemical) would be wrongly identified as inactive in two out of three assay systems.

The positive and negative controls. Each panel of Figure 2 illustrates specific positive and negative controls relevant to each experiment in Figure 1; this includes use of an antiestrogen (AE), which is a competitive antagonist of estrogen action (*90,91*). These controls allow one to interpret the absence of detectable low-dose effects in Figure 1B and C, either as the lack of cellular responsiveness to estrogen generally, or as the presence of a masking estrogenic contamination.

A concentration of E_2 that saturates the proliferative response in the low-dose range is used as a positive control. This treatment demonstrates the presence of estrogen responsiveness in the assay relative to the negative control that is estrogen free (Figure 2A). An antiestrogen such as raloxifene or ICI 182,780 is used to confirm a baseline for estrogen receptor activation in the negative control treatments; there should be no reduction in response by the antiestrogen because no receptor-mediated responses have been initiated in the absence of estrogen (Figure 2A). If an inhibition of response is observed in the presence of antiestrogen with no intentional addition of estrogen (Figure 2C), then the conclusion is that estrogenic stimulation is occurring in the system from contamination. Another important issue is that when high

doses of a chemical are being examined for estrogenic activity, after demonstrating that addition of antiestrogen inhibits the response, competitive reversal of this inhibition of response by co-incubation with an excess of estrogen (for example, 10 nM E_2) (Figure 2C) added with the antiestrogen is in turn used to distinguish antiestrogenic activity from toxicity due to the combined action of the test chemical and antiestrogen. This last step is the final element in discriminating between antiestrogenic activity of a compound and acute toxicity (*91*).

Interpretation of the controls. In Figure 2A, the positive control E_2 at 100 pM stimulated response, and of equal importance, exposure to an antiestrogen at 100 nM (AE) in the absence of any E_2 did not reduce the proliferative response below the control level of growth. The interpretation drawn from the controls in Figure 2A is that *a*) the MCF-7 cell system was estrogen responsive, and importantly, *b*) under the negative control growth conditions, there was no detectable background estrogenic contamination. In this system, both low- and high-dose effects of E_2 were observed (Figure 1A).

Figure 2B shows the same controls applied to C4-12-5 cells, a clonal variant of MCF-7 cells that lacks estrogen receptors. Positive control E_2 did not stimulate cell proliferation, and furthermore, the antiestrogen did not inhibit proliferation of the C4-12-5 cells (Figure 2B). The interpretation of these controls is that the C4-12-5 cells are estrogen nonresponsive, showing responses neither to low-dose estrogen nor to antiestrogen. Importantly, even though the cells were not responsive in the low-dose range of exposure, the proliferation of the estrogen receptor–negative C4-12-5 cells was still inhibited by E_2 in the same high-dose range that inhibited proliferation of the estrogen-responsive MCF-7 cells (Figure 1B); only high-dose toxic effects of E_2 were observed, and these are clearly not mediated by nuclear estrogen receptors.

Finally, as can be seen in Figure 2C, even in the same MCF-7 cells that were responsive within the low-dose range in the full dose response (Figure 1A), a very slight background level (contamination) of an estrogenic chemical was sufficient to eliminate detection of the low-dose stimulating effect of estradiol, if treatments are compared only with a negative control that is presumed, without testing, to be estrogen-free. In Figure 2C, it can be seen that the positive control E_2 added to the "Control" medium did not stimulate further growth, and without further information, the system would be incorrectly interpreted as nonresponsive in the low-dose range (Figure 1C). Incubating cells in the "Control" medium plus antiestrogen, however, inhibited cell proliferation, indicating the potential for an estrogen receptor–driven stimulation of cell growth. Competitive reversal of the antiestrogen effect with a surplus of E_2, indicated by the light blue bar in Figure 2C, confirmed that the inhibition was antiestrogenic and not due to nonspecific toxicity.

The interpretation of the dose–response experiment (Figure 1C) is now that the MCF-7 cells were fully responsive to E_2 in the low-dose range but were already maximally stimulated by background estrogenic contamination in the presumed negative control. DES at only 3 ppt was sufficient to fully mask the low-dose effects of E_2; only high-dose, toxic effects of E_2 could be observed (Figure 1C). In the absence of the appropriate controls, or if the controls were misinterpreted or ignored, E_2 itself, an unquestioned estrogen, would be incorrectly identified from Figure 1B or C as an inactive chemical in the low-dose range (its physiological range), but not in the high-dose range, with respect to estrogen-dependent cell proliferation.

Implications. Positive and negative controls such as those described above are needed for adequate interpretation of EEDCs in the context of low-dose effects, nonlinear saturation of response, and reversal of response that can generate a nonmonotonic dose–response relationship. Of great importance, research on low-dose effects requires a new level of understanding of ambient estrogenic activities, and controls are absolutely required to assess these activities experimentally. Ambient estrogenic activities for *in vitro* studies consist of contaminants in air, media, or plastic, whereas *in vivo*, ambient estrogenic activities could include variable background levels of endogenous hormone as well as activity from a variety of external sources such as feeds. Appropriate controls are not typically included in toxicological tests conducted for regulatory purposes.

Relevant to this discussion are findings that the concentration of E_2 in cell culture medium that results in proliferation at approximately 50% of maximum is very close to the concentrations of free serum E_2 during development in mouse and rat fetuses (0.2–0.3 pg/mL) (*23,53*). Even slight variations in the levels of estradiol have been related to differences in the course of development in mice, rats, and gerbils (*19,23,92–94*). For example, we experimentally increased the free serum estradiol concentration in male mouse fetuses from the control level of 0.2–0.3 pg/mL (via a Silastic capsule containing estradiol implanted in the pregnant dam). This 0.1 pg/mL increase in free serum estradiol resulted in a marked change in development of the urogenital system in the male fetuses (*23*).

Taken together, these findings indicate a very high degree of sensitivity (well below a part per trillion) of both human and rodent tissues to E_2 both *in vitro* and *in vivo*. This high degree of sensitivity to very small perturbations in E_2 provides the basis for concern about the use of appropriate controls to test for background contamination by estrogenic chemicals in studies with animals. Estrogenic contamination can occur via the food (*95,96*), caging (*97*), or bedding (*98*), as well as in studies with cultured tissue via components of media (*63*), or plastic tubes and cultureware (*99,100*). Although there have been studies that have examined the effects of components of diets on steroid synthesis in humans (*101*), this issue has not been a focus of toxicological studies involving EEDCs. Our recent findings show that in mice maintained on different types of commercial animal feeds during pregnancy, serum estradiol levels in fetuses are markedly different (unpublished observation).

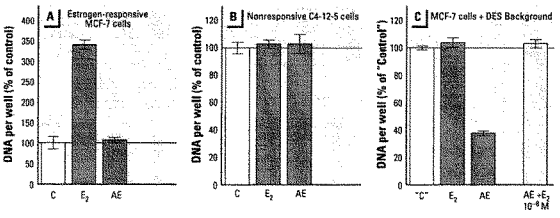

Figure 2. The relevant controls for the dose responses of Figure 1A–C. (*A*) Estrogen-responsive MCF-7 cells in estrogen-free medium. (*B*) Estrogen receptor–negative, estrogen-nonresponsive C4-12-5 cells derived from MCF-7 cells, in estrogen-free medium. (*C*) Controls. Estrogen-responsive MCF-7 cells in the presence of a background of 10 pM DES (3 ppt) added to the estrogen-free medium and present in all media and treatments including controls. Abbreviations: AE, 100 nM antiestrogen (raloxifene or ICI 182,780); AE + E_2 10^{-8} M, 100 nM antiestrogen (raloxifene or ICI 182,780) plus E_2 at 10^{-8} M; C, control estrogen-free medium; "C", estrogen-free medium plus 3 ppt DES; E_2, 100 pM E_2. Values are the mean and standard error of measurements in replicate wells; $n = 3$.

Endocrine Mechanisms Mediating Errors in Estimating Low-Dose Responses from High-Dose Studies

The default risk assessment assumes linearity of dose response. Major errors in assessing risk can be made when linearity of response and the preceding receptor occupancy is assumed across the entire dose range, which is the current assumption used in risk assessment. Although almost everyone involved in risk assessment recognizes that the assumption of linearity is invalid (even for cancer) (*102*), the application of safety factors that results in linear extrapolation across a wide dose range remains the default for current risk assessment. For example, safety factors (used to calculate a "safe" dose for human exposure) of 10-fold each are often used to estimate each of the following: human risk from animal studies, to account for variability within the human population, when the lowest dose tested results in an adverse response (termed the LOEL), and most recently, as an added safety factor for protecting children. Application of these 10-fold safety factors results in linear extrapolation from a LOEL or NOEL (determined by testing a few very high doses) to arrive at a safe dose. Thus, in practice, the model upon which risk assessment is practiced assumes that this linear extrapolation procedure is valid and will result in calculation of a dose that is safe for humans exposure.

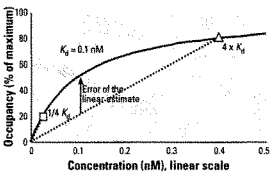

Figure 3. Error in predicting actual receptor occupancy based on linear estimation applied to a saturating test dose. Receptor occupancy (solid line) is graphed against a linear scale of ligand concentration from 0 to 0.5 nM, where the K_d for ligand binding = 0.1 nM. Linear estimations to zero concentration (dotted lines) are shown originating from single measurements at two test doses, one below the K_d (square point of origin, at 1/4 K_d = 0.025 nM) and one above the K_d (round point of origin, at 4 × K_d = 0.4 nM). This assumes no background-contaminating estrogenic activity from either endogenous or exogenous sources other than the chemical being tested. Where the test dose used as the origin of the linear estimation is below the K_d, the linear estimation is very close to actual occupancy. Where the lowest test dose used as the origin of the linear estimation to zero dose is above the K_d, the linear estimation deviates substantially from actual receptor occupancy, indicated as "Error of the linear estimate." The fold-underestimate of occupancy, and therefore underestimate of response for receptor-mediated events, increases as the origin of measurement increases above the K_d and is calculated in Table 2 for a number of EEDCs where the origin is 10,000-fold above the K_d, which could not be shown to scale on this figure.

Error of a linear estimate relative to actual receptor occupancy. When a linear extrapolation model is applied to a saturating, receptor-mediated response to estimate the risk of an adverse response, this linear estimate results in a false assumption concerning the actual reduction in response (and thus risk) that occurs with decreasing dose. The error we refer to is illustrated in the simplified graphic example in Figure 3. The use of 10-fold safety factors to estimate occupancy of receptors (and subsequent responses) on the basis of results from animal studies assumes a linear relationship between dose and response, even though this may not be overtly acknowledged. We will initially discuss the theory behind the error that occurs on the basis of extrapolation from very high to very low doses assuming a linear function and then provide examples from actual data for DES, genistein, and bisphenol A obtained from *in vitro* studies using MCF-7 cells. The error we refer to here based on receptor occupancy is in reality lower than the error based on actual responses, as responses can saturate at lower concentrations than those required to achieve receptor saturation (Table 1). Therefore, our calculations of error in Table 2 are, in fact, conservative.

For simplicity here, in the discussion below we will not discriminate between dose administered and dose at the estrogen receptor in target cells and will simply refer here to a test dose. The reason for this is that for *in vitro* studies conducted in serum-free medium, the administered dose and the dose available to bind to estrogen receptors are very similar (*4*). *In vivo* this is obviously not the case due to absorption, metabolism, clearance, plasma binding, etc., all of which are far more complicated to study in developing fetuses than in adults (*54*). It is nonetheless the basis of modern endocrinology that a dose at target does exist, whether or not it can be easily determined, and that this dose determines the response and its magnitude relative to the receptor occupancy it can generate. Our discussion here is meant to apply to the dose at target.

It is important to note that during fetal and early postnatal life, the pharmacokinetics of chemicals and drugs are markedly different relative to adulthood. In addition, pregnant and nonpregnant females also differ in this regard. Data from studies with adult animals thus cannot be used to predict the pharmacokinetics of chemicals in pregnant females and fetuses (*16,103,104*). Thus, evidence that a particular chemical is cleared rapidly in a nonpregnant adult cannot be used to discount the possibility of achieving a much higher dose at target in fetuses and neonates (*46*). Unfortunately, for most chemicals, there are no pharmacokinetic data and thus no basis for predicting dose at target for the most susceptible subpopulation: pregnant females and their fetuses.

The test dose for purposes of our discussion here is a high dose administered in toxicological experiments that is used to predict responses at much lower doses. As shown in Table 1 and Figure 3, the relationship between hormone concentration and receptor occupancy is approximately linear at low receptor occupancy (Figure 3, test dose example at 1/4 K_d). As the test dose exceeds the range of approximate linearity, for example, a test dose at 80% receptor occupancy (Figure 3 at 4 × K_d), the linear model (linear extrapolation from test dose to zero dose) will clearly underestimate actual receptor occupancy and will thus underestimate the actual responses that would occur at lower doses (Figure 3, arrow labeled "error of the linear estimate"). This deviation from linearity has great importance with regard to the strategy of using very high doses of EEDCs in toxicological studies and extrapolating to predict responses at much lower doses.

Table 2 presents specific quantitative information for a number of chemicals. With regard to understanding the error that can occur in estimating the potential for low-dose responses on the basis of extrapolating from high to low doses across a wide dose range, we will describe an *in vitro* experiment in which bisphenol A was examined in MCF-7 cells as an example. For our example here, the test dose for bisphenol A (shown in Table 2, row 1) is 844,000 ppb (844 mg/kg), chosen for its relation to K_d for ER-α and for proximity to test doses administered in prior *in vivo* toxicological studies of bisphenol A (again, using this as the dose at target) (*14*). Under the assumption that the test dose of 844,000 ppb is within a linear response range and therefore within a linear receptor occupancy range for direct hormonal effects, reducing the dose by 50% (to a dose of 422,000 ppb) would lead to the prediction that receptor occupancy would also drop by 50% (Table 2, row 2). In fact, because the test concentration is so much higher than the K_d, virtually no actual change in receptor occupancy occurs (the actual change in receptor binding in MCF-7 cells would be from 99.99 to 99.98% with this 50% reduction in dose), and no change in response mediated by these receptors would be detected.

When one administers a dose of bisphenol A that is 10-fold lower than the test dose (84,400 ppb or 84.4 mg/kg), receptor

occupancy still only drops from 99.98% to 99.90% in MCF-7 cells (Table 2, row 3), and again, this change is not likely to be a detectable decrease in binding. This decrease in dose also would thus not be likely to lead to a detectable decrease in response mediated by these receptors. Even at a dose of 844 ppb, which is a dose 1,000 times lower than the test dose of 844,000 ppb, 90.91% of receptors will still be occupied in MCF-7 cells. On the basis of the information presented in Table 1, one would not expect to approach the region of maximum detectability for a change in response until doses that resulted in less than 50% receptor occupancy (the K_d) were reached. In addition, on the basis of results in Table 1, it is apparent that responses can occur at concentrations in the range of 1% receptor occupancy. As shown in Table 2, at the concentration of bisphenol A that results in approximately 1% receptor occupancy (0.844 ppb), or 1 million times lower than our initial test dose, the linear extrapolation model would have predicted negligible receptor binding, and thus no response, based on a test dose of 844,000 ppb.

Nonmonotonic dose–response curve, response to endogenous hormone, and an assumed threshold dose all increase the magnitude of the error of a linear estimate. Our calculations are based on receptor occupancy, which is a physical chemical parameter subject to less between-species variation and greater precision of measurement than is the measurement of response. Cellular responses, however, occur at doses associated with very low receptor occupancy: the cell in essence amplifies the receptor signal. Therefore, use of receptor occupancy is in fact conservative relative to the ultimate physiological responses on which risk assessment would be based. For example, if these calculations were based on the EC_{50} (effective concentration 50%; 50% response) for a specific cell response such as cell proliferation that is 10- to 100-fold lower than the K_d (Table 1), then the underestimate of the potential for a response would be 10- to 100-fold higher, or up to 1,000,000-fold, instead of the 10,000-fold in this example.

Incorporation of additional features of real-world risk assessment will further add to the error, not reduce it. A nonmonotonic dose response, specifically the inverted U, can substantially increase the error of the linear estimate based on a high-dose reference point (that is well below the maximum response because of the inverted-U dose–response curve). This is illustrated qualitatively in Figure 4A, where the error of the linear estimate for response is compared with that for an inverted-U dose response from a reference point above the dose that results in the maximum response. To avoid the possibility of this type of error, it is necessary to examine a much wider range of doses than is typical in toxicological studies involving animals.

Finally, as illustrated in Figure 4B, the default risk assessment applied to EEDCs assumes the existence of a threshold. But when xenoestrogen activity is added to a natural system that is already responding to endogenous estrogen such as estradiol, any threshold in estrogenic response must already be exceeded by the endogenous hormone. This absence of a threshold in response to exogenous estrogen has been experimentally confirmed in an experiment concerning the regulation by estrogen of sex determination in reptiles (*22*). The assumption of no response up to an assumed threshold above the zero EEDC dose, when this is not the case, will result in a great, potentially infinite error if linear extrapolation is used instead of actually determining the shape of the dose–response curve (Figure 4B).

Figure 4B also depicts the error associated with examining a test chemical with estrogenic activity, such as bisphenol A, that adds to an existing background level of endogenous estradiol, which is variable because of endogenous and exogenous factors (*19*). Variation in endogenous estradiol is related to variation in phenotype in rodents (*105*), supporting the hypothesis that endogenous estrogen is already above threshold for estrogen-mediated responses (*22*). There can thus be no threshold for responses to exogenous EEDCs. This finding is important, as background levels of endogenous estradiol markedly alter the response of fetuses to endocrine disruptors administered to pregnant mice and rats, including EEDCs such as bisphenol A (*93,94*). This issue is also relevant with regard to comparing effects of EEDCs at different life stages. During fetal life in males and females, pregnancy, or proestrus in females, estradiol levels are significantly higher than during postnatal life in males or prior to puberty and during diestrus in females (*53*). These marked differences in the background levels of estradiol will obviously influence responses to low doses of EEDCs. The importance of endogenous estradiol levels in the response to low doses of EEDCs, which has been ignored in toxicological studies and in the models used in risk assessment, is covered in more detail below.

Implications for current risk assessment. For an EEDC such as bisphenol A, with a relative estrogenic activity approximately 10,000-fold less than E_2 in MCF-7 cells [but not necessarily other tissues where it is much more active; (*64*)], the range of estrogenic activity of this chemical equivalent to that of physiological E_2 would be approximately 0.05–30 ppb (0.05–30 ng/mL) within target cells. There are now numerous published reports that bisphenol A shows estrogenic activity at and below this concentration in a variety of cell culture systems (*4,28,100, 106–112*). For example, Gupta (*64*) reported that a 50-pg/mL (50 ppt) dose of bisphenol A significantly stimulated prostate gland formation and growth of the fetal mouse prostate in primary culture, similar to a 0.5-pg/mL dose of DES. Bisphenol A stimulated human prostate cancer cells to proliferate at a dose of 1 nM (~ 0.23 ppb) (*28*).

The currently accepted LOEL dose of bisphenol A of 50 mg/kg/day (*15*) was reported from high-dose toxicological studies (*14,113*). This study is typical in that it used doses 50,000–500,000-times higher than the

Table 2. Error in estimating responses to low doses, in the physiological range of estrogenic activity, for estradiol, DES, genistein, and bisphenol A as a result of assuming linearity across the entire dose–response curve with regard to predicted versus actual estrogen receptor occupancy.

Row	Estradiol (ppb)	DES (ppb)	Genistein (ppb)	Bisphenol A (ppb)	Actual receptors occupied (%)	Occupied receptors predicted by linear model (%)	= Fold underestimation of response by linear extrapolation[a]
1 test dose[b]	272	568	475,000	844,000	99.99	100	1
2	136	284	238,000	422,000	99.98	50	2
3	27.2	56.8	47,500	84,400	99.90	10	10
4	2.72	5.68	4,750	8,440	99.01	1	100
5	0.272	0.568	475	844	90.91	0.1	900
6 K_d[c]	0.0272	0.0568	47.5	84.4	50	0.01	5,000
7	0.00272	0.00568	4.75	8.44	9.09	0.001	9,000
8	0.000272	0.000568	0.475	0.844	0.99	0.0001	10,000

[a]Fold underestimation of response by linear extrapolation is the actual receptors occupied divided by the predicted receptors occupied. [b]The dose in row 1 is referred to in the text as the "test dose," at a dose 10,000-times higher than each K_d; calculated from K_d values of 0.1 nM (0.0272 ppb) for estradiol (approximate), 0.212 nM (0.0568 ppb) for DES, 176 nM (47.5 ppb) for genistein, and 370 nM (84.4 ppb) for bisphenol A (4,5). [c]Row contains concentrations at the respective K_d of each compound.

2- and 20-μg/kg/day doses we administered to pregnant mice on the basis of our calculation of an amount of bisphenol A that our preliminary findings accurately predicted would be bioactive in male mouse fetuses (*4*). The

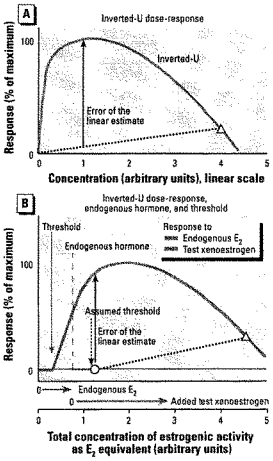

Figure 4. (*A*) The error due to assuming that the dose–response curve is linear (dotted line) when, in fact, the dose–response curve is nonmonotonic and forms an inverted U (solid blue line). The error in estimating actual responses that will occur at doses below the test dose in a toxicological study increases as the concentration of the test dose increases relative to a test dose that would result in a maximum response. This figure shows that the magnitude of the error in estimating responses at doses below the test dose for an EEDC (using linear extrapolation) is greater when the dose–response curve is nonmonotonic relative to the error when the dose–response curve is monotonic (Figure 3). (*B*) This figure depicts the error associated with examining a single dose of a test chemical (triangle) with estrogenic activity, such as bisphenol A, that adds to an existing background level of endogenous estradiol, which is variable because of endogenous and exogenous factors. In the current model used in risk assessment, a linear extrapolation (dotted line) from the test dose (triangle) to an assumed threshold dose (circle) is used, based on the assumption there will be an absence of response at this assumed threshold dose. In this figure, the assumption is that endogenous estrogen is already above threshold for the estrogen receptor–mediated response to the EEDC (vertical dashed line). There can thus be no threshold for the response to the exogenous EEDC. The assumption of no response at the assumed threshold EEDC dose, when this is not the case, will result in a great error, potentially infinite, in estimating the response at this dose, if linear extrapolation from a high test dose is used instead of actually determining the shape of the dose–response curve.

transplacental transport of bisphenol A has now been studied in greater detail in rodents (*103,114–116*), and the doses we used would result in unconjugated bisphenol A levels in mouse fetuses that are within the range measured in human umbilical cord blood (*16,103*).

Effects using low doses of bisphenol A, which are in the new low-dose range below the LOEL based on testing very high doses, have now been reported in rodent studies on mammary gland (*117*), vagina (*118*), prostate (*4,64,65,119,120*), sperm production (*121,122*), epididymis (*64,121*), rate of embryonic development (*123,124*), pituitary response to E_2 (*109*), and rate of growth and timing of puberty in females (*93,125*). There are also reports of effects of bisphenol A in mollusks, fish, and frogs at very low concentrations, including below 1 μg/L (1 ppb) (*126–132*). Even though a few studies have reported no effects of low doses of bisphenol A, the weight of the evidence now clearly supports that such effects occur in both vertebrates and invertebrates.

It is also interesting that in two highly publicized studies using low doses of bisphenol A (*133,134*), no effects of bisphenol A were found; in addition, no effects of their positive control chemical, DES, were found. Although DES at the dose used was questioned as a valid positive control by one of the groups (*135*), its validity as a positive control estrogen at the low doses used in these studies was fully endorsed by the National Institute of Environmental Health Sciences Low-Dose Peer Review Panel (*11*). In each of the two studies, the control animals were obese (30% over normal body weight) relative to mice used in prior studies that used the same strain and age and that had shown effects of fetal exposure to bisphenol A and positive control chemicals (*4,136*), including the same low dose of DES (*23,64,82*) used by Ashby et al. and Cagen et al. (*133,134*). The fact that the control animals in both the Ashby and Cagen studies were obese and had enlarged prostates and then did not respond to either bisphenol A or the positive control DES suggests that the interaction of components of the diet with manmade chemicals, such as bisphenol A, is an issue that requires further study; our recent studies have confirmed this prediction (unpublished data). This also serves as an example of the importance of attending to information provided by the appropriate negative and positive controls (Figure 2), which these authors ignored (*11*).

Conclusions

Information about the mechanism of action of EEDCs, together with information concerning mechanisms of hormone action, predict that current risk assessment assumptions can lead to a dramatic underestimation of responses (and thus risk) associated with exposure to low doses of EEDCs, particularly during development when the effects of very small changes in hormonal activity are permanent (*54,64*). The practice of examining only a few very high doses and then extrapolating to predict effects of doses thousands or millions of time below those being studied is especially problematic for endocrine disruptors. The necessity for including low doses in the physiologically relevant range of estrogenic activity, as opposed to only very high doses, when testing for effects of endocrine disruptors is dictated by *a*) evidence that estrogenic chemicals (as well as other hormone mimics or chemicals that otherwise interfere with endocrine function) can produce nonmonotonic dose–response curves where responses both increase and decrease across the dose range, and *b*) the theoretical absence of a threshold for environmental chemicals that operate via receptors (such as the estrogen receptor) for endogenous ligands, such as E_2; the threshold issue is covered in more detail elsewhere (*13,22*). In addition, controls valid for the positive determination of endocrine responsiveness must be included, and when included, interpreted appropriately, particularly when results that are apparently negative are obtained. The potential for error inherent in drawing strong positive conclusions from purely negative data has clearly not been appreciated by some toxicologists (*133,134*), as well as regulators responsible for assessing this information.

Taken together, the above *in vitro* findings show the substantial error that occurs as a result of extrapolating on the basis of findings using very high doses to predict effects at environmentally relevant doses, which are often thousands or millions of times lower than doses being tested. Responses to low doses of EEDCs should be determined by testing a much wider range of doses than the 50-fold range common in toxicological studies today (*13*), including doses in the environmentally relevant range, and by accounting for all sources of estrogenic activity (endogenous and exogenous) and their interactive effect (*137*).

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Accurate Prediction of the Response of Freshwater Fish to a Mixture of Estrogenic Chemicals

Jayne V. Brian,[1] Catherine A. Harris,[1] Martin Scholze,[2] Thomas Backhaus,[3] Petra Booy,[4] Marja Lamoree,[4] Giulio Pojana,[5] Niels Jonkers,[5] Tamsin Runnalls,[1] Angela Bonfà,[5] Antonio Marcomini,[5] and John P. Sumpter[1]

[1]Institute for the Environment, Brunel University, Uxbridge, Middlesex, United Kingdom; [2]Centre for Toxicology, School of Pharmacy, London, United Kingdom; [3]Department of Biology and Chemistry, University of Bremen, Bremen, Germany; [4]Institute for Environmental Studies, Vrije Universiteit, The Netherlands; [5]Department of Environmental Sciences, University of Venice, Venice, Italy

Existing environmental risk assessment procedures are limited in their ability to evaluate the combined effects of chemical mixtures. We investigated the implications of this by analyzing the combined effects of a multicomponent mixture of five estrogenic chemicals using vitellogenin induction in male fathead minnows as an end point. The mixture consisted of estradiol, ethynylestradiol, nonylphenol, octylphenol, and bisphenol A. We determined concentration–response curves for each of the chemicals individually. The chemicals were then combined at equipotent concentrations and the mixture tested using fixed-ratio design. The effects of the mixture were compared with those predicted by the model of concentration addition using biomathematical methods, which revealed that there was no deviation between the observed and predicted effects of the mixture. These findings demonstrate that estrogenic chemicals have the capacity to act together in an additive manner and that their combined effects can be accurately predicted by concentration addition. We also explored the potential for mixture effects at low concentrations by exposing the fish to each chemical at one-fifth of its median effective concentration (EC_{50}). Individually, the chemicals did not induce a significant response, although their combined effects were consistent with the predictions of concentration addition. This demonstrates the potential for estrogenic chemicals to act additively at environmentally relevant concentrations. These findings highlight the potential for existing environmental risk assessment procedures to underestimate the hazard posed by mixtures of chemicals that act via a similar mode of action, thereby leading to erroneous conclusions of absence of risk. *Key words:* concentration addition, estrogen, estrogen mimic, fathead minnow, mixture effects, *Pimephales promelas*, prediction. *Environ Health Perspect* 113:721–728 (2005). doi:10.1289/ehp.7598 available via *http://dx.doi.org/* [Online 14 March 2005]

Many environmental contaminants are capable of disrupting endocrine function in humans and wildlife. This phenomenon has been associated with reduced fecundity, reproductive failure, and population-level effects in a variety of aquatic organisms (Jobling et al. 2002; Matthiessen and Gibbs 1998; Nash et al. 2004). This highlights the urgent need to develop accurate methods of assessing the risk that these chemicals pose. Current methods usually focus on the assessment of single chemicals. This is in clear contrast to real-world exposure situations, which are generally to mixtures of endocrine-disrupting chemicals, many of which act via a common mode of action. This means that the overall risk posed in real exposure situations may be greater than that expected on the basis of the effects assessment of the individual mixture components, due to the potential for combined effects. Concerns over the ecological significance of these effects were heightened in the late 1990s after reports of spectacular synergisms between binary mixtures of estrogenic pesticides *in vitro* (Arnold et al. 1996). These results were subsequently withdrawn because of issues of reproducibility, leading many to question the overall significance of mixtures (Kortenkamp and Alterburger 1999). However, the issue has continued to attract interest in view of the fact that many of the estrogenic effects reported in the literature exceed expectations based on chemical-by-chemical assessments. A notable example of this is the discrepancy between the widespread distribution of reproductive abnormalities in wild fish populations relative to the low concentrations of estrogenic chemicals to which they are exposed (Jobling et al. 1998; van Aerle et al. 2001).

Many of the chemicals identified as endocrine disruptors are known to mediate their effects by binding with the estrogen receptor (Payne et al. 2000). Estrogenic chemicals include both the natural and synthetic steroidal estrogens, as well as a wide range of synthetic chemicals that mimic the actions of endogenous estrogen. The potencies of these different types of chemical vary over several orders of magnitude. For example, the steroidal estrogens, such as 17β-estradiol (E_2) and 17α-ethynylestradiol (EE_2), are capable of exerting estrogenic effects on fish when present in the water in the low nanograms per liter range (Thorpe et al. 2003). These chemicals pose a significant environmental risk, having been detected in effluents that discharge into rivers at concentrations that are individually capable of inducing a significant effect (Desbrow et al. 1998). In contrast, chemicals that mimic the actions of estrogen, such as the alkylphenols, exhibit much lower potencies and rarely occur at concentrations that are individually effective in the environment (Desbrow et al. 1998). Hence, the individual assessment of the hazard posed by these chemicals indicates a negligible risk. However, this approach does not account for the potential for endocrine disruptors to act in combination. This may lead to the underestimation of hazards that exist in real exposure situations, resulting in erroneous conclusions of absence of risk.

Increasing recognition of these shortcomings has prompted considerable efforts to investigate the combined effects of estrogenic chemicals (e.g., Ashby et al. 1997; Soto et al. 1994). However, many of these studies have been hampered by inadequate theoretical foundations on which to base the expected effects of mixtures of chemicals that exhibit nonlinear concentration–response curves (Kortenkamp and Altenburger 1999). More recently, however, the pharmacological concept of concentration addition (CA) has been applied to the assessment of estrogenic mixtures. This concept is based on the assumption that the components of the mixture act in a similar manner, such that replacing one or more chemicals totally, or in part, with the other mixture components can produce the same overall effect. The overall effect of the mixture can therefore be described quantitatively using a mathematical model, based on the concentration and potency of the individual mixture components (Bödeker et al. 1992). This means that potential hazards can

Address correspondence to J.V. Brian, Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, United Kingdom. Telephone: 44-1895-266-264. Fax: 44-1895-269-761. E-mail: jayne.brian@brunel.ac.uk

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be predicted from basic information about the components of the mixture and its composition (number and concentration of chemicals present), thereby negating the need for mixture testing. A number of studies have attempted to validate this concept by comparing the effects of the mixture with those expected on the basis of additivity. This has involved the single-substance testing of the individual mixture components in order to gain information for the modeling of mixture effects. The predictions made can then be tested experimentally. This approach has been used extensively in aquatic toxicology to demonstrate the validity of CA as a means of predicting the toxicity of multicomponent mixtures of similarly acting compounds in various assays with fish, daphnia, algae, and bacteria (e.g., Altenburger et al. 2000; Backhaus et al. 2000, 2004; Faust et al. 2001; Hermens et al. 1984a, 1984b; Könemann 1981).

There is considerable evidence that CA may also be used to predict the effects of mixtures of estrogenic chemicals. The validity of this approach has been demonstrated *in vitro*, using assays such as the yeast estrogenicity screen (YES) and the human breast cancer cell proliferation assay (E-SCREEN) (Payne et al. 2000, 2001; Rajapakse et al. 2002; Silva et al. 2002). Such studies have revealed the capacity for the components of the mixture to contribute to the overall effect by acting in relation to their potency, even at low-effect concentrations. For example, Silva et al. (2002) combined eight estrogenic chemicals at low-effect concentrations and demonstrated that the effects of this mixture were consistent with the predictions of CA. This highlights the capacity for these chemicals to act in combination, even when the individual components of the mixture are present at concentrations below the threshold of statistically detectable effects. This has become known as the "something from nothing" phenomenon (Silva et al. 2002).

In light of this *in vitro* evidence, there is now an urgent need to assess whether these mixture effects also occur in higher life forms, which reflect the net effects of complex chains of events involving the uptake, distribution, and metabolism of test agents until they reach their target sites. The induction of the egg yolk protein vitellogenin (VTG) is an established *in vivo* assay for analyzing estrogenic effects in fish. This protein is normally induced in the livers of female fish in response to stimulation by endogenous estrogen. However, it can be induced in both male and female fish exposed to extremely low concentrations of estrogenic chemicals (Sumpter and Jobling 1995). Although a causal relationship has not been established, a number of studies have demonstrated that VTG induction is associated with effects at higher levels of biological organization (e.g., Harries et al. 2000). It therefore offers a sensitive and integrated measure of estrogenic activity, which is relevant to the assessment of environmental risk. Recent evidence indicates that the induction of VTG can be used to assess the joint action of binary mixtures of estrogenic chemicals *in vivo* (Thorpe et al. 2001, 2003). Here, we have applied this assay to the analysis of multicomponent mixture effects.

The aim of this study was to investigate the predictability of the combined effects of five estrogenic chemicals on VTG induction in the fathead minnow (*Pimephales promelas*). We used CA as a concept on which to base the expectation of additivity. We tested the predictive power of CA by analyzing the estrogenic effect of each mixture component individually. Information on their potency was then used to make predictions, which were then tested by comparison with the observed mixture effects. Mixture effects at low-effect concentrations of the individual components were also investigated to analyze the applicability of CA under environmentally realistic conditions. All studies were conducted using an optimal experimental design that minimized the number of test organisms. Quality checks of the exposure conditions were conducted using analytical chemistry.

The work described in this article contributes to our current understanding of the combined effects of multicomponent mixtures of estrogenic chemicals at higher levels of biological complexity, as well as aiding in the development of methods that can be applied to the analysis of mixtures. Hence, the findings are of considerable relevance to the assessment of environmental risk.

Materials and Methods

Test organisms. A stock of fathead minnows was obtained from Osage Catfisheries (Osage Beach, MO, USA). These fish, and their offspring, were used to conduct 14 independent exposure studies. Before exposure, stock fish were held in communal holding tanks with a recirculating water supply. The exposure studies were conducted in 30-L glass aquaria (0.6 m × 0.3 m × 0.3 m), which were supplied with a continuous flow of water. The analysis of VTG induction focused on the responses of male fish. However, an equal number of females were included in each experiment to reduce the level of aggression between males. During the exposure, the fish were fed twice daily: once with frozen brine shrimp and once with flaked fish food. The photoperiod was maintained at 16-hr light/8-hr dark with 20-min dawn and dusk transition periods.

Test chemicals. We investigated the activity of five estrogenic chemicals. These were selected on the basis of previous reports of their presence in the environment and because of their likely association with intersexuality in wild fish (Desbrow et al. 1998). They included the natural steroidal estrogen E_2, the synthetic steroidal estrogen EE_2, and the estrogen-mimicking compounds 4-*tert*-nonylphenol (NP), 4-*tert*-octylphenol (OP), and bisphenol A (BPA). Stocks of E_2 (98% purity), EE_2 (98% purity), OP (97% purity), and BPA (99% purity) were purchased from Sigma Aldrich (Dorset, UK). NP (99% purity) was obtained from ACROS Organics (Leicestershire, UK). All chemicals were dissolved in HPLC-grade dimethylformamide (DMF) supplied by BDH Laboratory Supplies (Dorset, UK).

Water supply and test apparatus. We applied stock solutions to the tanks using a Watson-Marlow 205U multichannel peristaltic pump using silicon tubing (Watson Marlow, Falmouth, Cornwall, UK). Solutions were delivered at a rate of 0.02 mL/min into mixing vessels, which were supplied with dechlorinated water that had been heated to 25°C. Water entered the mixing vessels at a flow rate of 300 mL/min, resulting in a 1:15,000 dilution of the stock solution. The diluted stock solution then flowed into the tanks at a rate of 18 L/hr, which resulted in one complete water change every 100 min. Dissolved oxygen and water temperature were recorded daily, and the functioning of the delivery system was monitored throughout the study.

Delivery of the test chemical commenced 1 week before the start of each exposure. During this equilibration period, the fish were acclimatized to the experimental conditions in an identical set of undosed tanks. After 7 days, the fish were transferred into the tanks containing the chemical or chemicals, where they were maintained under exposure conditions for a period of 2 weeks. Three control tanks were run alongside each exposure. Two of these were negative controls (NCs), consisting of one undosed tank that received water only [water control (WC)] and one tank that was dosed with DMF [solvent control (SC)]. A positive control (PC) was also included in each study. The PC tank was dosed with EE_2 at a concentration of 10 ng/L, which has previously been found to induce a maximum VTG response (Panter et al. 2002).

Analytical chemistry. We determined exposure concentrations at three different time points during each experiment. We collected the first set of water samples after 1 week of dosing, immediately before the addition of the fish. The second set was taken 1 week after this, and the third set was taken after the final week, on the day that the exposure was terminated. Water samples were collected in solvent rinsed glass bottles. If the sample was to be analyzed for the presence of steroid estrogens, the bottles were silylated before use. The water samples were then analyzed according to the nature of the chemical in question, using one of the four following analytical techniques.

Water samples containing EE_2 were extracted onto preconditioned solid-phase C18 cartridges. Extracts were eluted into methanol, which was removed under a stream of nitrogen. The extracts were then resuspended in ethanol, and the EE_2 concentration was determined using an established radioimmunoassay technique (Lange et al. 2001). Samples containing E_2 also underwent solid-phase extraction (SPE) on a DVB Speedisk (Baker, Deventer, The Netherlands). After cleanup of the extracts with C18 cartridges, derivitization of E_2 was carried out using silyl reagents before analysis using gas chromatography combined with ion trap detection (adapted from Belfroid et al. 1999; Houtman et al. 2004). Samples containing BPA underwent SPE analogous to the E_2 procedure, after which the extracts were analyzed using HPLC coupled to diode array detection. This was carried out under isocratic elution conditions with methanol/water (60/40, vol/vol) (adapted from Belfroid et al. 1999). For the analysis of water samples containing NP and OP, the extraction step was omitted. After the addition of acetonitrile (5%), large sample volumes (300–800 μL) were injected onto a reversed-phase HPLC column, which was coupled with an ion trap mass spectrometer via an electrospray interface for on-column enrichment. Analytes were eluted using a fast gradient (Pojana et al. 2004).

Experimental design. We determined the complete concentration–response curve of each chemical in the test system in order to provide the information necessary to generate the prediction. The successful comparison of observed and predicted mixture effects was dependent upon the quality of these data. In order to generate a prediction of low uncertainty, that is, high accuracy and precision, it was necessary to minimize the chance of unknown systematic shifts in VTG sensitivity for each chemical within the study time that could result in a biased prediction (inaccuracy), and determine the concentration-effect information of each compound with a certain precision in order to maintain a given statistical variability of the prediction (precision). We achieved this by repeating each exposure at least once after a given time lag. Data from repeated studies were then pooled.

Slight differences in the absolute VTG levels between studies were accounted for by standardizing the absolute effects scale to relative effects of between 0 and 1. The mean VTG concentration in the fish from the NC (SC) and the PC tanks were used as the minimum and maximum responses, respectively. This scaling was carried out after the VTG effects data were $\log_{10}$ transformed, such that a median effective concentration (EC_{50}) corresponds to the concentration that produces a $\log_{10}$-transformed VTG induction, which is median in relation to the NCs and PC.

The aim of the single-chemical exposures was to produce the data necessary to predict the median effect concentration of the mixture without exceeding a given level of statistical uncertainty (the 95% confidence limits for the predicted EC_{50} were set at a maximum of ± 0.2 on the $\log_{10}$-transformed concentration scale). This relied on the premise that there was average effect data variability, determined on the basis of historical data sets produced under similar test conditions, and it required that the concentration range tested provided sufficient information on the VTG response curve. This information was based on results of the repeated preliminary exposure studies, each of which included six different concentrations, to which four male and four female fish were exposed.

In order to compare the mixture effects with the predictions of CA for a wide range of different VTG levels in the final mixture experiment, we used a "fixed-ratio" mixture design: a master stock was prepared, containing each of the chemicals at their EC_{50} concentrations. This was diluted to give a range of mixture concentrations of 100, 50, 30, 20, 10, and 5%, which corresponded with relative VTG responses between 0 and 100%, according to the CA expectations. Fish were exposed to this dilution series in two independent studies using the same methods and design as employed in the individual exposure studies. The concentration–response to the mixture was then determined and related to the effects predicted by CA.

In order to directly relate the effects of the compounds to the observed mixture effects, we performed a second mixture experiment. The design of this experiment involved the parallel testing of each chemical, both individually and in combination. Only one concentration of each chemical was tested. This approach aimed to investigate the potential for mixture effects to occur at low-effect concentrations of the components, that is, at concentrations that would not, individually, induce a statistical significant effect (Silva et al. 2002). The low-effect concentrations adopted were based on the EC_{50} of each chemical divided by 5. According to the principles of CA, it was predicted that this mixture would induce a 50% level of effect.

Fish sampling and analysis of plasma VTG. At the end of each exposure, the fish were sacrificed by overdosing with MS222 (Sigma Aldrich). The length and weight of each individual were recorded before bleeding. Blood samples were collected from the caudal peduncle using heparinized capillary tubes (Hawksley and Sons Ltd., Sussex, UK). These were centrifuged at 4,000*g* for 5 min. Plasma was then drawn off and stored at –20°C until required for analysis. Plasma VTG concentrations were determined using a carp-VTG enzyme-linked immunosorbent assay (ELISA) validated for the measurement of VTG in fathead minnows (Tyler et al. 1996).

Mathematical modeling and statistical analysis. We determined concentration–response curves for each of the five chemicals and for the mixture using pooled data from the repeated exposures. To account for the intra- and interexperimental variability associated with this nested data scenario, we used the generalized nonlinear mixed modeling approach in which both fixed and random effects are permitted to have a nonlinear relationship with the effect end point (Vonesh and Chinchilli 1997). As random effect, a shift parameter was included in the nonlinear regression model, which accounts for a shift of the whole curve based on the $\log_{10}$-transformed concentration scale. Furthermore, a best-fit approach was adopted: three different regression models (probit, logit, and Weibull) were fitted independently to the same pooled data set, and the best fit was selected on the basis of statistical criteria (Scholze et al. 2001). This approach was implemented using the NLMIXED function of the SAS statistical software package (SAS Institute, Cary, USA).

The expected concentration–response relationship of the mixture was calculated using CA, which is represented by Equation 1:

$$EC x_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC x_i} \right)^{-1}, \qquad [1]$$

where ECx_{Mix} is the concentration of the mixture that induces an overall effect x, ECx_i is the concentration of the ith chemical in an n-component mixture required to induce the same magnitude of effect, and p_i is the proportion of the ith component in the mixture (Backhaus et al. 2000). Hence, in addition to information regarding the exact composition of the mixture, knowledge of identical effect concentrations (ECx values) of the mixture components is all that is required to predict an ECx value for the mixture. CA was used to predict ECx values for the mixture in steps of 1% for effect levels from 10% up to 95%. These values were then connected using straight lines to give a graphical representation of the predicted curve. All predicted effect concentrations are estimates and are therefore subject to stochastic variability, which meant that the predicted effect concentration of the mixture also had to include a measure of statistical uncertainty. This was achieved using the bootstrap method (Efron and Tibshirani 1993), which enabled 95% confidence limits to be derived for the mean predicted effect.

Results

Analytical determination of exposure concentrations. Because of occasional technical problems that were encountered with the analysis of the water samples, we did not obtain full sets of reliable data for each chemical in all exposures. Inconsistencies between data sets for some chemicals created problems when plotting the concentration–response data by effectively shifting the position of the curve along the x-axis, thereby increasing the variability associated with the biological response. This reduced the accuracy and precision of the effect model for the chemicals concerned. In contrast, when the biological data (the VTG response) were plotted against the nominal concentrations, it proved to be highly reproducible. This strongly suggested that the occasional differences between nominal and measured concentrations were artifactual. For this reason, the concentration–response analyses were based on nominal, as opposed to measured, exposure concentrations.

The problems encountered with the chemical analyses were subsequently resolved, and good agreement between the nominal and actual exposure concentrations of each chemical was obtained in the mixture experiments. This is demonstrated in Table 1, which shows the measured concentration of all of the mixture components on the first day of each of the mixture experiments. These values were between 100 and 166% and 66 and 128% of the nominal value for EE_2 and E_2, and 64 and 128%, 50 and 110%, and 55 and 105% of nominal value for NP, OP, and BPA, respectively. Hence, the extent of the deviation from nominal concentrations did not vary consistently between chemicals, despite the differences between their exposure concentrations. The mean measured concentration of each chemical remained fairly constant over time: the measured concentrations of EE_2, E_2, NP, OP, and BPA 1 week and 2 weeks after the start of the exposure were an average of 99 and 77%, 89 and 92%, 92 and 96%, 84 and 97%, and 92 and 86% of those measured at the start of the exposure, respectively. Hence, the analytical data generally confirm that the exposure conditions were similar and reproducible for each of the chemicals used.

Biological effects data. All exposure studies ran to completion. The rate of mortality did not differ between treatments, which indicated that the chemicals tested were not acutely toxic and that the fish were not unduly stressed. The baseline concentrations of VTG determined for control males and females were consistent with the literature (Harries et al. 2000; Panter et al. 1998; Tyler

Table 1. Nominal and measured exposure concentrations at the beginning of each mixture experiment.

Concentration (mixture dilution)	EE_2 (ng/L)		E_2 (ng/L)		NP (µg/L)		OP (µg/L)		BPA (µg/L)	
	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured
First mixture experiment										
10.1 mg/L (5%)	0.03	0.03, 0.05	1.25	< 0.8, 1.3	0.35	0.4, 0.7	2.25	1.5, 2.4	7.5	4.1, 6.1
20.2 mg/L (10%)	0.06	0.07, 0.08	2.5	< 1.5, 2.6	0.7	0.7, 0.8	4.5	2.5, 5.1	15	9.6, 12
40.4 mg/L (20%)	0.12	0.14, 0.19	5	3.9, 4.9	1.4	0.9, 1.4	9	4.5, 8.2	30	19, 22
60.6 mg/L (30%)	0.18	0.23, 0.23	7.5	6.2, 9.0	2.1	2.3, 2.0	13.5	11, 12	45	43, 32
101 mg/L (50%)	0.3	0.31, 0.42	12.5	13, 16	3.5	3.5, 2.8	22.5	20, 14	75	79, 41
202 mg/L (100%)	0.6	0.6, 1.0	25	25, 28	7	7.1, 5.5	45	35, 32	150	150, 110
Second mixture experiment										
40.4 mg/L (20%)	0.12	0.13	5	6	1.4	1.8	9	9.4	30	20

The measured values given for the first mixture experiment represent the concentrations determined during two independent exposure studies.

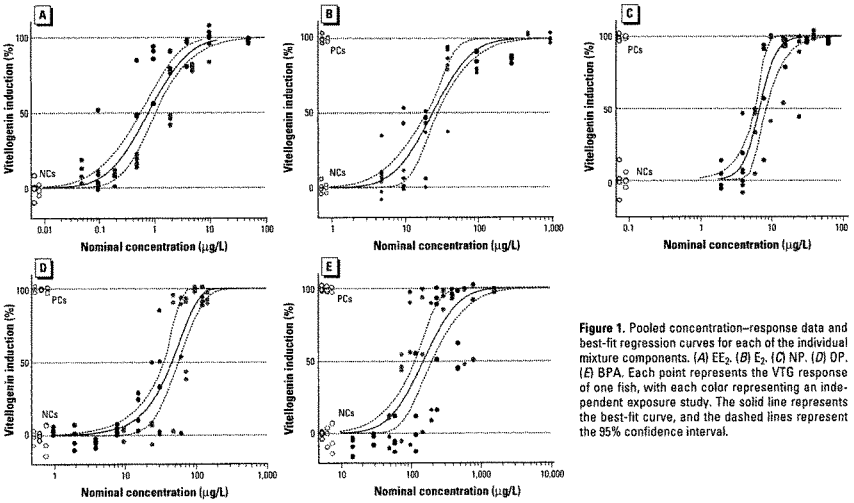

Figure 1. Pooled concentration–response data and best-fit regression curves for each of the individual mixture components. (*A*) EE_2. (*B*) E_2. (*C*) NP. (*D*) OP. (*E*) BPA. Each point represents the VTG response of one fish, with each color representing an independent exposure study. The solid line represents the best-fit curve, and the dashed lines represent the 95% confidence interval.

et al. 1996), and there were no significant differences between the VTG levels of WC and SC fish in any of the exposures. Clear concentration–response curves could be determined for male fish in response to each of the single chemicals as well as to the mixture. In contrast, female VTG levels exhibited extensive variability, depending on their stage in the spawning cycle (data not shown). For this reason, only the data from male fish were suitable for inclusion in the analyses.

Concentration–response analysis for individual chemicals. The analysis of the concentration–response data for each chemical was based on data pooled from at least two independent exposure studies. In the case of OP and BPA, a third smaller-scale study was conducted. This was necessary because the first two exposures did not cover the full extent of the VTG response curve. In general, data from repeated studies showed excellent agreement, although there was some disparity between the positions of the curves for EE_2 and, to a lesser extent, E_2. This is likely to reflect the increased potential for error when working in the nanograms per liter concentration range. These findings support the need to base the prediction of mixture effects on more than one set of data using the means of repeated and pooled data sets.

Each of the chemicals tested induced VTG in a concentration-dependent manner. Figure 1 shows the concentration–response data for each chemical and their estimated regression curves. The corresponding best-fit models with estimated parameters are given in Table 2, together with the estimated EC_{50} values and the confidence limits, which were always below the planned tolerance benchmark of ± 0.2 on the $\log_{10}$-transformed concentration scale. It was possible to determine the 100% effect (relative to the PC) for each chemical, and the lowest tested concentration did not provoke effects significantly different from the untreated controls. This allowed the estimation of full concentration–response curves without needing to extrapolate to untested effect levels. Figure 2 shows the concentration–response curves for each chemical plotted on the same concentration scale, thus highlighting the magnitude of variations in potency. EE_2 was the most potent chemical tested, with an EC_{50} of 0.9 ng/L, which was between 25 and 30 times more potent than E_2. The EC_{50} of the natural steroid E_2 was 25 ng/L. NP and OP were 280 and 1,800 times less potent than E_2, with EC_{50} values of 7 and 45 µg/L, respectively. BPA was the least potent chemical tested, with an EC_{50} of 150 µg/L. This was 6,000 times less potent than E_2.

Concentration–response analysis for the mixture. The VTG response induced by the mixture is shown in Figure 3, together with the line of best fit and the curve predicted by CA. The variability associated with the best-fit estimate is shown in Table 2. A concentration–response curve was evident, and there was excellent agreement between the results of the two independent exposures. The pooled data sets provide sufficient information for EC estimates of low statistical uncertainty and thus a good basis for the comparative assessment of observed and predicted mixture effects. The comparison of the observed VTG response and the corresponding regression fit with the prediction curve yielded excellent agreement, independently of the effect level. No statistical deviation could be detected, with the prediction lying within the narrow 95% confidence limits along the full length of the curve. These findings provide evidence that estrogenic chemicals act in an additive manner *in vivo* and that their effects can be predicted accurately using CA.

Mixture effects at low-effect concentrations. The results of the investigation into mixture effects for compounds at low-effect concentrations are shown in Figure 4. Analysis of the data revealed that, individually, each of the chemicals failed to provoke a response that was statistically different from that of the controls at a concentration that was equivalent to one-fifth of their EC_{50}. In contrast, when fish were exposed to the same dose of all five chemicals in combination, VTG was significantly induced. In line with the first experiment, there was good agreement between the observed effect of the mixture and the prediction of CA, with the prediction falling within the confidence limits of the observed effects. This confirms that the combined action of estrogenic chemicals does not deviate from additivity even in the low-effect concentration range.

Table 2. VTG induction by the individual compounds and the mixture.

	Concentration–response function				
Compound	Model[a]	$\hat{\beta}_1$	$\hat{\beta}_2$	$\hat{\sigma}^2_{\text{between exp}}$	EC_{50} (95% CI)
EE_2	Probit	5.03	1.65	0.29	0.0009 (0.0005–0.001)
E_2	Probit	3.75	2.33	0.11	0.025 (0.020–0.029)
NP	Logit	−7.10	8.40	$< 10^6$	7.02 (6.05–8.56)
OP	Weibull	−6.37	3.57	$< 10^6$	48.2 (36.2–58.0)
BPA	Probit	−5.61	2.55	0.06	158 (119–205)
Mixture					
Observed	Weibull	−6.61	3.71	$< 10^6$	48.0 (40.9–61.4)
Predicted	CA	—	—	—	44.3 (38.6–47.1)

CI, confidence interval. $\hat{\beta}_1$ and $\hat{\beta}_2$ are statistical estimates of model parameters; 95% CIs are approximate confidence intervals for effect concentrations given in µg/L; $\hat{\sigma}^2_{\text{between exp}}$ is the statistical estimate for variance between experiments; and EC_{50} values are in relation to the NCs and PC, calculated from the given concentration–response function (rounded values). [a]Concentration–response functions as defined by Scholze et al. (2001).

Discussion

Exposure concentrations. The decision to determine the concentration–response relationships on the basis of nominal, as opposed to measured, exposure concentrations was made in order to overcome problems that were initially encountered with the analytical chemistry (discussed above). In theory, the measured concentrations should provide a more accurate reflection of the exposure conditions, because they account for experimental errors that may have arisen because of inaccuracies in the preparation of stock solutions and/or the dosing of tanks. As a result, the measured concentrations should provide the basis for the mathematical modeling of mixture effects.

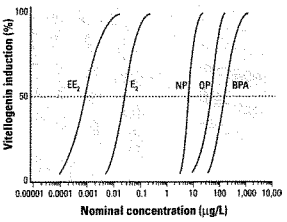

Figure 2. Best-fit regression curves for the individual mixture components plotted on the same concentration scale.

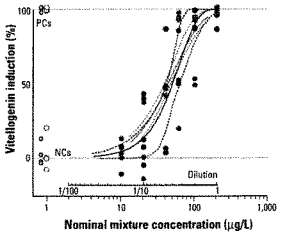

Figure 3. Comparison between the observed and CA-predicted mixture effects of five estrogenic chemicals in the male fathead minnow. Each point represents the VTG response of one fish, with each color representing an independent exposure study. The solid black line represents the best-fit of the observed effect data, and the solid red line represents the CA prediction. Dashed lines represent the 95% confidence intervals. The predicted effect of the mixture falls within the 95% confidence interval of the observed data across the entire dose–response curve.

However, if problems occur when measuring the exposure concentrations, these can add more variability than they remove. This, in turn, reduces rather than improves the accuracy of the prediction. Hence, in the absence of a full set of reliable measured concentrations, it was more accurate to base the mathematical model on the nominal values.

This approach did not appear to reduce the reproducibility of the concentration–response analysis of NP, OP, and BPA. In contrast, the agreement between the concentration–response curves determined for E_2 and EE_2 in each of the repeated exposures was slightly reduced when the VTG response data were plotted against nominal, as opposed to measured, concentrations. However, these differences were marginal. This indicates that the nominal values provided a reliable indication of the real exposure concentrations and validates their use in the concentration–response analyses. This approach may not have used the chemical analytical data to their full potential. However, the determination of exposure concentrations was extremely useful in confirming the accuracy of the dosing system. Without this, it would not have been possible to validate the methods employed.

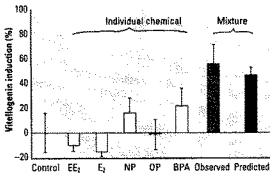

Figure 4. Mixture effects at low-effect concentrations (one-fifth of EC_{50}) of five estrogenic chemicals. Error bars indicate SEM. Individual concentrations were 0.12 ng/L EE_2, 5 ng/L E_2, 1.4 µg/L NP, 9 µg/L OP, and 30 µg/L BPA. The mixture treatment contained all five chemicals at the aforementioned concentrations, resulting in an overall mixture concentration of 40.4 µg/L. Analysis of variance detected a significant difference between treatments ($F_{6,19} = 4.05$, $p < 0.01$). Post hoc tests revealed no difference between the response of fish exposed to each of the chemicals individually and that of the control fish. In contrast, the mixture elicited a response that was significantly different from that of the controls.

Single-substance effects. Despite the plethora of published data describing the potency of the chemicals tested in this study, comparisons between studies are complicated by apparent differences between the species tested, the end points analyzed, and the assay systems used. However, comparable studies involving the analysis of VTG induction in male fathead minnows exposed to estrogenic chemicals under flow-through conditions have yielded results that are consistent with the effects reported here. For example, Panter et al. (1998) reported the induction of VTG in response to between 32 and 100 ng/L of E_2 after a 3-week exposure, which is in the same order of magnitude as the potency observed in this study. EE_2 has previously been found to induce VTG at concentrations between 0.1 and 1 ng/L (Pawlowski et al. 2004). This is consistent with the EC_{50} of 0.9 ng/L reported here. The potency of NP is also consistent with previous evidence that this chemical is effective at concentrations between 1 and 10 µg/L in fathead minnows after a 2- to 3-week exposure (Harries et al. 2000; Pickford et al. 2003). Studies by Sohoni et al. (2001) suggest that BPA is less potent, although the effects reported were of a similar order of magnitude as those observed in this study. Concentration–response data from comparable studies on the test species were not available for OP.

Differences between the relative potencies of each of the compounds tested in this study are also described in the literature. These data are reviewed in Table 3, which reflects the differences in the potency of each of the chemicals tested. The potency of each chemical relative to E_2 also varied extensively between studies. The cause of this variability is unknown, but is likely to reflect differences between the exposure systems, the concentrations tested, and the effect levels used to determine potency. Differences in species sensitivity may have also influenced the patterns observed.

Mixture effects. The results of the first mixture experiment demonstrate that mixtures of estrogenic chemicals have the capacity to act in combination and that their effects can be accurately predicted on the basis of the concentration–response curves of the individual mixture components according to the principles of CA. The predictions were in close agreement with the observed effects across the entire range of effects. Thus, we can conclude that the combined effect of the mixture does not deviate from additivity. This is consistent with the *a priori* assumption of this concept, which is dependent upon the components of the mixture acting via a common mechanism to contribute to the overall mixture effect. Although the validity of this concept has been demonstrated for estrogenic chemicals in assays involving unicellular organisms and mammalian cells (Payne et al. 2000, 2001; Rajapakse et al. 2002; Silva et al. 2002), these results provide the first evidence that the principles of CA hold true for multicomponent mixtures of estrogenic chemicals at higher organizational levels, despite the increased biological complexity of the assay system and the greater potential for toxicokinetic effects.

Similar additive effects have previously been reported in response to binary mixtures of estrogenic chemicals *in vivo*. Thorpe et al. (2001) investigated the effects of two-component mixtures on VTG induction in rainbow trout. Concentration–response curves were determined for fixed-ratio binary mixtures of E_2 and NP (1:1,000) and of E_2 and methoxychlor (MXC; 1:1,000), and these were related to the predictions of CA. The mixture of E_2 and NP induced effects that were in agreement with the predictions of CA across the entire range of concentrations tested. In contrast, the mixture of E_2 and MXC induced effects that were less than additive. This was attributed to the fact that MXC may act via a mechanism different from that of E_2 and NP. Nevertheless, the effects observed provide strong evidence of the capacity for mixtures of similarly acting chemicals to behave in an additive manner according to the principles of CA. However, this conclusion was not confirmed in a subsequent investigation into the

Table 3. Relative potencies previously reported for the five mixture components in terms of VTG induction.

Test organism	Sex	Exposure system	Exposure duration (days)	Effect level	EE_2	E_2	NP	OP	BPA
Roach (*Rutilus rutilus*)[a]	Male	Flow-through	21	LOEC	—	1	—	1,000	—
Rainbow trout (*Oncorhynchus mykiss*)[a]	Male	Flow-through	21	LOEC	—	1	—	100	—
Zebrafish (*Danio rerio*)[b]	Male	Flow-through	8	LOEC	0.06	1	—	—	—
Sheepshead minnow (*Cyprinodon variegatus*)[c]	Male	Flow-through	16	LOEC	0.53	1	50	—	—
Killifish (*Fundulus heteroclitis*)[d]	Male	Injection	8	LOEC	—	1	20	200	100
Rainbow trout (*Oncorhynchus mykiss*)[e]	Female (juvenile)	Flow-through	14	EC_{50}	0.04–0.09	1	1,000	—	—
Zebrafish (*Danio rerio*)[f]	Male	Semistatic	21	LOEC	> 0.25	1	25,000	5,000	50,000
Rainbow trout (*Oncorhynchus mykiss*)[f]	Juvenile	Semistatic	21	LOEC	> 0.25	1	5,000	1,500	50,000
Fathead minnow (*Pimephales promelas*)[g]	Male	Flow-through	14	EC_{50}	0.036	1	280	1,800	6,000

LOEC, lowest observed effect concentration. These data are scaled relative to the E_2 potency observed in each study.
[a]Routledge et al. (1998). [b]Rose et al. (2002). [c]Folmar et al. (2003). [d]Pait and Nelson (2003). [e]Thorpe et al. (2001). [f]Van den Belt et al. (2003). [g]Present study.

combined effects of E_2 and EE_2 (Thorpe et al. 2003). The effects of this mixture were consistent with CA at low-effect concentrations, but a divergence occurred with increasing effect level, with the predicted effects exceeding those that were observed. This was attributed to the limitations of the experimental design rather than being the result of a real deviation from additivity (Thorpe et al. 2003).

The problems encountered by Thorpe et al. (2003) were attributed to the fact that only three concentrations of the mixture were tested. This may have reduced the accuracy of the concentration–response relationship. An additional problem arose because of difficulties in defining the maximum response to the individual test compounds, as well as the maximum response predicted by CA. These difficulties were overcome in the present study by testing a wider range of mixture concentrations and by standardizing the response across exposures according to the minimum and maximum response of the controls. The accuracy with which these methods allowed the effects of the mixture to be predicted undoubtedly reflects the power of the mathematical modeling and statistical analyses. It also demonstrates the capacity for the VTG induction assay to produce high-quality, reproducible data for analyzing the mixture response.

Low-dose implications. The additive nature of the combined effects observed in the first mixture experiment demonstrates that all components contribute to the overall effect of a mixture. This implies that the overall effects will always exceed the highest individual effect of the mixture components. By this line of reasoning, low-effect concentrations of the individual components may give rise to considerable mixture effects. This phenomenon is of particular importance for the environmental hazard assessment of chemicals because it indicates that concentrations of chemicals that show no effect when applied singly may provoke substantial effects when acting in combination. The second mixture experiment investigated whether these theoretical conclusions from the CA concept also hold true in the real world, by analyzing the combined effect of the mixture components when they were present at low, noneffective concentrations. Even under these circumstances, a highly significant mixture effect of more than 50% was observed. These *in vivo* results were consistent with the "something from nothing" effects reported by Silva et al. (2002), which were produced using *in vitro* techniques.

More recently, the potential for estrogenic mixture effects at low concentrations has been explored *in vivo* using an assay based on an increase in rat uterotrophic weight (Tinwell and Ashby 2004). Concentrations that individually induced low effects were determined for seven estrogenic chemicals. Equipotent concentrations were tested, both individually and in combination, at various concentrations. The highest concentration of the mixture induced a significant increase in uterine weight in relation to the effects produced by the individual chemicals (although this difference was marginal). At 5- and 10-fold dilutions, few of the individual chemicals induced a significant response, and at a 50-fold dilution, no significant responses were observed. However, the same dilutions of the mixture were found to induce a significant response, thereby demonstrating the potential for mixture effects, even when the effects of each individual chemical cannot be detected. Although these findings were not related to expectations based on additivity, they are in perfect agreement with the results of the present study. This provides strong evidence of the capacity for estrogenic chemicals to act in combination at higher levels of biological organization, even at the type of low-effect concentrations encountered in the environment.

Regulatory context. Our findings in this study, combined with those of Tinwell and Ashby (2004), highlight the limitations of existing approaches to environmental (and human) risk assessment when considering the hazard posed by mixtures of endocrine-disrupting chemicals. Estrogenic chemicals, such as the alkylphenols, which are generally present in the environment as mixtures and at concentrations below those required to individually induce an effect, may therefore add to the overall risk when present with other chemicals that act via a similar mechanism. The failure to account for the combined effects of these chemicals will undoubtedly lead to the underestimation of potential hazards and hence erroneous conclusions regarding the risk that they pose. In demonstrating the inadequacy of the chemical-by-chemical approach to risk assessment, these findings represent a significant step toward achieving a more realistic means of assessing the environmental hazard posed by estrogenic chemicals. In addition to their regulatory implications, these findings indicate that CA may be a valuable tool for predicting the hazard posed by this type of mixture.

Research needs. It is important to recognize that CA can be applied only when the mixture is completely defined in terms of the number of chemicals present and the mixture ratio. A predictive risk assessment of combination effects will therefore depend heavily on the generation of robust tools for analyzing the type of mixtures that occur in real exposure situations. It should also be acknowledged that the scope of these findings is limited to the assessment of chemicals that act via the same mechanism to induce a common effect. The next major challenge will be to consider the endocrine-disrupting effects of mixtures of chemicals that act via different modes of action, or that have both agonistic and antagonistic effects. Potential interactions with non-endocrine-active compounds, such as solvents and surfactants, should also be considered, along with the influence of additional stresses incurred via changes in the environment and organismal physiology. Although the task of integrating this body of knowledge into hazard assessment procedures presents a formidable challenge, these improvements will be essential in ensuring the adequate protection of wildlife populations and human health.

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Research

Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening

C. DESBROW,[†] E. J. ROUTLEDGE,*[,‡] G. C. BRIGHTY,[§] J. P. SUMPTER,[‡] AND M. WALDOCK[†]

Centre for Environment, Fisheries and Aquaculture Science, Burnham Laboratory, Remembrance Avenue, Burnham-on-Crouch, Essex, CMO 8HA, U.K., Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K., and Environment Agency, National Centre for Ecotoxicology and Hazardous Substances, Hawberry Park, Wallingford, Oxon, OX10 8BD, U.K.

A fractionation system, combined with an in vitro assay for detecting estrogenic activity, was developed in order to isolate and identify the major estrogenic chemicals present in seven sewage-treatment works (STW) effluents, receiving primarily domestic effluent, discharging into British rivers. Three sterols were isolated from estrogenic fractions of sewage extracts; these were the natural hormones 17β-estradiol and estrone and the synthetic hormone 17α-ethynylestradiol. 17β-Estradiol and estrone were present in all the effluents at measured concentrations ranging from 1 ng/L to almost 50 and 80 ng/L, respectively. The concentration of 17α-ethynylestradiol was generally below the limit of detection but was positively identified in three of the effluent samples at concentrations ranging from 0.2 to 7.0 ng/L. These data suggest that natural and synthetic hormones may be responsible for the observed induction of vitellogenin synthesis in male fish placed downstream of effluent discharges from STWs that receive mainly domestic inputs.

Introduction

In 1994, Purdom and colleagues reported that sewage-treatment work (STW) effluent was estrogenic to fish (*1*). The effluents tested were mainly domestic (rather than industrial) in source, indicating that the estrogenic component(s) were likely to be domestic in origin and were probably common to most of them. For this reason, this research program concentrated on effluents receiving primarily domestic input, with limited industrial inputs. At present, no specific examples have been reported where an estrogenic effect on wild fish has been conclusively linked to a particular chemical emanating from STW effluents, although many chemicals, including 17α-ethynylestradiol (the main component of the oral contraceptive pill) and alkylphenolic chemicals (breakdown products of one group of nonionic surfactants), have been implicated (*1*, *2*). STWs receiving domestic and industrial waste release a complex (and ill-defined) mixture of natural and synthetic chemicals into the aquatic environment, due to their partial or complete resistance to biodegradation during the treatment process. Although effluents have been tested for their toxicity to aquatic organisms, usually in order to determine safe discharge levels, few chemicals within an effluent have been tested for toxicity or hormone disrupting activity on an individual basis, either in vivo or in vitro. As it was clearly not practical to identify, quantify, and test all the individual substances present in effluent, a bioassay-directed fractionation procedure was adopted in which STW effluent was chemically separated into fractions of decreasing complexity. Each fraction was analyzed for estrogenic activity using a yeast-based estrogen screen (*3*). Fractions identified as active in the bioassay were separated further until they could be analyzed by gas chromatography–mass spectrometry (GC–MS), leading to identification of the active chemicals. This procedure, through a series of steps involving separation and resolution, simultaneously eliminates inactive compounds and isolates chemicals that are biologically active without using any preconceived ideas about the identity of the compounds responsible for the activity in the mixture.

Several schemes have been developed in order to isolate and identify toxins from complex mixtures based on a Toxicity Identification and Evaluation (TIE) approach (*4*). Such procedures have been successfully used for the analysis of pollution-related fish mortalities (*5*), industrial effluents (*6*, *7*), urban runoff (*8*), STW effluents (*9*), and sediments (*10*, *11*). To assess the biological activity (or toxicity) of a complex mixture, a selective end point must be established. In toxicity studies, bioassays based on the life cycle of the cladoceran *Daphnia* are commonly used. In such studies, the *Daphnia* were first exposed to the whole sample to evaluate the toxicity of the mixture, which was then fractionated using a series of chemical separation techniques. At each separation stage, the toxicity of the individual fractions was re-assessed using the bioindicator organism, and the results obtained were used to direct the fractionation procedure toward the most toxic components. This was repeated until an extract was generated containing a small number of toxins. These fractionation procedures are time-consuming and have a number of drawbacks. For example, an increase in toxicity could result with each chemical manipulation of the sample, due to alterations in the bioavailability of toxins. Conversely, interactive effects may be reduced as the mixtures were resolved into single components.

The fine details of each TIE procedure vary, depending on the characteristics of the target toxins, but solid phase extraction (SPE) columns containing reverse-phase C18 have been shown to reversibly bind a broad range of organic compounds, which may be subsequently eluted with a range of solvents according to their polarity. The C18 step is not universally successful, however, as certain compounds are either poorly retained or are retained with such great affinity to the C18 phase or to the plastic components of the cartridge (e.g., tributyltin and some polycyclic aromatic hydrocarbons) that they are difficult to remove subsequently (*12*, *13*). Finer fractionation of the C18 SPE eluent is usually performed using high-performance liquid chromatography (HPLC), with a C18 stationary phase (*4*). At each stage, fractions collected are tested for toxicity, and the active fractions are analyzed using

* To whom correspondence should be addressed. Telephone: 01895 274 000; fax: 01895 274 348; e-mail: edwin.routledge@brunel.ac.uk.
† Burnham Laboratory.
‡ Brunel University.
§ National Centre for Ecotoxicology and Hazardous Substances.

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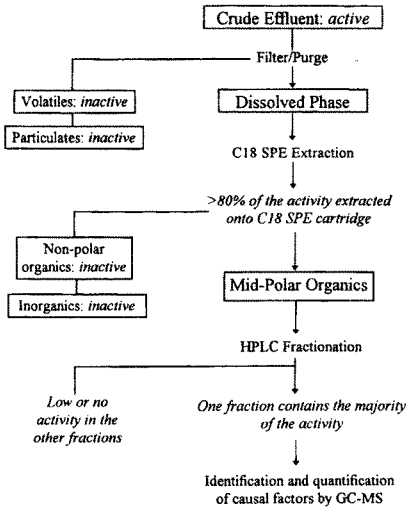

FIGURE 1. Overview of the scheme used for fractionating domestic sewage effluent together with the findings from preliminary studies.

an unambiguous technique such as GC–MS or HPLC–MS. We have employed the TIE approach, modified for our specific needs, to identify and quantify the main estrogenic chemicals in domestic STW effluent.

Materials and Methods

The final methodology employed for the detection and quantification of estrogenic compounds in domestic STW effluent was based on the procedures developed and refined throughout the research program, using various volumes of Southend STW effluent. A summary of the overall fractionation scheme for domestic STW effluent, which also shows the findings from the in vitro bioassay for estrogenic activity, is illustrated in Figure 1.

Site Selection. The data presented here were the product of the analysis of seven STW effluents. Sites were selected to provide a range of treatment processes (i.e., primary, secondary, or tertiary treated effluents containing largely domestic waste rather than effluent containing large amounts of industrial waste), convenience of sample collection, continuity with previous sampling programs, and known alkylphenol content. Effluents from the River Lea catchment (Harpenden STW, Rye Meads STW, Deephams STW) as well as Horsham STW (discharges into River Arun) were selected as these effluents had been previously shown to be estrogenic to fish using the in vivo caged male trout bioassay (*2*, *14*). Billing STW (discharges into the River Nene) and Naburn STW (discharges into the River Ouse, Yorkshire) were also analyzed. The sites chosen for investigation are summarized in Table 1. Southend STW effluent was used for the majority of the method development because a preliminary assay showed that it contained relatively high levels of estrogenic activity as compared to other effluents and therefore provided a readily measurable response to enable isolation of the estrogenic fraction(s).

Sampling Procedure. Discrete samples were taken from each of the STWs in preference to a bulked composite sample (taken throughout a 24-h cycle), as the stabilities of the estrogenic compounds at this stage were unknown. Large numbers of effluent samples were not processed because the methodology was too time-consuming. However, the consistency of the estrogenic activity in each effluent sample was determined by assay on three separate occasions. All the sampling equipment was treated with a weak bleach solution (for disinfection), which was then removed using distilled water. The equipment was rinsed with methanol prior to use to remove any organic contaminants. A 20-L sample of effluent was collected upstream of the point of discharge into the receiving river water using a stainless steel bucket, funnel, and aluminum churn (or stainless steel pressure vessel). A total of 100 mL of methanol was added to the effluent sample on return to the laboratory to prevent bacterial growth and to aid subsequent solid-phase extraction.

Assay for Estrogenic Activity. A yeast-based screen for estrogenic activity was employed; full validation of the assay was published previously (*3*). The bioassay detects "real" estrogens (such as 17β-estradiol, estrone, and estriol) as well as all known xenoestrogens (such as alkylphenolic chemicals, Bisphenol A, *o,p'*-DDT, diethylstilbestrol, phytoestrogens); thus, we expected the bioassay to produce quantitative measurements of estrogenic compounds regardless of the identity of the chemical (or chemicals) responsible for the estrogenic activity of STW effluent.

In this system, the human estrogen receptor is expressed in yeast in a form capable of binding to estrogen-responsive sequences (ERE) situated within a strong promoter sequence on expression plasmids. Upon binding an active ligand, the occupied receptor now binds to the EREs and interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene *Lac-Z*, and the enzyme produced (β-galactosidase) is secreted into the medium where it metabolizes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm (*3*). Preliminary crude effluent samples (whole, glass wool (GW) filtered, purged, GW filtered and purged, after C18 extraction) were also filter sterilized through 0.2 μm pore size Whatman Puradisc filters before testing. A volume of approximately 4 mL of the each crude effluent fraction was passed through the 0.2-μm filter prior to the collection of a separate sample used for testing. Varying amounts of this resulting aqueous filtrate (for example 10, 25, and 50 μL) were then added to wells on a microtiter plate to which 200 μL of seeded assay medium (assay medium plus yeast) was added. Methanol and LC fractions from the subsequent rounds of effluent fractionation were added directly to the assay plates (without the need to filter sterilize) and were allowed to evaporate to dryness before adding the seeded assay medium. The plates were incubated at 32 °C for 4 days or until a response was resolved above the background absorbance of the medium, after which the final absorbance of the medium was read at 540 nm.

SPE Extraction of Effluent Samples. A 20-L effluent sample was placed inside a container, which was then pressurized in order force the effluent through a Teflon filter cartridge packed with glass wool (previously rinsed with acetone and dried) at a flow rate of 10 mL/min. A 5-g (20 mL volume) and a 2-g (6 mL volume) octadecylsilane (C18) solid-phase extraction (SPE) cartridge (IST, Hengoed), previously conditioned with 15 mL of methanol followed by 15 mL of water, were placed in series after the glass wool filter. The function of the second cartridge was to collect any compounds 'breaking through' the first cartridge. The C18 cartridges were disconnected from the extraction apparatus after all the effluent had passed through. The cartridges were then dried under vacuum with air, using a VacMaster system

TABLE 1. Details of the Sites Used in the Effluent Fractionation Project Including Population Numbers, Daily Flow through Sewage-Treatment Plant, and the Type of Treatment Process

site	population equiv	daily flow (m^3)	treatment type
Southend STW	197 749	45 000	primary settlement treatment (from April to October, 50% of the high flow receives Vitox treatment)
Harpenden STW	31 200	8 250	percolating filters and sand filters
Rye Meads STW	357 000	88 500	diffused air-activated sludge, final settlement and tertiary lagoons
Deephams STW	796 000	160 000	diffused-air activated sludge
Naburn STW	388 000	20 000	screening, primary settlement, biological filtration, secondary humus settlement
Horsham STW	107 250	18 000	biological filtration and settlement lagoons
Billing STW	285 959	60 000	extended aeration

(IST, Hengoed). When all the water had been dispelled, the cartridges were wrapped in hexane-rinsed foil and were stored at −20 °C until required. For the control, the same procedure was carried out using distilled deionized water. This was termed the 'procedural blank', which was analyzed alongside the effluent fraction at each stage.

Elution of Material from SPE Cartridges. After thawing for 2 h in a fume hood at room temperature, the C18 cartridges were connected to the Vacmaster system for elution. Initial fractionation of the dissolved phase was performed using a method based on the U.S. EPA toxicity-based fractionation procedure developed by Mount and Anderson-Carnahan (*4*). SPE columns containing C18 reversibly bind a broad range of compounds. These can then be eluted according to their polarity, enabling the selective removal and concentration of organic compounds from complex mixtures. By controlling the elution sequence of the compounds retained on the C18 matrix, using solvents of varying polarities, the biologically active components can be isolated within discrete fractions appropriate for higher resolution procedures. The compounds retained on the C18 cartridges from the effluent sample were eluted from the column using a series of 5 mL (5-g cartridge) or 2.5 mL (2-g cartridge) volumes of methanol/water mixtures (0%, 25%, 50%, 75%, 80%, 85%, 90%, 95%, and 100% methanol), which were collected in separate vials. The same SPE cartridges were then sequentially eluted with solvents of low polarity to nonpolar solvents (diethyl ether, 50/50 diethyl ether/hexane, and finally hexane) to elute compounds of the widest polarity range that had not been removed previously by the 100% methanol. These samples were blown down to incipient dryness under nitrogen and made up in methanol. All these samples were subsequently assayed for estrogenic activity.

Reconcentration of Active Fractions. Following their assessment for estrogenic activity, the active fractions were combined and reconcentrated prior to the next fractionation stage using HPLC. Active SPE fractions were combined in a clean, solvent-rinsed glass Winchester bottle (2.7-L capacity). The sample vials were then rinsed twice with 1 mL of Nanopure water, which was also added to the bottle. The bottle was then filled with 2.5 L of Nanopure water to ensure that the methanol from the samples was diluted sufficiently to allow re-extraction onto the new C18 cartridge. This procedure was necessary because of the difficulty in blowing down large volumes of methanol containing trace amounts of water. However, this method of concentration also reduced the risk of evaporative loss of the semivolatile components.

The diluted sample was re-extracted under vacuum (maximum flow 10 mL/min), and the cartridge was dried as described previously. The sample was eluted from the cartridge into a beaker using 2 × 15 mL of hot methanol (60 °C). The sample was then transferred to a round-bottomed flask along with the contents of the beaker following a 2 × 1 mL methanol rinse. The sample, concentrated by evaporation to a volume of approximately 1 mL using a rotary evaporator, was then transferred to a graduated test tube together with the methanol used to rinse the flask (2 × 0.5 mL of methanol). The test tube sample was then blown down to a volume of 1.5 mL at room temperature under nitrogen, and the sample was placed in a sealed 2-mL vial and stored at 4 °C until required.

Fine Fractionation by Semipreparative HPLC. Half of the concentrated 1.5-mL sample was chromatographed by injecting 150 μL (5 times) through a 25 cm × 10 mm × 5 μm Spherisorb ODS2 C18 semipreparative HPLC column (Fisher Scientific, Loughborough, U.K.) at a flow rate of 5 mL/min, with a UV detector (210 nm) using HPLC-grade water and methanol as mobile phase solvents.

Gradient elution was employed using a water and methanol mixture. The standard gradient started with 40% methanol for the first 3 min following the injection onto the column (40%, 0−3 min), after which the polarity was gradually decreased to 100% methanol by 30 min (40−100%, 3−30 min). The carrying solvent was maintained at 100% methanol for a further 10 min (100%, 30−40 min), after which time it was returned to 40% methanol (100−40%, 40−41 min), which was maintained for the remaining duration of the run (40%, 41−45 min).

As the sample components were eluted from the separating column by the carrying solvent, they were monitored through an ultraviolet (UV) detector, and discrete fractions were collected at 1.5- or 1-min intervals (30 × 7.5 mL fractions collected in 45 min or 45 × 5 mL fractions collected in 45 min, respectively) into vials. All the fractions produced were then assessed for estrogenic activity.

Further Separation of the Active Fraction Using a Shallow HPLC Gradient. A finer fractionation procedure based on reversed phase HPLC was also developed in order to further purify (separate) the estrogenic fraction already isolated from the first HPLC run. An identical HPLC column was used, but a 'shallower' gradient of solvent mixtures (methanol/water) was employed. The shallow gradient started with 55% methanol for the first 3 min after the injection onto the column (55%, 0−3 min) and was then increased to 60% methanol by 30 min (55−60%, 3−30 min), after which time it was gradually returned to 55% methanol (60−55%, 30−40 min) for the remaining duration of the run (55%, 40−45 min).

Extraction of Estrogenic HPLC Fractions into Dichloromethane. The active fractions from each final HPLC run were combined in a 500-mL separating funnel (together with the Nanopure water used to rinse out the sample vials), and the contents of the separating funnel were then diluted to a volume of 300 mL using Nanopure water. A 500-μL aliquot of a 2 μg/mL d_2-17β-estradiol internal standard (dissolved in dichloromethane) was also added to the sample. The combined sample was then liquid/liquid extracted three times, using 50 mL of dichloromethane (DCM). The DCM extracts were then dried using anhydrous sodium sulfate and rotary evaporated to a volume of 1 mL. The sample was then re-

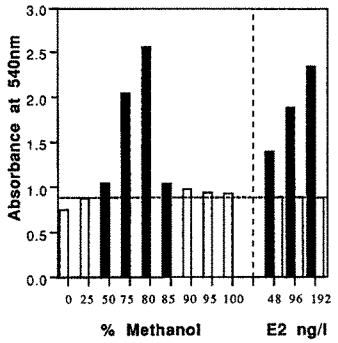

FIGURE 2. Typical estrogenicity profile obtained from a 20-L sample of domestic effluent following concentration and elution from C18 SPE cartridges using methanol/water mixtures of varying polarity. The bars depict the absorbance of the yeast screen medium following incubation with a 20-μL aliquot of sample: an absorbance of ~0.8 indicate no estrogenic activity, whereas an absorbance value above this (up to a maximum of 2.8) indicates estrogenic activity. In each case, the activity was eluted from the column using 50–85% methanol. For comparison, the results obtained with various concentrations of 17β-estradiol (the positive control) are shown.

duced in volume using a gentle stream of nitrogen to 150 μL. The fractions eluting immediately before and after the active fractions (bracketing fractions) were prepared in the same way.

Analysis of Active Fractions by GC–MS. All the samples as well as the full procedural blank and reference standards were analyzed in triplicate by GC–MS using a Finnigan MAT Magnum ion trap mass spectrometer run in E1 mode at 70 eV with a Varian GC system and Finnigan MAT A200S autosampler. The ions selected for quantitation were those that produced the greatest signal-to-noise ratio. These were 213 270 (estrone), 213 272 (17β-estradiol), 215 274 (d_2-17β-estradiol internal standard) and 213, 296 (17α-ethynylestradiol).

Determination of Extraction Efficiencies and Detection Limits. A 20-L tap water sample was spiked with 200 ng of 17β-estradiol, estrone, 17α-ethynylestradiol, and d_2-17β-estradiol, so that a final concentration of 10 ng/L of each steroid was present in the sample. This sample was extracted as before using two preconditioned 5-g C18 cartridges (arranged in series), which were then rinsed with 40 mL of 25% methanol solution (discarded), followed by 2 × 50 mL of 85% methanol solution. The 85% methanol eluate was diluted in 1 L of Nanopure water and reconcentrated using a preconditioned 5-g C18 cartridge. The cartridge was eluted using 20 mL of 100% methanol, which was blown down to a final volume of 1.5 mL. Half of this sample was fractionated by semipreparative scale HPLC, and the resulting fractions were tested for estrogenicity. Active fractions were combined, diluted in water, and liquid/liquid extracted using DCM (3 × 25 mL of DCM). The extracts were dried and concentrated to 150 μL before analysis by GC–MS.

Results

Preliminary Findings. During initial studies, effluent was filtered through glass wool to remove particulates, purged with nitrogen to remove volatiles, and filtered and purged together. The same procedure was performed on the procedural blank (distilled deionized water). The 'whole' effluent sample was found to be estrogenic (results not shown).

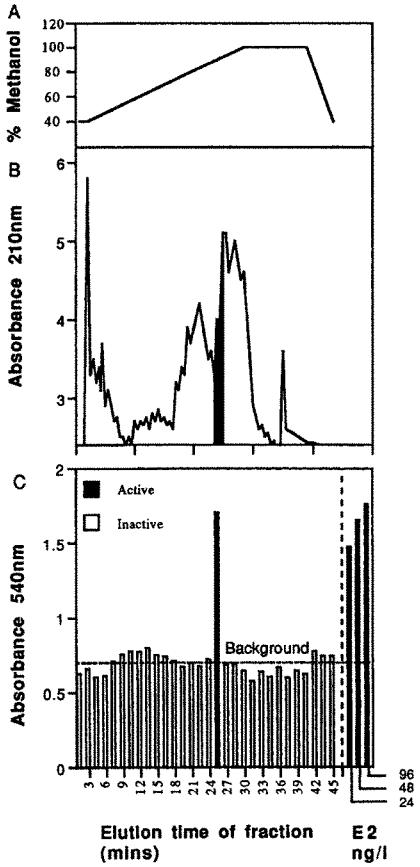

FIGURE 3. Fine fractionation of Southend effluent using reverse-phase HPLC: (A) methanol gradient, (B) UV absorbance profile, and (C) estrogenic activity in the yeast screen following incubation of a 10-μL aliquot of each HPLC fraction. See legend for Figure 2 for a fuller explanation of the results obtained from the yeast-based estrogen screen. The figure shows that the estrogenic activity appeared as a single fraction, which was eluted between 24 and 25.5 min after injection of the sample. The shaded section on the UV profile depicts the position where the activity was eluted.

Samples of effluent were filtered using glass fiber filters, and the material removed by the filters was extracted sequentially using a series of solvents of increasing polarity. These extracts and the filtered effluent were assessed for estrogenic activity in the yeast screen. The biological activity was not removed by filtering (results not shown), and the extracts of the particulate matter were also devoid of estrogenic activity (results not shown). Therefore, the estrogenic component(s) were not retained by the filters but were present in the dissolved phase of the effluent samples. Furthermore, the biological activity was not removed by purging, indicating that the estrogenic component was

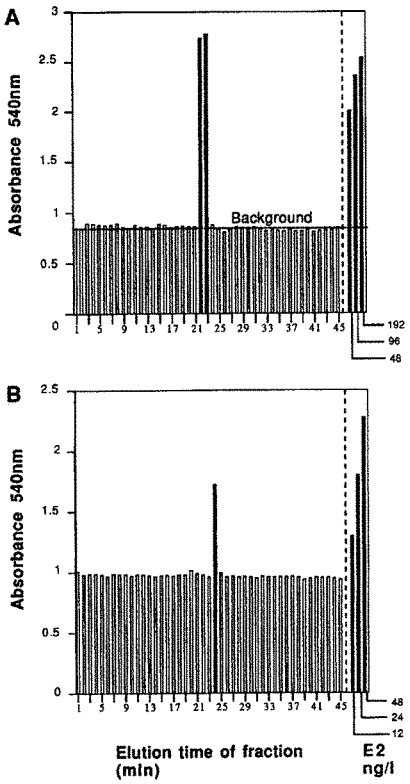

FIGURE 4. Estrogenic activity profiles produced from domestic effluents following fine fractionation using reverse-phase HPLC. These figures depict the color change of the medium following incubation with a 10-μL aliquot of each fraction in the yeast screen: (A) Naburn STW effluent and (B) Harpenden STW effluent. In each case, the activity occurred over 1−2 fractions, which were eluted between 22 and 24 min following injection of the sample. See legend for Figure 2 for a fuller explanation of the results.

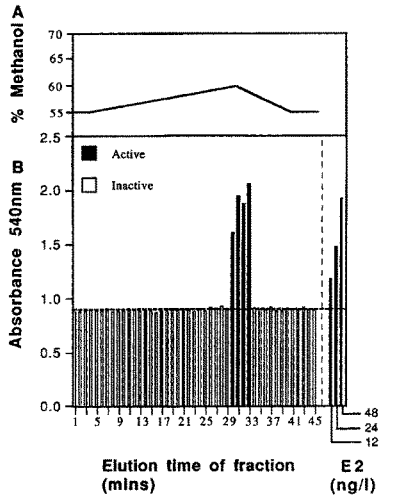

FIGURE 5. Further separation of the active fractions produced previously during the fine fractionation of Southend STW effluent using reverse-phase HPLC: (A) methanol gradient and (B) estrogenic activity in the yeast screen following incubation of a 10-μL aliquot of each HPLC fraction. The figure shows that the estrogenic activity, previously in a single fraction, was resolved into at least two peaks of activity occurring over four fractions eluting between 30 and 33 min after injection of the sample. See legend for Figure 2 for a fuller explanation of the results.

unlikely to be volatile (results not shown).

Passing the filtered effluent through a SPE cartridge caused a >80% reduction in estrogenic activity (results not shown). Since most of the activity was retained on the cartridge, it appeared that the active compounds were not ionic substances, as these would have passed through the C18 column and produced a positive estrogenic response in the cartridge eluent. These results indicated that most of the estrogenic activity contained in the effluent was due to organic substances that bound to the C18 phase of the SPE cartridge. The details of these developmental stages, which determined the full direction of the fractionation procedures used subsequently on 20-L effluent samples, are published in a Government report (*15*).

Results of C18 Fractionation. Figure 2 illustrates a typical C18 elution profile produced when the compounds retained on the column were sequentially eluted using solvents of decreasing polarity. The figure shows that the majority of the estrogenic activity was eluted from the column between 50% and 85% methanol.

Estrogenic Activity of HPLC Fractions from Southend STW Effluent. Figure 3 depicts the results of the reversed-phase HPLC fractionation of Southend STW effluent. Figure 3C illustrates that the estrogenic activity occurred as a single peak (which eluted between 24.0 and 25.5 min) and was equivalent in activity to approximately 90 ng of E2/L. This suggested that the activity was due to a single compound or a set of closely related compounds that were eluted concurrently using the HPLC conditions employed. The UV profile of the chromatographic run (Figure 3B) indicated that the sample loaded remained a highly complex mixture and reverse-phase HPLC, at this stage, was unable to resolve the mixture into fractions composed of a few identifiable compounds. It was therefore impossible to determine whether the activity within the fraction was due to a highly abundant component or whether it was due to minor components concealed within the UV profile.

The estrogenic components present in the HPLC fraction were found to be liquid/liquid extractable into dichloromethane (result not shown), enabling their identification by GC−MS. Therefore dichloromethane (DCM) extracts of the active fraction as well as the bracketing fractions were prepared and analyzed separately using the ion-trap GC system. The major components of the extracts were found to be isomers of α-terpineol, a terpenoid alcohol widely used in detergents and cosmetics, which has been identified previously in sewage effluent (*16*). There were also around 20 unidentified minor components that occurred in all three extracts, but the highest concentration of α-terpineols

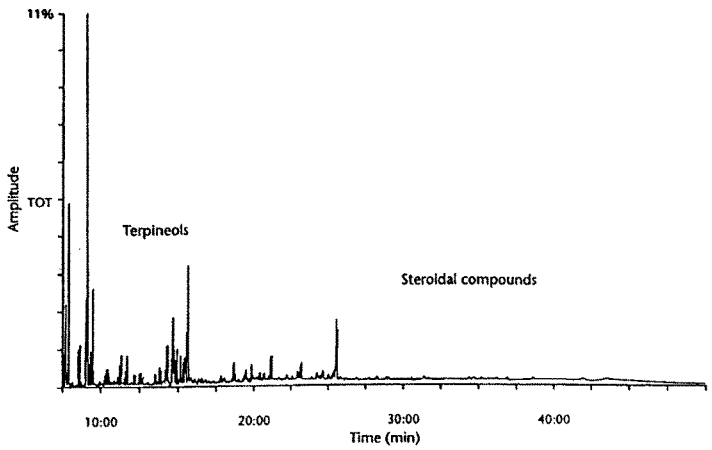

FIGURE 6. Total ion chromatogram of the estrogenic fraction from Southend STW. The profile shows that the fraction contained mainly terpineols, with minor components tentatively identified as steroids.

occurred in the active fraction. Standards of the identified terpineol, of both pure and technical (an isomeric mixture) grade, were purchased (Aldrich, Sigma, Fluka), and solutions spanning the concentration range present in the fraction were used to confirm the identifications by GC–MS and to test for estrogenic activity in the yeast screen. None of the terpineols tested were found to be active in the yeast screen. An additional fractionation step was therefore considered necessary to reduce the sample complexity further.

Estrogenic Activity of HPLC Fractions from Other Domestic Effluent Samples. Figure 4 depicts the reverse-phase HPLC profiles of two of the other effluents investigated using the fractionation conditions described previously. In contrast to Southend STW effluent, samples were collected at 1-min intervals to produce 45 5-mL fractions. In all cases, the HPLC profiles were similar, and the findings were consistent with the Southend STW effluent result. That is, the estrogenic activity appeared as a single fraction that eluted between 22 and 24 min after the sample injection. Small shifts in the retention time of the active fraction may be explained by differences in the composition (in terms of both the chemicals present and their quantity) of the extracts, which altered the chromatographic resolution of the column.

Further HPLC Separation of Southend STW Effluent Using a Shallow Gradient. Following the elimination of the terpineols as the estrogenic components in the active fraction, it was necessary to focus on the large number of potentially active trace compounds. An additional fractionation step was developed in order to reduce the sample complexity further.

Using the shallow elution gradient, the active fraction from the first HPLC run was resolved into two separate peaks of estrogenic activity, eluting between 29 and 31 min and between 31 and 33 min, respectively (Figure 5). A 50-μL aliquot of each active fraction was tested, producing an average response equivalent to between 25 and 50 ng of 17β-estradiol/L in the yeast screen (Figure 5). At this stage, it was also noted that the active fraction behaved in the yeast screen in a manner similar to steroid standards, in which the color development occurred rapidly as compared to the many xenoestrogens we have tested. On the basis of this observation, it was postulated that the estrogenic component could be a steroidal compound. As steroidal estrogens are very potent compounds, it was possible that the effects observed were caused by extremely low levels of these compounds in the effluent that were difficult to detect. Thus, it was at this stage in the developmental process that the methodology was up-scaled to handle larger volumes (20 L) of effluent in order to ascertain whether steroidal estrogens were present.

Behavior of Steroids in the Fine Fractionation Procedure. The behavior of steroids in the fractionation system was evaluated using the synthetic estrogen 17α-ethynylestradiol (EE2). If steroids (or steroid-like compounds) were responsible for the activity in the effluent, we expected their chromatographic retention time to coincide with that of the active fraction. Therefore, in separate chromatographic runs, 20 ng of EE2, procedural blanks, and the active effluent fraction were injected onto the HPLC column, and fractions were collected at 1-min intervals using both gradients (40–100% methanol and 55–60% methanol). In the 40–100% gradient run, the EE2 standard coeluted with the estrogenic activity of the effluent sample. In the 55–60% gradient run, the EE2 standard coincided with the second peak of estrogenic activity in the effluent sample. These results indicated that estrogenic steroids may be responsible for the estrogenic activity in domestic STW effluent. In both cases, the blanks were inactive, and a range of alkylphenolic standards did not coelute with the active fraction (result not shown).

Identification of Estrogenic Chemicals in Effluent by GC–MS. Figure 6 illustrates the mass chromatogram of the active fraction from Southend STW effluent. The standard GC–MS library searching routine identified potent natural and synthetic steroidal estrogens in the extract, namely, estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2). This result was confirmed using a standard solution containing E1, E2 and EE2, which was analyzed under the same GC–MS conditions. The elution times of the standard compounds coincided with retention time of the peaks identified in the effluent extract, as is shown in Figure 7A,B.

Extraction Efficiency and Detection Limits. The extraction efficiencies for estrone, 17β-estradiol, d_2-17β-estradiol, and 17α-ethynylestradiol following liquid/liquid extraction

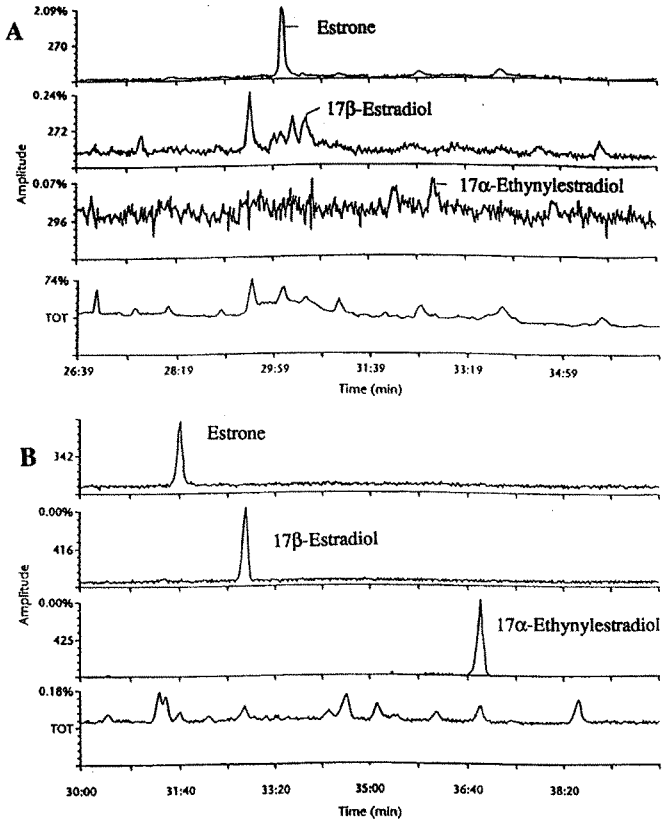

FIGURE 7. Gas chromatograms comparing the retention times of the minor components (present in the 30–40 min range of Figure 6) of the estrogenic fraction from two STW effluents (A and B) with those of standards of natural and synthetic steroidal estrogens. The traces show that in both cases the retention times of the minor components (bottom traces), tentatively identified as steroids by GC–MS, correspond chromatographically to estrone, 17β-estradiol, and 17α-ethynylestradiol standards (top three traces) under both derivatized and underivatized conditions and were unambiguously identified by MS library searching routines: (A) underivatized extract from Naburn STW collected in December 1995 with low 17α-ethynylestradiol concentrations (B) derivatized sample from Southend STW collected in May 1995 with higher 17α-ethynylestradiol concentrations.

into dichloromethane were 85.4%, 83.3%, 88.6%, and 78.8%, respectively. Detection limits varied, depending on the performance of the GC–MS and the quality of the sample, but for a 20-L sample were generally around 0.2 ng/L in the original effluent sample. Potential improvements to the GC–MS method, by creating silyl derivatives of the sterols (less polar compounds) which were more amenable to gas chromatography, resulted in an improved peak shape for the steroids. However, this advantage was largely offset by a loss of sample during the additional manipulation.

Concentration of 17β-Estradiol, Estrone, and 17α-Ethynylestradiol in Domestic Effluent Samples. The results from the effluent fractionations are listed in Table 2. The results indicate that estrone and 17β-estradiol were present in the effluents at concentrations ranging from 1 ng/L up to 80 and 50 ng/L, respectively. 17α-Ethynylestradiol was not detected in two-thirds of the samples collected, but was detected in effluent collected from Southend STW (up to 7 ng of EE2/L) and in other effluents, for example, from Naburn STW (up to 4.3 ng of EE2/L), that contained a higher steroid load than some of the other effluents.

Discussion

In this project the TIE approach, modified for our specific needs, was used to identify the estrogenic components in domestic STW effluent. The scale of the problem of isolating causal compounds can be gauged from reported estimates that 63 000 chemicals are in common use worldwide, of which 3000 account for 90% of the total global production. In addition, anywhere between 200 and 1000 new synthetic

TABLE 2. Concentrations (ng/L) of Natural and Synthetic Steroidal Estrogens Detected in Domestic STW Effluents Discharging into British Waters[a]

site	date	estrone	17β-estradiol	17α-ethynylestradiol
Southend STW	15/5/95	48.0 ± 1.3	48.0 ± 6.0	7.0 ± 3.7
	17/5/95	45.0 ± 2.5	42.0 ± 1.4	nd
	22/5/95	32.0 ± 1.2	29.0 ± 1.2	nd
Harpenden STW	17/7/95	5.2 ± 0.6	3.7 ± 0.6	nd
	24/7/95	8.5 ± 0.6	7.1 ± 1.0	nd
	1/8/95	8.9 ± 0.8	4.4 ± 0.5	nd
Rye Meads STW	17/7/95	3.6 ± 0.4	2.7 ± 0.1	nd
	24/7/95	1.8 ± 0.5	5.5 ± 0.5	nd
	1/8/95	2.1 ± 0.4	6.3 ± 0.2	nd
Deephams STW	17/7/95	13.0 ± 4.6	12.0 ± 2.6	nd
	24/7/95	2.0 ± 0.05	4.9 ± 0.4	nd
	1/8/95	9.4 ± 0.9	4.3 ± 0.5	nd
Naburn STW	28/11/95	76.0 ± 10.3	10.0 ± 1.6	4.3 ± 0.5
	4/12/95	15.0 ± 1.0	6.5 ± 1.2	0.6 ± 0.2
	16/1/96	48.0 ± 2.9	9.8 ± 1.0	1.9 ± 0.2
Horsham STW	30/11/95	6.1 ± 0.6	4.9 ± 0.4	0.2 ± 0.1
	4/12/95	10.0 ± 0.3	5.7 ± 0.7	0.6 ± 0.1
	15/1/96	12.0 ± 0.5	4.0 + 0.4	0.8 ± 0.1
Billing STW	29/11/95	6.4 ± 0.5	6.1 ± 0.04	nd
	7/12/95	1.4 ± 0.15	7.4 ± 0.6	nd
	11/1/96	9.9 ± 1.2	6.9 ± 0.9	nd

[a] nd, not detected. Quantitation was based on *m/z* 213 270 (estrone), 213 272 (estradiol), 215 274 (d_2-17β-estradiol internal standard) and 213 296 (17α-ethynylestradiol). Each value represents the mean and standard deviation of three replicate injections.

chemicals enter the market each year (*17*). Despite the complex composition of the effluents tested, only natural and synthetic steroidal estrogens were identified as candidate compounds responsible for the estrogenic activity observed in STW effluents with principally domestic inputs. This conjecture was supported by the very low concentrations of alkylphenolic compounds (NP and OP) measured in the effluents studied (*15*), which were generally less than 1 μg/L.

As the effluents tested contained little or no agricultural input (they came from STWs located in urban areas), we assume that the natural and synthetic steroidal estrogens detected were human in origin. Hepatic metabolism of natural steroidal estrogens occurs by 2-hydroxylation and 16α-hydroxylation pathways (*18*). For example, 16α-hydroxylation of 17β-estradiol (the main natural estrogen) eventually leads to the production of estriol, which was reported to be over 300-fold less active than E2 in vitro (*19*). Studies on the estrogen profile of urine samples indicate that women can excrete around 7 μg of estrone, 2.4 μg of 17β-estradiol, and 4.6 μg of estriol per day (*20*). Moreover, approximately 0.5 μg of estrone, 0.4 μg of 17β-estradiol and 1.25 μg of estriol is eliminated in the feces per day (*18*). Natural steroids are eliminated in the urine as inactive glucuronide or sulfated conjugates. In contrast, fecal elimination of steroids is reported to occur mainly as unconjugated forms (*21*), because the gut contains high levels of the bacteria *Escherichia coli*, which are able to deconjugate steroid metabolites due to their capacity to synthesize large quantities of the enzyme β-glucuronidase (*22*). As feces contain high levels of *E. coli*, it was postulated that STWs must also contain a large population of the bacteria, actively producing β-glucuronidase. Therefore it is possible that conjugated natural and synthetic steroidal estrogens eliminated from the body are deconjugated during the sewage-treatment process into the parent compounds, which we detected in the effluent, due to their restored biological activity. It is also interesting to note that the ratio of the levels of estrone to estradiol reported in urine (3.5 parts E1:1 part E2) is fairly similar to the ratio observed in the effluent (1.5 parts E1:1 part E2). Estriol (E3) was not detected among the minor components present in the active fraction, despite the fact that it was reported to be excreted in about the same quantity as 17β-estradiol. However, based on its potency in vitro, a concentration of 11 ng of E3/L (the mean concentration of E2 found in the effluents) would be equivalent in biological activity to approximately 0.03 ng of E2/L. On the basis of this calculation, if E3 was present, we would not have detected it, and it was unlikely to have contributed significantly to the estrogenic activity of the effluents. Moreover, E3 may not have been present in the same HPLC fraction as the other active steroidal compounds.

17α-Ethynylestradiol (EE2), the main estrogenic component of the combined oral contraceptive pill, was also detected in some of the effluent samples. The oral contraceptive pill contains between 30 and 50 μg of EE2 per pill (*23*). In excretion studies, EE2 was identified as the principal component of the glucuronide and arylsulfate fraction of bile and urine (*24*). As a large proportion of the EE2 ingested was excreted as unmetabolized glucuronide conjugates (*24*), exposure to β-glucuronidase activity during the STW process may also release the biologically active form of EE2 into the aquatic environment. Reports from laboratory biodegradation studies indicated that EE2 was highly stable and persistent in activated sludge, with no detectable degradation occurring after 120 h of treatment as compared with gestagens, which were completely metabolized within 48 h of treatment (*23*). The relationship between sewage-treatment process and steroid concentration could not be accurately determined from this study as a real assessment would require measurements of concentrations in the influent and the effluent. However, there were indications that the better effluent treatments resulted in lower steroid concentrations in the effluent. The solubility of EE2 in pure water and sewage-treatment water was reported to be 4.2 and 4.7 mg/L, respectively, which was 3-fold less soluble than natural steroidal estrogens (*23, 25*). This fact is believed to contribute to the increased resistance of EE2 to biodegradation as compared with natural steroidal estrogens. Based on the limited data set in Table 2 it appears that, when EE2 was detectable, the ratio of E2 to EE2 was 9:1. Therefore, the mean concentration of EE2 was probably 0.6 ng/L, with a median concentration around half this value. These values correspond well with measurements taken in The Netherlands, which reported concentrations in river water of 0.3 ng of EE2/L, with concentrations 5-fold lower in drinking water (*26*). These concentrations of EE2 were at or just below the

best detection limits observed during the effluent fractionation method, indicating that, although EE2 was not always detected, it may still be present in the other effluent samples.

In the last two decades, a number of studies have reported the presence of natural and/or synthetic steroidal estrogens in sewage-treatment effluent, river water, or drinking water samples. In these studies, the objective of the research was to determine whether natural and synthetic steroids were present in the aquatic environment. In the present study, the objective was to identify the estrogenic components in effluent, without any preconceived ideas of their identity; they were found to be natural and synthetic steroidal estrogens. In 1970, Tabak and associates reported concentrations of 2000 ng of EE2/L, 25 ng of E1/L, 60 ng of E3/L, and 10 ng of E2/L (parent compounds plus conjugates) in STW effluents from the United States. Low levels (between 0.1 and 3.0 ng/L) of natural and synthetic estrogens were also reported in drinking water in southern Germany (*27*). In a British study, using an immunoassay procedure, EE2 was tentatively detected in STW effluent, river water, and potable water at concentrations in the low nanogram per liter range and below (*28*). Natural steroidal estrogens were detected in raw sewage from the Tel Aviv area (Israel) using a radioimmunoassay procedure at concentrations from 48 to 141 ng/L (depending on drought conditions) and in storage water (used for drinking) taken from Lake Kinneret (Northern Israel) at concentrations between 14 and 22 ng/L. Moreover, estrogen concentrations in treated sewage water (used for irrigation) discharged from small farm-based sewage-treatment units and municipal STWs in Israel were reported to be between 153 and 39 ng/L, respectively, with levels 2–3-fold higher in the summer months (*29*). In a more recent German study (employing sophisticated modern techniques), concentrations of up to 20 ng of E2/L and up to 62 ng of EE2/L were reported in effluents from STW plants, and EE2 was also occasionally detected at levels below 5 ng/L in river water (*30*). However, unlike the present study, the steroidal extraction procedures employed by Tabak (1970), Rurainski (1977), Shore (1993), and Stumpf (1996) included a pH adjustment step that may deconjugate the steroids present in the water samples (*25, 27, 29, 30*). Consequently, the figures derived from these studies may depict the total combined concentration of free and conjugated hormones and not necessarily the environmental concentration of unconjugated (active) steroids present in the samples.

In summary, in this study, the TIE approach was used to fractionate domestic effluent into samples of decreasing complexity, which were subsequently assessed for estrogenic activity in vitro. In all the effluents tested, the most active fraction (>80% total activity in domestic effluent) was found to contain low levels of natural and synthetic steroidal estrogens. This supports the earlier suggestions by Purdom et al. that the estrogenic activity was likely to be due to a common contaminant(s) and source (*1*). The results presented in Table 2 indicate that the concentrations of EE2 detected in the samples were generally too low to fully account for the magnitude of the vitellogenin response observed when male fish were exposed to the effluent (*31*). For example, in laboratory tank trials, exposure of male rainbow trout to 10 ng of EE2/L was required to produce a response of a similar magnitude to those observed following exposure to STW effluents (*1*). However, very little data address the issue of sensitivity of fish exposed to natural estrogens via the water. Hence, it was not possible to conclude whether the concentrations of E2 and E1 reported in this study would or would not be estrogenic to fish. Moreover, little, if any, information is available on whether different species, sexes, or life stages of fish differ in their sensitivity to estrogens. To address some of these issues and thus put the results obtained from the fractionation studies into an environmental context, in vivo laboratory tank trials were conducted in which rainbow trout and roach were exposed to low concentrations of estrogenic chemicals via the water. Therefore, as the second part to this project, in vivo tank trials were conducted in which trout (males) and roach (males and females) were exposed to environmentally relevant concentrations of E2 and E1 (*32*) to determine if the concentrations reported in this study were able to induce vitellogenin synthesis.

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ORIGINAL ARTICLE

A mixture of seven antiandrogens induces reproductive malformations in rats

Cynthia V. Rider,*†[1] Johnathan Furr,† Vickie S. Wilson† and L. Earl Gray Jr†[1]

*Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, and †Endocrinology Branch, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, North Carolina, NC, USA

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Correspondence:
L. Earl Gray, Jr, Endocrinology Branch, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, MD-72, Research Triangle Park, North Carolina, NC 27711, USA.
E-mail: gray.earl@epa.gov

[1]Both authors contributed equally to this work.

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Summary

To date, regulatory agencies have not considered conducting cumulative risk assessments for mixtures of chemicals with diverse mechanisms of toxicity because it is assumed that the chemicals will act independently and the individual chemical doses are not additive. However, this assumption is not supported by new research addressing the joint effects of chemicals that disrupt reproductive tract development in the male rat by disrupting the androgen signalling pathway via diverse mechanisms of toxicity [i.e. androgen receptor (AR) antagonism in the reproductive tract vs. inhibition of androgen synthesis in the foetal testis]. In this study, pregnant rats were exposed to four dilutions of a mixture containing vinclozolin, procymidone, linuron, prochloraz, benzyl butyl phthalate, dibutyl phthalate and diethylhexyl phthalate during the period of sexual differentiation and male offspring were assessed for effects on hormone sensitive endpoints including: anogenital distance, infant areolae retention and reproductive tract tissue weights and malformations. The ratio of the chemicals in the mixture was based upon each chemical's ED_{50} for inducing reproductive tract malformations (hypospadias or epididymal agenesis). The observed responses from the mixture were compared with predicted responses generated with a toxic equivalency approach and models of dose addition, response addition or integrated addition. As hypothesized, we found that the mixture of chemicals that alter the androgen signalling pathway via diverse mechanisms disrupted male rat reproductive tract differentiation and induced malformations in a cumulative, dose-additive manner. The toxic equivalency and dose addition models provided the best fit to observed responses even though the chemicals do not act via a common cellular mechanism of action. The current regulatory framework for conducting cumulative risk assessments needs to consider the results, including those presented herein, which indicate that chemicals that disrupt foetal tissues during sexual differentiation act in a cumulative, dose-additive manner irrespective of the specific cellular mechanism of toxicity.

Introduction

Environmental chemicals have been implicated in the increasing occurrence of male reproductive dysfunction over the past 50 years (Skakkebaek *et al.*, 2001; Damgaard *et al.*, 2002; Swan *et al.*, 2003, 2005). Strong evidence from animal studies supports the link between in utero exposure to endocrine disrupting compounds (EDCs) and abnormal development of the male reproductive tract (Foster, 2006; Gray *et al.*, 2006). Until recently, toxicological studies and risk assessment primarily focused on the effects of individual chemicals. However, humans, fish and wildlife are exposed to a complex milieu of chemicals from sources including food and water, personal care products, occupation and the environment (Kolpin *et al.*, 2002; Squillace *et al.*, 2002; Silva *et al.*, 2004; Wolff *et al.*, 2007). Mixtures of EDCs have the potential to interact with hormone signalling pathways and elicit dose additive, synergistic, or antagonistic effects that differ from those resulting from individual chemical exposures.

Developing a scientific framework to assess these mixtures is fundamental to protecting humans, fish and wildlife from risk associated with exposure to mixtures.

A structurally diverse array of compounds has the potential to disrupt the androgen signalling pathway via multiple mechanisms (Kelce *et al.*, 1994; Ostby *et al.*, 1999; Parks *et al.*, 2000; Gray *et al.*, 2001; Mylchreest *et al.*, 2002; Hotchkiss *et al.*, 2004; Wilson *et al.*, 2004) and there is mounting evidence that mixtures of endocrine active chemicals display cumulative, dose additive or synergistic effects even though they do not act via a common mechanism of toxicity. Previously, most of this research focused on oestrogenic mixtures. In vitro studies as well as in vivo studies have found that mixtures of xenoestrogens elicit additive mixture responses that conform to a dose addition model (Payne *et al.*, 2000; Rajapakse *et al.*, 2002; Silva *et al.*, 2002). It has been reported that androgen receptor (AR) antagonists display additive toxicity in vitro (Nellemann *et al.*, 2003) and when administered in utero or to young adult male rats (Gray *et al.*, 2001; Nellemann *et al.*, 2003). Birkhoj *et al.* (2004) assessed a more complicated mixture containing five pesticides and found that they displayed dose additive inhibition of AR transcriptional activation. Recently, we found that in utero exposure to a binary combination of diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP), two chemicals that inhibit foetal testis hormone production including testosterone, elicited additive effects on differentiation of the male rat reproductive tract causing hypospadias, epididymal agenesis and undescended testes (Howdeshell *et al.*, 2007).

Cumulative effects, however, are not limited to only chemicals with common mechanisms of toxicity. Two in utero studies with mixtures of pairs of chemicals with different mechanisms of action found cumulative, dose additive effects of the mixtures. For example, mixtures of benzyl butyl phthalate (BBP) and linuron (Hotchkiss *et al.*, 2004), or procymidone and DBP (Gray *et al.*, in prep) induced hypospadias in a dose-additive manner (study 1: procymidone = 1.5% hypospadias, DBP = 0% and DBP plus procymidone = 49%; study 2: BBP = 0%, linuron = 0% and linuron plus BBP = 56%). Taken together, these results indicate that the phthalates and pesticides did not interact independently of one another in a response additive manner but rather they behaved as if they acted via a common mechanism of toxicity in a dose-additive manner. The responses were dose additive even though the individual chemicals act directly on different foetal tissues (the testis vs. the tract) at different points in the androgen signalling pathway (steroid hormone synthesis vs. AR antagonism).

Currently, the concepts of independent action (also known as response addition) and dose addition are used to describe the action of chemical mixtures (Cassee *et al.*, 1998). Dose addition has been validated extensively for use with mixtures of chemicals that have the same mechanism of toxicity (Könemann, 1981; Berenbaum, 1985; Altenburger *et al.*, 2000). According to the dose addition model, chemical doses are added together after first accounting for their individual potencies. The overall mixture response is then based on this total mixture dose. The toxic equivalency approach is a subcategory of dose addition often associated with dioxin-like compounds (Safe, 1990). The toxic equivalency approach utilizes relative potency factors, calculated by dividing the ED_{50} of a reference compound by the ED_{50} of each chemical in the mixture, to put all chemical doses in terms of a reference chemical. After adding these doses, the total expected mixture response is inferred from the reference chemical dose-response curve. Alternatively, response addition has been applied with varying success to mixtures containing chemicals with different mechanisms of toxicity (Hermens & Leeuwangh, 1982; Hermens *et al.*, 1984; Broderius *et al.*, 1995; Backhaus *et al.*, 2000; Faust *et al.*, 2003). According to response addition, the mixture response is calculated by derivation of the sum of the responses elicited by each of the mixture components. Recently, integrated addition, which combines dose and response addition concepts into a single model, has been successfully used to calculate the cumulative toxicity of complex mixtures containing components with both same and different mechanisms of toxicity (Teuschler *et al.*, 2004; Altenburger *et al.*, 2005; Olmstead & LeBlanc, 2005).

There are major differences in predictions based upon response addition and dose addition that have significant implications for risk assessments. The response addition model predicts that exposure to several chemicals at once will not produce any adverse effects if each chemical is administered at a dose level at or below the dose that produces no effect. In contrast, predictions based upon the assumption of dose addition indicate that exposure to combinations of several chemicals at once can cause adverse responses even though the chemicals are administered at or below their no observed adverse effect levels (NOAELs) (Silva *et al.*, 2002; Gray *et al.*, 2006). Based upon the fact that the phthalate DBP does not bind AR and procymidone does not inhibit foetal testosterone synthesis, a mixture of DBP and procymidone would be expected to induce a 1.5% incidence of hypospadias if they truly acted as predicted by response addition modelling. Instead, the mixture group displayed a 49% incidence of hypospadias (Gray, in prep), a level predicted with dose addition modelling.

Given the above cited failure of dissimilar chemicals in the two phthalate-pesticide mixture studies to behave independently as expected, having produced malformation

rates that were consistent with dose addition rather than response addition or integrated addition, the current study was designed to examine if dose addition models could predict the in utero effects of a larger number (seven) of diverse chemicals. The generally accepted concept is that these seven chemicals would act independently and produce effects that could be predicted by either response or integrated addition models. However, based upon our two studies, discussed above, we hypothesized that response and integrated addition models would grossly underestimate the malformations induced by the mixture and that dose addition models would provide a more accurate assessment of the developmental toxicity of the mixture.

In this study, we used individual chemical dose-response data (Fig. 1) in mixture toxicity models of toxic equivalency, dose addition, response addition and integrated addition. We then compared our modelled predictions to data generated from in utero exposure of rats to a seven chemical mixture composed of vinclozolin, procymidone, linuron, prochloraz and the phthalates: BBP, DBP and DEHP.

Although we refer to all seven chemicals in the study as 'antiandrogens', we are using the term broadly to include any chemical that disrupts the foetal androgen signalling pathway, as opposed to referring only to AR antagonists.

The pesticides vinclozolin, procymidone, linuron and prochloraz are all AR antagonists which act directly on the foetal reproductive tissues by inhibiting the masculinizing action of androgens within the tissues directly; whereas, the phthalates do not act in this manner at relevant concentrations. The three phthalates and the pesticides linuron and prochloraz act on the foetal testis by different molecular mechanisms to inhibit hormone production. Linuron inhibits both androgen and progestin synthesis directly in the Leydig cells (Wilson *et al.*, 2004); whereas, prochloraz blocks the conversion of progestins to androgens (Blystone *et al.*, 2007). In contrast, it is generally agreed that the phthalates do not inhibit steroidogenesis directly at relevant concentrations but rather they

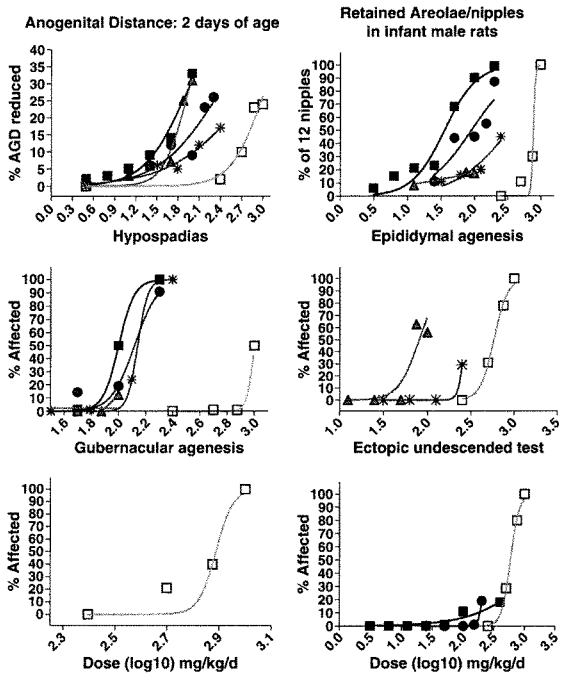

Figure 1 Logistic regression models of the data from the individual chemicals used in this study. The ED_{50}s and slopes from these analyses were used to generate the relative potency factors for the toxic equivalency analysis and to generate predicted effects with the dose, response and integrated addition models. The three phthalates used in this study were considered to be equipotent for modelling purposes, an approach supported by several studies on each of these chemicals. Dibutyl phthalate and diethylhexyl phthalate data were used for the models shown.

disrupt foetal Leydig cell migration, maturation, proliferation and distribution with the testis (Parks *et al.*, 2000; Barlow *et al.*, 2004; Mahood *et al.*, 2006) resulting in inhibition of insulin-like-3 (insl3) and androgen levels (Wilson *et al.*, 2004). As a consequence of these multiple and diverse mechanisms of toxicity the phenotypic profiles of the seven chemicals used in the mixture fall into four overlapping syndromes [(i) vinclozolin and procymidone, (ii) prochloraz, (iii) linuron and (iv) the three phthalates]. While there are common effects among them (i.e. AGD, infant and permanent nipples, reduced sex accessory tissue weights and hypospadias, for example) there also are some significant differences in the types of effects they produce (for example, only phthalates cause gubernacular agenesis).

The main objectives of this study were to: (i) determine whether dissimilar 'antiandrogenic' compounds induced male reproductive tract malformations in a dose additive effects manner when present in combination and (ii) to assess the ability of modelling approaches to accurately predict the effects of the mixture based on data from single chemical exposures. Results of this study demonstrate that toxic equivalency or dose addition, not response addition or integrated addition are more appropriate models for predicting the effects of this group of 'antiandrogenic' chemicals on the development of male rat reproductive tract malformations.

Materials and methods

Animals

Timed pregnant Sprague-Dawley rats were purchased from Charles River Breeding Laboratory (Raleigh, NC, USA) on gestation day (GD) 2. Animals were housed individually in clear polycarbonate cages (20 cm × 25 cm × 47 cm) with a bedding of heat-treated laboratory-grade pine shavings (Northeastern Products, Warrensburg, NY, USA). Animals were fed Purina Rat Chow 5008 (pregnant and lactating females) or Purina Rat Chow 5001 (weanling and adult rats) and provided with access to filtered municipal drinking water (Durham, NC, USA) ad libitum. This study was conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee.

Treatment and chemicals

Our goal in dose selection was to use doses of each chemical that would contribute equally to the induction of malformations by the mixture. The mixture of each chemical at 1/7th of its respective ED_{100} for inducing reproductive tract malformations [15 mg/kg/day vinclozolin (Riedel-de-Haen, Seelze, Germany), 15 mg/kg/day procymidone (ChemServices, West Chester, PA, USA), 35 mg/kg/day prochloraz (Riedel-de-Haen), 20 mg/kg/day linuron (Crescent Chemical Company, Islandia, NY, USA) and 150 mg/kg/day each of benzyl n-butyl phthalate (BBP; Aldrich Chemical Company, Milwaukee, WI, USA), (DBP; Sigma, St Louis, MO, USA) and DEHP (Sigma)] represented the high dose. Pregnant rats were dosed with the vehicle (corn oil at 2.5 mL/kg; Sigma; n=3), 25% (n=2), 50% (n=3), 75% (n=2) or 100% (n=2) of the high dose by oral gavage on GD14-18. The two lowest dose groups contained individual chemicals at or below their NOAELs for inducing male reproductive tract malformations.

Male offspring assessment

On postnatal day (PND) 2, all pups were sexed, weighed and anogenital distances (AGD) were measured with a dissecting microscope with an ocular micrometer (Hotchkiss *et al.*, 2004) by an observer blinded to treatment group. At PND 14, each male rat was examined in a blinded manner for areolae or nipples. At 175–195 days of age, all male rats were anaesthetized with CO_2, decapitated and examined for reproductive tract alterations. The ventral surface of each male rat was shaved and examined for the presence of nipples. Rats were then assessed for abnormalities including cleft phallus, hypospadias, epididymal agenesis, testicular atrophy, undescended testes, fluid filled testes, ventral prostate or seminal vesicle agenesis and malformations of the gubernacular cords (elongated or absent). Weights were taken on body, glans penis, ventral prostate, seminal vesicles, testes, epididymis and levator ani bulbocavernous muscle (LABC).

Mixture modelling

Methods used to model mixture effects have been described in detail previously (Howdeshell *et al.*, 2007; Olmstead & LeBlanc, 2005; Rider & LeBlanc, 2005). Software for predicting mixture responses using the models described below is available on-line at: http://www.ncsu.edu/project/toxresearch/model5/.

Individual chemical assessment

The mixture modelling was based upon historical data (for details see Gray *et al.*, 1994, 1999a,b; Hotchkiss *et al.*, 2004; McIntyre *et al.*, 2000; Noriega *et al.*, 2005; Ostby *et al.*, 1999) and new unpublished data from our laboratory (Fig. 1). Each of the aforementioned studies included exposure to the chemicals during sexual differentiation

and provided a comprehensive assessment of the postnatal development of the male offspring through adulthood. Dose-response relationships were characterized for every chemical and each reproductive endpoint and the data were fit with a sigmoidal line using a four parameter logistic equation. The ED_{50}s and slopes generated in the individual chemical analyses were used in equations to predict the overall mixture response:

$$R = \frac{1}{1 + (ED_{50}/D)^{\rho}} \quad 1$$

where R is the response, D is the chemical dose, ρ is the power or Hill slope of the curve, and ED_{50} is the exposure dose eliciting a 50% response. The ED_{50}s and slopes generated in the individual chemical analyses were used in equations to predict the overall mixture response.

Dose addition

Dose addition is based on the assumption that all chemicals in a mixture share a common mechanism of action. In the dose addition model chemical doses are converted into equivalent units using a ratio of dose to ED_{50} and the doses are then added to get a final mixture dose. The mixture dose is then converted to mixture response using a modified logistic equation (Olmstead & LeBlanc, 2005):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{D_i}{ED50_i}\right)^{\rho'}}} \quad 2$$

where R is the response to the mixture, D_i is the concentration of chemical i in the mixture, $ED50_i$ is the concentration of chemical i that causes a 50% response, and ρ' is the average power (Hill slope) associated with the chemicals.

Toxic equivalency factor (TEF)

A reference chemical was selected for each endpoint based on the strength of the individual chemical dose-response data for the endpoint. Vinclozolin was the reference chemical for all the data except for epididymal and gubernacular agenesis, for which the phthalates served as the reference chemicals. Based upon earlier observations in our laboratory (Gray *et al.*, 2000) and upon unpublished data (Howdeshell, in prep; Hotchkiss, in prep) we considered these three phthalates DBP, BBP and DEHP as equipotent and the models are based upon studies with DBP and DEHP. Relative potency factors were calculated by dividing the ED_{50} of the reference compound by the ED_{50} of each of the other chemicals. The dose of each chemical present in the mixture was then multiplied by the corresponding potency factor in order to calculate the reference chemical equivalents of each chemical. The converted doses were then added to get the mixture dose in terms of the reference chemical. This dose was used to ascertain the predicted mixture response based on the reference chemical dose-response curve.

Response addition

Response addition describes the independent action of multiple chemicals. This approach assumes that each chemical has a unique mechanism of action. The response addition equation is based on probability theory and is expressed mathematically as:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i) \quad 3$$

where R represents the response to the mixture and R_i is the response to individual chemical i.

Integrated addition

In the integrated addition approach, chemicals are first categorized based upon their mechanisms of action. In this study, the AR antagonists vinclozolin and procymidone were put into one mechanism-based group; the phthalates BBP, DBP, and DEHP were put into a second group; linuron and prochloraz made up groups three and four respectively. The toxicity of each chemical group was calculated using a dose addition model and the mechanism-based groups were then combined using a response addition model. The equation for integrated addition represents a merging of the dose addition and response addition equations (Olmstead & LeBlanc, 2005).

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i}\right)^{\rho'}}} \right\} \quad 4$$

Statistical analyses

Statistical analyses were performed using PROC GLM in SAS (Cary, NC, USA). Statistically significant ($p < 0.05$) differences between control and treated groups were detected using PROC GLM followed by LSMEANS on litter means.

Several methods of analysis were used to identify the 'best' model for the observed data. For quantitative endpoints (AGD, areolae and organ weights), the variance in the observed litter mean data were used in *t*-tests comparing the predicted mean values to the observed values. For incidence data (i.e. % hypospadias) the observed malformation rates (numbers of affected males per group/total numbers of males) were compared with the predicted values using Fishers Exact test.

In addition, we compared logistic regression models from the observed and four mixture models by comparing the ED_{50} (±99% CL) from the logistic regressions. We also fit the responses predicted by the dose addition, toxic equivalency, response addition and integrated addition models to the four parameter logistic regression model using the parameters from the observed data from all the dose groups to determine which set of modelled effects most closely fit observed responses. This allows identification of the 'best' model by identifying the model yielding the highest R^2 or coefficient of determination (Zar, 1996).

Results

Individual chemical analyses

Dose-response analyses were performed for individual chemicals with each endpoint (Fig. 1; data shown for AGD, infant nipples, hypospadias, epididymal agenesis, gubernacular agenesis and undescended testes). Individual chemical dose-response parameters [dose eliciting 50% effect (ED_{50}) and slope] were derived from a logistic fit to the data. As expected, individual chemicals displayed different response profiles. For example, at the doses used in our studies, vinclozolin or procymidone exposures did not elicit epididymal or gubernacular lesions. However, vinclozolin and procymidone were considerably more potent than the phthalates in inducing hypospadias or vaginal pouch. Parameters acquired from individual chemical dose-response analyses were incorporated into mathematical models of mixture toxicity to predict overall mixture effects.

Mixture analyses

At the time of necropsy on PND 175–195 all of the androgen sensitive endpoints examined displayed dose-dependent effects following in utero exposure to the mixture of seven chemicals (Table 1). Mixture exposure significantly decreased the weight of androgen sensitive organs including ventral prostate, seminal vesicles, testes, epididymis and LABC (Table 1). There was a trend towards decreased glans penis weight, although this was not significant as severe malformations precluded weight measurement at the highest dose (Table 1).

Predicted vs. observed

Experimentally derived mixture toxicity responses were compared with values predicted by toxic equivalency,

Table 1 Male rat reproductive tract effects resulting from in utero exposure to a mixture of antiandrogens. Male rats were exposed in utero to four dilutions of a mixture composed of vinclozolin, procymidone, linuron, prochloraz, benzyl butyl phthalate, dibutyl phthalate and diethylhexyl phthalate on GD14–18 and necropsied on PND175–195

Tissue	Control	25% of top dose	50% of top dose	75% of top dose	100% of top dose
AGD (mm)	3.59 ± 0.09	3.20 ± 0.05	2.93 ± 0.10	2.31 ± 0.11**	2.02 ± 0.04**
(number of litters/males examined at PND 2)	(3/9)	(2/14)	(2/16)	(2/9)	(2/10)
Areolae at 13 days of age (# per male)	0	0	3.07 ± 0.62*	9.80 ± 1.20**	11.67 ± 0.22**
Body weight (g)	626 ± 45	661 ± 16	670 ± 78.5	675 ± 16	678.5 ± 23.4
Glans penis (mg)	128.8 ± 5.6	129.6 ± 7.7	123.7 ± 0.5	114.5 ± 1.5	all malformed
Ventral prostate (mg)	792 ± 57	608 ± 8.1	746 ± 20	499 ± 384	129 ± 75*
Seminal vesicles (mg)	1954 ± 160	2053 ± 16	2207 ± 19	1573 ± 526	846 ± 42*
Paired testes (mg)	3532 ± 145	3556 ± 280	3791 ± 25	4038 ± 318	2617 ± 834
Paired epididymides (mg)	1249 ± 46	1239 ± 95	1324 ± 33	1349 ± 152	844 ± 352
LABC (mg)	1525 ± 84	1420 ± 77	1459 ± 30	1071 ± 252*	800 ± 23**
Hypospadias %	0	0	0	75**	100**
Undescended testes %	0	0	0	0	80**
Epididymal agenesis %	0	0	0	25	80**
Gubernacular agenesis %	0	0	0	0	10

Organ weight values are presented as litter means ± SE, an *indicates value differs from control by $*p < 0.05$ or $**p < 0.01$ by litter means analysis. The malformation rates are the numbers of malformed males/total numbers of males per group, an ** indicates that the value differs significantly from control by Fishers exact test by $p < 0.01$.

dose addition, response addition and integrated addition models. As the phenotypic profiles differ significantly among the seven chemicals, relative potency factors for the toxic equivalency analyses were calculated from the dose response curves for each chemical for every endpoint rather than using a single potency factor for each chemical for all the effects.

Anogenital distance and retained areolae

Both AGD reduction and areolae retention were highly sensitive endpoints. Increasing doses of the mixture resulted in a linear reduction in AGD in male offspring of treated rats (Fig. 2, top panel). Observed mixture AGD data were reported previously to be well predicted by toxic equivalency (Gray *et al.*, 2006). In the current assessment of the AGD data which utilized additional individual chemical data, the toxic equivalency approach again provided the best fit to the observed decrease in AGD. Dose addition was slightly better than response addition and integrated addition models, which provided similar predicted responses to one another, under-predicting the observed responses (Fig. 2, Table 2).

Infant male offspring exposed to the three highest doses of the mixture displayed female-like nipples/areolae at 13 days of age (Fig. 2, bottom panel). The toxic equivalency approach provided the 'best' fit to the observed increase in areolae retention in the 50% and 75% mixture groups whereas toxic equivalency and dose addition both adequately predicted the observed response in the 100% group. Integrated addition and response addition models under-predicted the observed incidence of retained nipples at day 13 by about 60% (Fig. 2, Table 2). However, even though the toxic equivalency model provided the 'best' fit of the observed data, it tended to over-predict low dose and under-predict high dose mixture effects indicating that the logistic models had significantly different slopes (as judged by lack of overlap of the confidence limits; logistic regression slopes of 6.3 vs. 1.9 for observed and the toxic equivalency models respectively).

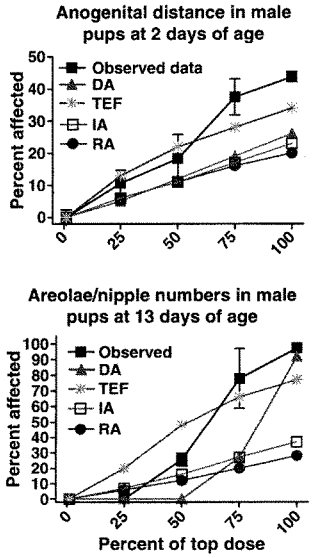

Figure 2 Comparison of responses predicted with toxic equivalency, dose addition, response addition and integrated addition models to observed mixture responses following in utero exposure of male rats to a seven chemical mixture on GD14-18. Anogenital distance (AGD) reduction data (upper panel) are expressed as per cent reduced from control mean. Observed AGD results were reported previously (Gray *et al.*, 2006) without accompanying modelled predictions. Retained areolae data (lower panel) represent the per cent of maximum (12) possible areolae. Observed data are mean and standard error values for each treatment group.

Malformations

The toxic equivalency model was a slightly better predictor of the incidence of hypospadias (100% affected) than the dose addition model (it had a significantly higher ED_{50} than the observed), while the response addition and integrated addition models grossly under-predicted this malformation ($p < 0.001$ lower than observed by Fisher Exact test) (Fig. 3 and Table 2).

Epididymal agenesis was induced in 80% of males in the high dose group and this response was well predicted by both toxic equivalency and dose addition models, with response and integrated addition models again greatly under-predicted the incidence of this malformation ($p < 0.001$ lower than observed by Fisher Exact test) (Fig. 3 and Table 2).

The incidence of undescended testes was 80% in the high dose of the mixture and the toxic equivalency and dose addition models provided the best fits to this response in the high dose group, although the ED_{50}s were slightly higher than observed. The predictions for these two models in the high dose were not significantly lower than observed by Fisher Exact, $p < 0.35$. In contrast, response and integrated addition models grossly under-predicted ($p < 0.001$ by Fisher Exact) the incidence of this malformation (Fig. 3, Table 2).

Table 2 Logistic regression models were run on the observed (OBS) data and on the values generated for the dose addition (DA), toxic equivalency (TEF), integration addition (IA) and response addition (RA) models of the mixture data

	AGD		Nipples day 13		Hypospadias		Epididymal agenesis		Undescended testes	
ED_{50} rank	MODEL	ED_{50}	MODEL	ED_{50}	MODEL	ED_{50}	MODEL	ED_{50}	MODEL	ED_{50}
1	OBS	65a	*TEF*	*52a*	OBS	74a	OBS	85a	OBS	93a
2	*TEF*	*81a*	OBS	59b	*TEF*	*83b*	*TEF*	*87a*	*TEF*	*99b*
3	DA	120b	DA	82c	DA	90b	*DA*	*87a*	*DA*	*99b*
4	IA	147c	IA	142d	IA	none	IA	132b	IA	134c
5	RA	187c	RA	198d	RA	none	RA	none	RA	none

	Ventral prostate weight		Testis weight		Epididymal weight		Seminal vesicle weight		Gubernacular agenesis	
Ed_{50} rank	MODEL	ED_{50}	MODEL	ED_{20}	MODEL	ED_{25}	MODEL	ED_{50}	MODEL	ED_{20}
1	OBS	70a	OBS	93a	DA	78a	OBS	92a	OBS	113
2	*TEF*	*103a*	*DA*	*108a*	OBS	95ab	*DA*	*93a*	TEF	none
3	DA	155a	TEF	124b	*TEF*	*98b*	*TEF*	*94a*	DA	none
4	IA	253b	IA	187d	IA	104b	IA	none	IA	none
5	RA	303b	RA	398e	RA	5011c	RA	none	RA	none

The ED_{50} values for each model are ranked and compared with one another using 99% confidence limits. ED_{50} values with confidence limits that do not overlap have different letters (a–d) and are significantly different from one another using this metric. The values used to generate these models are shown in Figures 1-3. The 'best' model(s) for predicting the observed effects with the highest coefficient of determination is italicized. ED_{50} with different letters differ from one another based upon a lack of over lap of their confidence limits. Testis, epididymal weights and gubernacular agenesis models are compared using ED_{20}, ED_{25} and ED_{20} values respectively, since the observed data did not approach an ED_{50}.

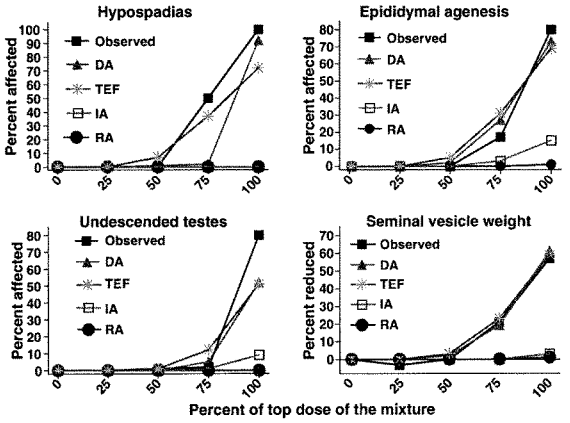

Figure 3 Comparison of the observed malformation rates for hypospadias, undescended testes and epididymal agenesis and seminal vesicle weight reductions with toxic equivalency (TEF), dose addition (DA), response addition (RA), and integrated addition (IA) models with predicted malformation rates following in utero exposure of male rats to a seven chemical mixture on GD14-18. As hypothesized in the study, the mixture of seven antiandrogens induced malformations of the male reproductive tract in a manner that was well predicted by the TEF and DA models but not by IA or RA. Seminal vesicle weight was similarly affected.

Lastly, a 10% incidence of gubernacular agenesis was observed in the high dose treatment group (Fig. 3, Table 2), but observed and predicted incidence values were all too low to discriminate amongst the models. This occurred because higher doses of phthalates are required to induce this lesion in the SD rat strain (Wilson *et al.*, 2007) than we used in our studies (as phthalates also induce pregnancy loss) and the four pesticides used in this study do not induce this malformation, having no effect on foetal testis insl3 levels (Wilson *et al.*, 2004).

Organ weights

The mixture of seven 'antiandrogens' reduced the weights of all androgen responsive organs (Table 1). The toxic equivalency and dose addition models best described the observed reductions in seminal vesicle and ventral prostate in the high dose group, while the response addition and integrated addition models were significantly lower than the observed value in the high dose group (Table 2). Ventral prostate weight was reduced in rats exposed in utero to all doses of the mixture (Fig. 3, Table 2). Seminal vesicle weight was reduced in the two higher dose groups and both toxic equivalency and dose addition models predicted this effect; whereas, the response and integrated addition models significantly under-predicted the effect in the high dose group (Table 2).

Testis weight was reduced by the highest dose of the mixture by 28% but, this effect was not statistically significant because of the marked increase in variability in the high dose groups and the small numbers of litters used (Table 2).

Epididymal weight also was reduced in the highest dose group by 34%. The reduction in epididymal weight was best predicted by the toxic equivalency model, with dose addition and integrated addition being slightly less precise. However, none of the predictions were significantly lower than the observed value in the high dose group (Fig. 3, Table 2).

The observed effects were compared with the model predictions by comparing the ED_{50} values of the logistic regressions, comparing the coefficients of determination of the modelled data and, for the high dose group, with Fisher Exact test for malformation and by *t*-tests for organ weights, AGD and areolae. Overall, the toxic equivalency and dose addition models were more consistently the 'best' predictors of the observed effects of the mixture on the androgen-dependent organ weights (Table 2). The response addition model often grossly under-predicted the effects of the mixture on infant male rat nipple retention, malformation rates and several of the organ weights. The integrated addition model was rarely better than response addition.

Discussion

The results of this study demonstrate that adverse reproductive tract responses to a mixture of seven diverse chemicals conformed best to toxic equivalency or dose addition models of mixture toxicity, while models of response addition and integrated addition often grossly under-estimated mixture effects in the higher dose groups. This finding suggests that current regulatory guidelines for assessing the risk associated with chemical mixtures should consider a broader definition of 'mode of action' or 'mechanism of toxicity' based upon common effects rather than limiting cumulative risk assessments to only chemicals that act via identical cellular and molecular mechanisms. Herein, we show again that chemicals that disrupt differentiation of the same target tissue during a critical period in utero induce dose additive responses even though they act directly on different foetal tissues and on different molecular targets (testis and steroidogenesis vs. reproductive tract and AR antagonism). We first reported dose additive responses with a binary mixture of the pesticide linuron (one mechanism of action is AR antagonist) and the phthalate BBP (an inhibitor of foetal testosterone and insl3 synthesis) (Hotchkiss *et al.*, 2004).

In this study, in which we increased the number of chemicals in the mixture from two to seven it is clear that the effects of this mixture in the high dose groups could not be explained by models of response addition (also referred to as independent action) or integrated addition. Only the toxic equivalency analysis and dose addition model provide reasonable predictions of high incidences of hypospadias, undescended testes and epididymal agenesis. Whereas these malformations were displayed by 80–100% of the high dose group male rat offspring, response and integrated addition models predicted incidences of 0–15%. We propose that toxic equivalency or dose addition models provide the best fit to observed mixture responses because the individual chemicals in the mixture target a common pathway, the androgen signalling pathway, identically by preventing AR-dependent gene activation in the reproductive tract. Therefore, at the tissue level, these chemicals are not acting independently.

The chemicals in this study disrupt a common pathway during sexual differentiation, but they do so by disrupting different cellular and molecular targets. Vinclozolin and procymidone are AR antagonists (Kelce *et al.*, 1994; Gray *et al.*, 1999a; Ostby *et al.*, 1999). The phthalates elicit developmental disruption in androgen dependent tissues (Mylchreest *et al.*, 1998; Gray *et al.*, 2000; Parks *et al.*, 2000; Barlow *et al.*, 2004; Foster, 2006), although they do not display significant binding to the AR (Parks *et al.*, 2000; Satoh *et al.*, 2001). Instead, phthalates disrupt the androgen signalling pathway by inhibiting foetal testosterone synthesis and decreasing expression of insl3 (Wilson *et al.*, 2004). Linuron and prochloraz disrupt androgen signalling via multiple mechanisms (Gray *et al.*, 2006). Both bind weakly to the AR in-vitro (Lambright *et al.*, 2000; Vinggaard *et al.*, 2006). Additionally, both linuron and prochloraz inhibit foetal testosterone synthesis (Lambright *et al.*, 2000; Wilson *et al.*, 2004). Clearly, the chemicals assessed in this study display different specific mechanisms of toxicity and directly affect different foetal

tissues which results in alterations of common 'downstream' signalling events in the androgen signalling pathway during the critical period of male rat sexual differentiation in utero.

In a recent review of endocrine disrupting chemical mixture work, the study of mixtures containing chemicals with diverse mechanisms of endocrine action was identified as an important area requiring further study (Kortenkamp, 2007). There is long standing debate on the most appropriate methods for assessing mixtures of chemicals that have different mechanisms of toxicity (Greco *et al.*, 1992). Mixture modelling convention dictates that chemicals with different mechanisms of toxicity should theoretically display independent action and adhere to a model of response addition. The integrated addition model was developed to accommodate complex mixtures containing components with a common mechanism of toxicity (e.g. vinclozolin and procymidone), as well as components with different mechanisms of toxicity (e.g. vinclozolin and BBP). Accordingly, the integrated addition model should have provided the best fit to responses generated from the seven-chemical mixture assessed in this study. To the contrary, we found that in general, the toxic equivalency or dose addition models provided the best fit to observed mixture responses, especially in the case of reproductive tract malformations (Table 2, Fig. 4).

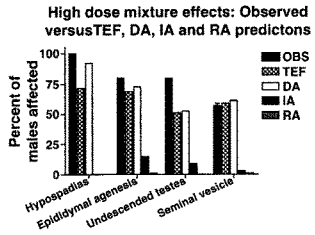

Figure 4 Statistical comparison of the high dose effects of the mixture effects observed in the study with the toxic equivalency (TEF), dose addition (DA), response addition (RA) and integrated addition (IA) model predictions. The observed malformation rates were compared with the model predictions using Fishers exact test and the observed per cent reduction in seminal vesicle weight was compared with the model predictions using a *t*-test based upon the litter-mean error variance from the anova of the effect of the mixture dose on seminal vesicle weight. TEF and DA model predictions did not differ significantly from observed for hypospadias, undescended testes and epididymal agenesis and the seminal vesicle weight reduction, whereas RA and IA predictions were significantly lower than the observed values.

We propose that the toxic equivalency or dose addition models are the only mathematically and biologically plausible models to describe the joint action of the mixture on the androgen-sensitive endpoints assessed in this study. The arguments supporting this conclusion are that (i) the individual chemicals display complex modes of action that are not entirely distinct and (ii) we are assessing endpoints that are far downstream of the biochemical mechanisms of the individual chemicals and represent integrated biological responses. In simple terms, androgen-sensitive tissues cannot distinguish between decreased androgen signalling resulting from testosterone synthesis inhibition (i.e. phthalates) vs. that resulting from antagonists (i.e. vinclozolin) binding to ARs. When phthalates reduce testis testosterone production, lower concentrations of androgens will reach the tissues, fewer AR are bound, AR-dependent genes are not transcribed at normal rates and the tissue is demasculinized. Although AR antagonism is a distinctly different mechanism of toxicity from inhibition of steroidogenesis, and in fact the AR antagonists even act directly on a different foetal tissue than the phthalates (differentiating tract tissue vs. testis) the effect on AR-dependent gene expression is identical; there is less activated AR bound to an androgen and AR-dependent gene expression is attenuated. Microarray analyses of the effects of AR antagonists vs. removal of androgens support our thesis that these diverse chemicals disrupt AR-dependent gene expression identically. When we examined how the gene expression profiles of the rat ventral prostate were altered by either exposure to AR antagonists (vinclozolin and procymidone) or androgen ablation we found that the profiles were indistinguishable except for the fact that complete removal of androgens was more effective than the partial AR antagonism seen with pesticide treatments (Rosen *et al.*, 2005).

There is precedent for mixtures of diverse-acting chemicals eliciting mixture responses that resemble dose addition more than response addition (Hermens & Leeuwangh, 1982; Hermens *et al.*, 1984; Broderius *et al.*, 1995; Birkhoj *et al.*, 2004). Birkhoj *et al.* (2004) assessed the combined in vitro antiandrogenic effects of a group of five pesticides with diverse structures and mechanisms of action. They found that the three pesticides deltamethrin, methiocarb, and prochloraz, which elicited antiandrogenic effects individually, contributed to mixture responses that conformed to a dose addition model (Birkhoj *et al.*, 2004). Hermens & Leeuwangh (1982) and Hermens *et al.* (1984) discussed the possibility that independence of action among chemicals with diverse mechanisms of toxicity becomes increasingly unlikely as the measured endpoints move from specific to non-specific. For example, the reproductive tract endpoints assessed in this study, such as hypospadias, represent the culmination

of a cascade of complex and integrated molecular events, which have yet to be fully elucidated.

To date, cumulative risk assessments have generally been conducted only for chemicals that have identical cellular and molecular mechanisms of toxicity. In this regard, the US EPA 'has determined so far that four groups of pesticides each have a common mechanism of toxicity and require cumulative risk assessments because exposure to these pesticide groups may pose potential risks to human health and the environment. The four groups are: the organophosphates, N-methyl carbamates, triazines and chloroacetanilides' http://www.epa.gov/pesticides/cumulative/. In addition to these groups, the USEPA is considering whether or not an assessment of the toxicity of dibutyl phthalate should include other phthalates with 'antiandrogenic' activity. Even though several phthalates share this common mechanism of toxicity and affect common tissues in the developing male rat reproductive tract, regulatory agencies have not yet conducted cumulative risk assessments on this class of chemicals.

In addition, in the 2000 risk assessment on vinclozolin, the USEPA (http://www.epa.gov/oppsrrd1/REDs/2740red.pdf) recognized that 'vinclozolin is a member of the imide group of the dicarboximide class of fungicides, as are iprodione and procymidone...' and that 'all of these fungicides appear to be antiandrogenic...' but based upon the data available at that time the Agency concluded that '...although there are data suggesting that these dicarboximide fungicides induce some of the same antiandrogenic effects, the mechanism by which they cause these toxic effects has not been adequately evaluated'. Since then, however, it is clear that both of these chemicals are AR antagonists capable of inducing common malformations of the male rat reproductive tract independently with in utero exposure. Furthermore, it has been shown that these fungicides induce dose additive effects when co-administered in the young male rat (Gray *et al.*, 2001; Nellemann *et al.*, 2003; Metzdorff *et al.*, 2007) or with in utero exposure (reviewed by Gray *et al.*, 2001, 2006; Metzdorff *et al.*, 2007).

Although regulatory agencies are beginning to consider conducting cumulative assessments on chemicals like the phthalates or the dicarboximide fungicides like vinclozolin and procymidone, considerably more research will need to be executed to provide a framework and guidance for conducting cumulative assessments on chemicals with diverse modes of toxicity, like those used in the current mixture study. Additional research is needed to determine how broadly the definition of 'mode of action' needs to be to accurately reflect the ability of mixtures of diverse chemicals to disrupt development and to repeat these observations given the relatively small sample sizes used herein. Although our sample sizes are large enough to determine that the dose addition and toxic equivalency models predicted the observed data better than the integrated or response addition models for some of the endpoints, like hypospadias, a larger study would enable clearer discrimination among the models for some other endpoints and, of equal import, to identify NOAELs for the mixture. Our working hypothesis is currently that endocrine disrupting mixtures that disrupt a common developmental pathway in a differentiating tissue, like the androgen signalling pathway, will produce dose-additive effects even if the chemicals in the mixture do not all share a common cellular and molecular mechanism of toxicity.

In summary, this study found that a mixture of seven antiandrogenic compounds with diverse structures and mechanisms of toxicity acted in a dose additive manner to disrupt male rat reproductive tract development. Furthermore, these in-vivo effects were reasonably well predicted by toxic equivalency or dose addition models using individual chemical dose-response data from our laboratory. As hypothesized, the mixture of seven antiandrogens induced malformations of the male reproductive tract in a manner that was well predicted by the TEF and DA models but not by IA or RA. Seminal vesicle weight was similarly affected. These findings indicate that cumulative risk assessments may need to be expanded beyond mixtures of chemicals that act by precisely the same cellular and molecular mechanism to include mixtures of diverse chemicals like the phthalates combined with AR antagonists that share a common 'mode of action'; disrupting differentiation of common signalling pathway in the foetal reproductive tract in utero.

Disclaimer

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, ORD, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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Identification of Metabolites of Trenbolone Acetate in Androgenic Runoff from a Beef Feedlot

Elizabeth J. Durhan,[1] Christy S. Lambright,[2] Elizabeth A. Makynen,[1] James Lazorchak,[3] Phillip C. Hartig,[2] Vickie S. Wilson,[2] L. Earl Gray,[2] and Gerald T. Ankley[1]

[1]Mid-Continent Ecology Division, National Health and Environmental Effects Laboratory, U.S. Environmental Protection Agency, Duluth, Minnesota, USA; [2]Reproductive Toxicology Division, National Health and Environmental Effects Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA; [3]Ecological Exposure Research Division, National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, USA

Little is known concerning the potential ecological effects of hormonally active substances associated with discharges from animal feeding operations. Trenbolone acetate is a synthetic anabolic steroid that is widely used in the United States to promote growth of beef cattle. Metabolites of trenbolone acetate include the stereoisomers 17α- and 17β-trenbolone, both of which are stable in animal wastes and are relatively potent androgens in fish and mammals. Our purpose in this study was to evaluate the occurrence of 17α- and 17β-trenbolone in a beef cattle feedlot discharge and in river water upstream and downstream from the discharge. In conjunction with that effort, we measured *in vitro* androgenic activity of the discharge using CV-1 cells that had been transiently cotransfected with human androgen receptor and reporter gene constructs. Samples were collected on nine different occasions during 2002 and 2003. Whole-water samples from the discharge caused a significant androgenic response in the CV-1 cells and contained detectable concentrations of 17α- and 17β-trenbolone. Further work is needed to ascertain the degree to which synthetic androgens such as trenbolone contribute to androgenic activity of feedlot discharges. *Key words:* environmental androgen, feedlot runoff, trenbolone. *Environ Health Perspect* 114(suppl 1):65–68 (2006). doi:10.1289/ehp.8055 available via *http://dx.doi.org/* [Online 21 October 2005]

Environmental contaminants that adversely affect fish and wildlife through disruption of the hypothalamic–pituitary–gonadal (HPG) axis or that have direct effects on the reproductive tract are currently of interest. Although most early work focused on changes in the HPG axis caused by chemicals that bind to and activate estrogen receptors, recent studies have highlighted the variety of contaminants that can affect HPG function through interactions with the androgen receptor. For example, investigations of pulp and paper mill effluents from different locations in North America as well as an examination of a beef feedlot discharge from one site (Nebraska), have associated morphological alterations in fish collected from the field with *in vitro* androgenic activity in water samples from the affected sites (Hewitt et al. 2003; Jenkins et al. 2001; Orlando et al. 2004; Parks et al. 2001). Partly because of sample complexity, chemicals responsible for androgenic activity in pulp and paper mill effluents have not been identified successfully (Durhan et al. 2002; Hewitt et al. 2003). Similarly, little is known concerning the identity of chemicals that might be responsible for androgenic activity in feedlot discharges; however, with these types of samples, insights may be gained through consideration of chemicals used for livestock production. Specifically, much of the beef production in the United States utilizes anabolic androgenic materials to promote production of muscle mass in the animals. One of the most commonly used chemicals for this purpose is the synthetic androgen precursor trenbolone acetate (Meyer 2001; Neumann 1976; Raloff 2002; Roche 1986). Two metabolites of that acetate, 17α- and 17β-trenbolone, are relatively stable in animal waste and in the environment (Schiffer et al. 2001). These metabolites also bind with high affinity to fish androgen receptor(s) (Ankley et al. 2003; Bauer et al. 2000; Pottier et al. 1981; Wilson et al. 2004). *In vivo* studies with both isomers have shown that they are highly potent in fish; they masculinize females and decrease fecundity at water concentrations in the low nanogams per liter range (Ankley et al. 2003; Jensen K, unpublished data).

The present study had two objectives. First, we sought to evaluate whether the observation of Orlando et al. (2004) concerning androgenic activity in beef cattle feedlot discharge was an isolated or a more generalized (widespread) phenomenon. To achieve this goal, we first collected samples from several sites adjacent to a beef cattle feedlot in Ohio over the course of more than a year. We then determined androgenic activity in the samples using a cell-based assay with a transiently transfected (human) androgen receptor and promoter reporter gene constructs (Orlando et al. 2004; Parks et al. 2001). The feedlot operator indicated that trenbolone acetate was used in the production facility, so our second objective was to evaluate the samples with instrumental analyses to determine the possible presence and concentrations of 17α- and 17β-trenbolone in the site samples.

Methods and Materials

Study site. The beef cattle feeding operation was located in southwest central Ohio. It was constructed in the mid-1960s and consists of eight cattle buildings on about 96 ha. The cattle barns have a compacted clay floor, which is cleaned periodically and lined with fresh hardwood chips to absorb urine and provide comfort to the animals as insulation from the bare ground. Manure and urine fall on the bedding material. The waste is handled as a solid material with typical moisture content of 40–50%. Shallow drains from the buildings collect groundwater, including wastes that seep from the chips. As of early 2002, the feeding operation had a capacity to hold and feed 9,800 head of cattle. Revalor S implants (Hoechst-Roussel Agri-Vet Co., Sommerville, NJ), which contain both trenbolone acetate and 17β-estradiol, are used at this facility.

Three locations on a river adjacent to the feedlot were chosen for sample collection: *a*) 572 m upstream from all drainage from the facility, *b*) a discharge drain that collects run-off from the two sets of buildings, and *c*) 381 m downstream from the discharge drain. Samples were collected in amber 1- or 4-L (methanol rinsed) glass containers, placed on ice, and shipped overnight from the U.S. Environmental Protection Agency (EPA) laboratory in Cincinnati, Ohio, to U.S. EPA laboratories in Research Triangle Park, North Carolina, and Duluth, Minnesota. Upon arrival, the samples were stored at 4°C in the dark until analyzed. Samples were collected

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Address correspondence to E.J. Durhan, Mid-Continent Ecology Division, National Health and Environmental Effects Laboratory, U.S. EPA, 6201 Congdon Boulevard, Duluth, MN 55804 USA. Telephone: (218) 529-5167. Fax: (218) 529-5003. E-mail: durhan.elizabeth@epa.gov

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on nine different occasions and were designated by sample identifier and date as follows: A-February 2002, B-March 2002, C-June 2002, D-July 2002, E-September 2002, F-October 2002, G-March 2003, H-April 2003, and I-June 2003.

CV-1 assay. Androgenic activity in water samples was determined as described by Parks et al. (2001). Briefly, CV-1 cells (monkey kidney cell line; American Type Culture Collection no. CCL-70) were transiently cotransfected with human androgen receptor vector [pCMVhAR; Wong et al. (1995)] and MMTV (mouse mammary tumor virus)-luciferase reporter using Fugene reagent (Boehringer, Mannheim, Germany). Twenty-four hours after transfection, the original medium (Dulbecco's modified Eagle's medium; Gibco Invitrogen Corp., Carlsbad, CA, USA) with 5% dextran charcoal-stripped serum (Hyclone, Logan, UT, USA) was removed from the cell culture and replaced with medium prepared from water samples that had been passed through a 0.2-μm nylon filter. After 24 hr, cells were harvested, and relative light intensity was determined using a luminometer. Relative luciferase light data were analyzed and expressed as fold response relative to media. Hence, a fold induction of one is a negative response, equivalent to the media-only control. Each group of CV-1 assays of site samples was conducted simultaneously with a dihydrotestosterone (DHT) positive control (27 ng/L) and an untreated media negative control.

The CV-1 assay was also performed on two separate occasions with varying concentrations of both 17α- and 17β-trenbolone to estimate the detection limit of the assay for each compound. These studies were conducted using solutions of 17α- and 17β-trenbolone prepared in water under the conditions described above. In these studies, CV-1 cells were incubated with 17α-trenbolone (at 0, 2.7, 13.6, 18.9, 27, and 54 ng/L) or with 17β-trenbolone (at 0, 0.27, 5.4, 13.6, 27, and 40 ng/L), again with the positive DHT control. The chemicals for the CV-1 assays were obtained from Hayashi Pure Chemical Industries [Osaka, Japan; 17α-trenbolone (80657-17-6), 99.9% purity] and Sigma Chemical Co. [St. Louis, MO, USA; 17β-trenbolone (10161-33-8), 98% purity].

Because of logistical constraints, many of the CV-1 assays with the site samples were performed only once. However, with four samples (samples A, B, F, and G), the CV-1 assays were performed on three different occasions, together with a set of co-treatments with the androgen receptor antagonist 2-hydroxyflutamide (292 μg/L). Data from the A, B, F, and G sampling periods were used for statistical analyses of the data. In these analyses, CV-1 luciferase activity data were $\log_{10}$ transformed, and analyzed using PROC GLM available in SAS, version 8.2 (SAS Institute, Cary, NC, USA). Overall statistical significance was determined using the *F*-value from analysis of variance, followed by a least squares means analysis, which compares the means of individual groups with one another by *t*-tests. Differences between treatments were considered significant at $p \leq 0.05$.

Instrumental analyses. Water samples for the instrumental analyses were processed by filtration (1-μm glass fiber filter) followed by C_{18} solid-phase extraction (SPE) concentration. A Bakerbond 6-mL HC C18 SPE column (JT Baker, Philipsburg, NJ, USA) was prepared by washing with 20 mL of acetonitrile and then conditioned with 20 mL of methanol followed by 20 mL of deionized water. One liter of filtered site water was pumped onto the conditioned SPE column at a rate of about 5 mL/min. The SPE column was then rinsed with 20 mL of 50% methanol–water (discarded) and allowed to vacuum dry for 2 min. The SPE column was eluted with two aliquots of 2 mL of methanol. The two aliquots were combined and, at this point, used for chemical analyses.

Trenbolone (both 17α and 17β) was measured in concentrated site samples by HPLC with fluorescence detection and, in a subset of samples, by gas chromatography–mass spectrometry (GC-MS). The HPLC determinations of trenbolone were made by injecting 40-μL aliquots onto a Nucleosil 100-Å, 5-μm C_{18} AB (Macherey-Nagel Inc., Easton, PA, USA) column (4.6 mm × 250 mm) on an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA) at a flow rate of 0.9 mL/min. The solvent gradient started at 35% methanol–water and changed linearly over 3 min to 65% methanol–water, followed by isocratic elution with 93% methanol–water. Fluorescence detection was used with excitation and emission wavelengths at 359 and 458 nm, respectively. External standard quantitations were made. Detection limits were usually about 4 ng/L in the water samples. Standards used for the HPLC and GC–MS analyses were obtained from Aventis Pharma S.A. (Antony, France; 17α-trenbolone, 99.6% purity) and Sigma (17β-trenbolone).

Trenbolone measurements were made using GC/MS by injecting a 10-μl Aliquot of the SPE extract onto a 30 m × 0.25 mm Delta-3 GC column (Machery-Nagel, Oensingen, Switzerland) with a 0.25-μm film using an Apex large-volume injector (Apex, Philadelphia, PA, USA). The injector was equipped with a 2-mm quartz liner and a plain glass insert and was heated ballistically from 80°C to 250°C in 50 sec. The column oven was programmed from 80°C (3-min hold) to 250 °C at 15°C/min, followed by 250°C to 325°C at 5 °C/min, then held at 325°C for 5 min. A mass spectrometer (Agilent Technologies) was used in the single ion monitoring (SIM) mode at mass 270 and in the full scan mode for structural confirmation with an external standard method of quantitation. Detection limits for the GC-MS analyses were rather variable, ranging from slightly < 10 to about 100 ng/L.

Results

Androgenic activity of 17α- and 17β-trenbolone in the CV-1 cell line is shown in Figure 1A,B. In the case of 17α-trenbolone, androgenic activity was increased to about 3-fold over media at test concentrations of 12–30 ng/L, but the concentration–response relationship for the assay was comparatively flat above that, increasing to about 4-fold over media above 50 ng/L (Figure 1A). Androgenic activity in the cell line was increased by 5- to 8-fold over media by 17β-trenbolone concentrations of 6–40 ng/L (Figure 1B). The concentration activity curve of 17β-trenbolone in the CV-1 cells had an initial rise followed by a plateau at about 8-fold above media at a test concentration of 27 ng/L (Figure 1B). Statistical significance (above the media control) was achieved with ≥ 5.4 ng/L of 17β-trenbolone and ≥ 13.6 ng/L of 17α-trenbolone.

When we assessed the samples, we found the positive control DHT (27 ng/L) consistently produced a significant induction of

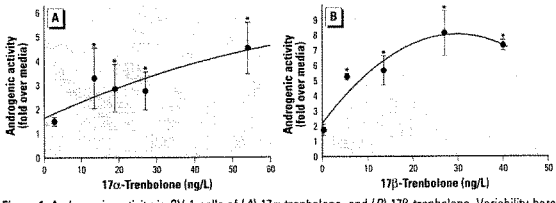

Figure 1. Androgenic activity in CV-1 cells of (*A*) 17α-trenbolone, and (*B*) 17β-trenbolone. Variability bars (± SE) reflect duplicate determinations, expressed as fold over media. The trend lines on *A* and *B* are a polynomial regression of the second order.
*Samples that have statistically significant androgenic activity compared with the media control.

androgenic activity in the CV-1 assay, with a mean value (± SE, n = 9) over the course of the 16-month study of 16.2 ± 1.1-fold over media (Figure 2). Figure 2 also presents the effects of the environmental samples on the CV-1 cells. For four of the nine sampling periods (E, F, H, I), no clear difference was observed among the upstream, downstream, or discharge samples. For the other sampling periods (A, B, D, G), the discharge sample had elevated androgenic activity in the CV-1 cells, while neither the upstream nor the downstream samples (A, B, G) displayed consistently elevated androgenic activity (Figure 2). Based on statistical analysis of the four sampling periods for which CV-1 assays were conducted on multiple occasions (A, B, F, G), androgenic activity from the discharge samples but not from the upstream and downstream sites was significantly elevated. Co-treatments of the CV-1 cells with 2-hydroxyflutamide on these dates blocked increases in luciferase activity caused by either DHT or discharge samples from the feedlot, indicating that both responses were mediated via the androgen receptor (data not shown).

Concentrations of chemicals in the samples with elution times (and fluorescence properties) similar to those of 17α- and 17β-trenbolone are indicated in Figure 3A,B. As was true for the androgenic activity, in most instances when the two analytes were detected by HPLC, they were in the discharge rather than in the upstream or downstream samples. The 17α-trenbolone was detected more frequently and at higher concentrations than the 17β-trenbolone, which is consistent with a greater excretion of the 17α- versus 17β-isomer by cattle treated with trenbolone acetate (Schiffer et al. 2001). Overall, 17α-trenbolone was detected in six of nine samples from the discharge with concentrations ranging from < 10 to about 120 ng/L. Two of the nine discharge samples had detectable 17β-trenbolone at concentrations of about 10 and 20 ng/L. There were small amounts of 17α-trenbolone and/or 17β-trenbolone detected in some of the upstream (A, B, E, F) and in some of the downstream samples (A, E, F), but these concentrations were predominantly much lower than those found in the discharge samples.

Figure 4A–C depict confirmatory GC-MS data for the two trenbolone acetate metabolites in water collected from the feedlot discharge. The top panel of the figure shows a GC-MS scan for the chemical standards, the middle panel depicts GC-MS data for 17α- and 17β-trenbolone spiked into water from the discharge (at 50 ng/L) of sample E, and the bottom panel shows data for the two metabolites detected in the discharge of sample E. Quantitation of 17α- and 17β-trenbolone via GC-MS indicated concentrations of 8 and 12 ng/L, respectively, in the sample. HPLC analysis of this same sample produced respective concentrations of 30 and 10 ng/L. Neither 17α- nor 17β-trenbolone was detected in samples analyzed by GC-MS from other dates (sample ID:detection limit; F: 10 ng/L, G: 30 ng/L, H: 25 ng/L, and I: 40 ng/L).

Discussion

Schiffer et al. (2001) reported that half-lives for degradation of 17α- and 17β-trenbolone in liquid cattle manure were on the order of 260 days. Hence, it is not unreasonable to expect that the two trenbolone acetate metabolites would be detectable in runoff or discharges from a feedlot that uses the material as a growth promoter. However, this finding is, to our knowledge, the best- documented example of the occurrence of trenbolone in surface waters of the United States. Both trenbolone metabolites bind with high affinity to androgen receptors in mammals and fish (Wilson et al. 2002, 2004), and they are potent androgens *in vivo;* for example, they cause masculinization and decreases in fecundity in fish at nanograms per liter concentrations comparable to those detected in the discharge sample from this study (Ankley et al. 2003; Jensen K, unpublished data).

Assays of the water with the CV-1 cell line indicated an elevated level of androgenic activity in several samples collected over time from the discharge. The fact that hydoxyflutamide blocked the increase in gene expression in the

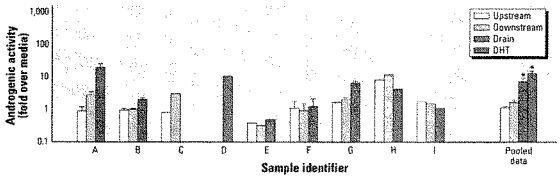

Figure 2. Androgenic activity in CV-1 cells of upstream, downstream, and discharge samples expressed relative to media. Only a discharge sample was collected on D, and only upstream and downstream samples were collected on C. Pooled data are from samples A, B, F, and G, for which the CV-1 assays were conducted in triplicate. Variability bars for these samples reflect the SE of individual replicate analyses, while for the pooled sample set, the bars indicate variability of all data for samples A, B, F, and G. The mean (± SE) DHT positive controls from all assays are also shown with pooled data.
*Samples that have statistically significant androgenic activity compared with the media control.

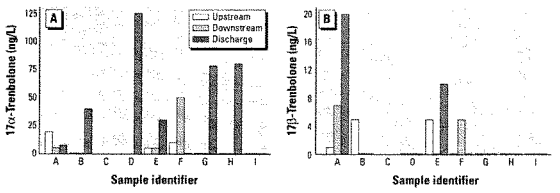

Figure 3. Concentrations (nanograms per liter) of 17α-trenbolone (*A*), and 17β-trenbolone (*B*) in upstream, downstream, and discharge samples as determined by HPLC.

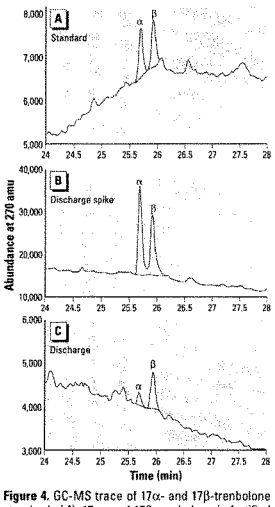

Figure 4. GC-MS trace of 17α- and 17β-trenbolone standards (*A*), 17α- and 17β-trenbolone in fortified discharge sample E (*B*), and discharge sample E (*C*).

CV-1 cells demonstrates that this induction was androgen-receptor mediated. Orlando et al. (2004) also reported that water from a pond adjacent to a beef feedlot located in Nebraska stimulated androgenic activity in the cell line, but they were unable to identify chemicals responsible for the activity. It would be tempting to speculate that 17α- and/or 17β-trenbolone were responsible for the androgenic activity observed in the present study; however, the data collected in our experiments do not permit this type of extrapolation. First, because of variations in efficiency of transfection of the receptor/reporter gene construct from experiment to experiment, even with the DHT positive control, the CV-1 assay is probably best considered a semiquantitative measure of androgenic activity. Second, given the detection limit for 17β-trenbolone (5 ng/L) in the CV-1 assay, it is possible that a positive androgenic response caused by 17β-trenbolone may not be consistently verifiable even by HPLC. Finally, in the present study, while performance of the HPLC method remained reasonably constant over time, the varying GC-MS detection limits (i.e., 10–100 ng/L) did not allow a sufficient number of confirmatory analyses to validate the HPLC data from a quantitative perspective. Further work at this site and/or at other feedlots is needed to ascertain the identity of chemicals (including natural steroids, such as testosterone, that may be excreted by the animals) that may contribute to androgenic activity in water associated with the sites. A number of variables, including sex, age, and reproductive status of the livestock, can affect androgenic activity in animal wastes (Lorenzen et al. 2004). A biologically based fractionation approach, based on a system such as the CV-1 cells, would be a logical method to employ for further studies (Durhan et al. 2002; Hewitt et al. 2003; Jenkins et al. 2001). Given the seeming variability we observed in the nature of samples from the site, this type of work might be most productively conducted at a location(s) where inputs/discharges can be better controlled (or documented).

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Senator LAUTENBERG. Thank you very much, Mr. Grumbles.
Dr. Hirsch, please.

STATEMENT OF ROBERT M. HIRSCH, ASSOCIATE DIRECTOR FOR WATER, U.S. GEOLOGICAL SURVEY

Dr. HIRSCH. Mr. Chairman and members of the Subcommittee, thank you for the opportunity to provide the views of the U.S. Geological Survey on pharmaceuticals in the environment. I am Robert Hirsch, the Associate Director for Water at the USGS.

The observed presence of pharmaceuticals in the environment has prompted public interest regarding potential adverse ecological effects and potential contamination of drinking water. The interest has already increased public awareness of the ways we handle and dispose of our medications and has resulted in interest by industries in treatment technologies and best management practices that are most effective at removing pharmaceuticals and other trace organic chemicals from our waters and our solid and liquid wastes.

The USGS studies a wide range of chemicals of emerging environmental concern. Human and veterinary pharmaceuticals are only one class of chemicals in a group of contaminants that enter the environment via human and animal waste. These contaminants include many chemicals used in our homes, businesses and industries, including detergents, fragrances, fire retardants, disinfectants, plastics and insect repellants.

Many of these chemicals are a new focus for environmental research because they are used in relatively small quantities and therefore were not expected to be of significant environmental concern. Recent findings have demonstrated that, for example, the manner in which we handle and dispose of our wastes can concentrate those chemicals in some environmental settings to levels that may be an ecological or health concern, and that pharmaceuticals have been entering the environment for as long as we have used them.

In 1999, the USGS broadened its water quality science programs by initiating research on pharmaceuticals and other human and animal waste-related chemicals. By 2002, a USGS study had documented the presence of pharmaceuticals and other waste-associated chemicals in the Nation's streams, and this report largely defined the issue in the United States.

Since 2002, the USGS has published more than 160 reports that provide additional information on the occurrence of pharmaceuticals in various environmental settings, the sources of these chemicals to the environment, and to a lesser degree, the potential environmental health effects.

The ecological effects of some pharmaceuticals found in the environment have been documented in the scientific literature. For example, it was not a surprise when antibiotics, which are designed to act as anti-bacterials, were found to have adverse effects on soil microorganisms at environmentally relevant concentrations.

Testing also has found that some pharmaceuticals do not cause adverse effects in some organisms tested. In one study, three freshwater invertebrates were exposed to an anti-convulsant drug commonly found in the environment. Only one of the three species demonstrated an adverse effect. Significant uncertainty remains re-

garding the effects of long-term exposure to levels found in environmental settings.

Similarly, the potential human health effects of long-term exposure at the low levels of pharmaceuticals are not well understood and they do warrant continued study.

The USGS has developed the capability to analyze for approximately 70 pharmaceuticals in environmental samples. We have collected and analyzed samples from approximately 1,500 sites across the Nation. Our current research focuses on four key priorities: one, assessing chemical loads of various sources, including wastewater treatment plants, animal feeding operations, landfills and other industrial facilities; two, evaluating ecological effects, for example fish endocrine disruption in streams enriched with wastewaters or anti-microbial resistance in setting where antibiotics are released to the environment; three, assessing the occurrence of pharmaceuticals in waters that are a source of drinking water and, to a lesser extent, in treated drinking water; and four, defining the comparative performance of varying water and wastewater treatment processes to remove pharmaceuticals and other chemicals.

The USGS works with a number of Federal partners, including collaborations with the U.S. EPA, the CDC, NOAA and the Fish and Wildlife Service. As co-chair, along with U.S. EPA and the Food and Drug Administration, of the Federal Interagency Work Group on Pharmaceuticals in the Environment, we have seen increased coordination of Federal research, including discussions with the FDA to use their information from the drug approval process to prioritize the thousands of pharmaceuticals for environmental study.

Similarly, results of USGS studies of environmental occurrence are used by many scientists to guide health-effect studies to assure that actual environmental conditions are being tested.

We welcome the opportunity to provide any further information or assistance to the Subcommittee. Thank you, Mr. Chairman, for the opportunity to present this testimony. I will be pleased to answer any questions you or other members of the Subcommittee might have.

[The prepared statement of Dr. Hirsch follows:]

STATEMENT OF
DR. ROBERT M. HIRSCH
ASSOCIATE DIRECTOR FOR WATER
U.S. GEOLOGICAL SURVEY
U.S. DEPARTMENT OF THE INTERIOR
BEFORE THE
COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS
SUBCOMMITTEE ON TRANSPORTATION SAFETY, INFRASTRUCTURE
SECURITY AND WATER QUALITY

April 15, 2008

Mr. Chairman and Members of the Subcommittee, thank you for the opportunity to provide the views of the U.S. Geological Survey, Department of the Interior, on pharmaceuticals in the environment. The observed presence of pharmaceuticals in the environment has prompted public interest regarding potential adverse ecological effects and potential contamination of drinking water. The interest has already increased public awareness of the ways we handle and dispose of our medications and has resulted in interest by industries in waste treatment technologies and best management practices that are most effective at removing pharmaceuticals and other trace organic chemicals from surface and ground waters and solid and liquid wastes.

The USGS studies a wide range of chemicals of emerging environmental concern. Human and veterinary pharmaceuticals are only one class of chemicals in a new group of contaminants that enter the environment via human and animal wastes. These contaminants include many chemicals used in our homes, businesses, and industries, including detergents, fragrances, fire retardants, disinfectants, plastics, and insect repellants.

Many of these chemicals are a new focus for environmental research, because they are used in relatively small quantities, and therefore, were not expected to be of significant environmental concern. In recent years, they have been detected increasingly in the environment at very low levels (less than one part per billion). Despite these extremely low levels, investigation is warranted to determine if there are any potential adverse

environmental and human health effects. Research and monitoring by the USGS and others have demonstrated that
(1) the manner in which we handle and dispose of our wastes can concentrate these chemicals in some environmental settings to levels that may be an ecological health concern, and
(2) pharmaceuticals have been entering the environment for as long as we have used them.

In 1998, the USGS broadened its water-quality science programs by initiating research on pharmaceuticals and other human- and animal-waste related chemicals. We were spurred by the findings of European colleagues, who, looking for a pesticide, detected a heart medication in the North Sea (Buser et al., 1998). The realization that chemical-use and waste-handling practices had resulted in detectable concentrations of a drug in such a large water body suggested the need for further research. By 2002, a USGS study (Kolpin et al., 2002; Barnes et al., 2002; and Buxton and Kolpin, 2002) had documented the presence of pharmaceuticals and other waste-associated chemicals in the Nation's streams, and largely defined this issue in the United States.

Since 2002, the USGS has published more than 160 reports that:

- Document the occurrence, concentration, and mixtures of these chemicals in various environmental compartments, including stream water, well water, stream sediment, and soil amended with manure and biosolids (the solid byproduct of wastewater treatment plants);
- Demonstrate the comparative contributions from various sources, including wastewater treatment plants, livestock production and animal feedlot wastes, aquaculture, onsite septic systems, combined sewer overflows, and other industrial discharges; and
- Demonstrate that some of these chemicals are assimilated by organisms (Kinney et al., 2008) or cause adverse ecological health effects (Vajda et al., 2008).

The bibliography of USGS reports that support these findings on pharmaceuticals and other emerging contaminants in the environment is available on the Internet at: *http://toxics.usgs.gov/bib/bib-Emerging.html*.

A recent example of USGS research is described in a series of reports on the levels and mixtures of human- and animal-waste related chemicals that are found in wastewaters, biosolids, and manures, and the soils to which they are applied for fertilization, as well as the earthworms found in those soils (Kinney et al., 2006a, 2006b, and 2008).

USGS investigations at a conventional drinking water treatment facility in New Jersey described the changes in pharmaceutical concentrations from the source water through multiple stages of the treatment process (Stackelberg et al., 2004, 2007). Additional investigations like this one will inform decisions on improving existing and developing new treatment works that are more efficient at removing these compounds from source waters (the sources of drinking water).

Two USGS papers, anticipated to be published in the coming weeks in the peer-reviewed journal *Science of the Total Environment,* summarize the occurrence of these chemicals in ground water (Barnes et al., in press) and in raw (untreated) sources (streams and wells) of drinking water (Focazio et al., in press). The paper surveying source waters includes results from 74 sites near drinking water intakes in 25 states and Puerto Rico. All data from these reports will be made available to the public in accompanying data reports available on the USGS web site.

The ecological effects of some pharmaceuticals found in the environment have been documented in scientific literature For example, it was not a surprise when antibiotics, which are designed to act as antibacterials, were found to have adverse effects on soil microorganisms at environmentally relevant concentrations (Thiele-Bruhn and Beck, 2005). Testing also has found that some pharmaceuticals do not cause adverse effects on some organisms tested. In one study, three freshwater invertebrates were exposed to an anticonvulsant drug commonly found in the environment. Only one of the 3 species

demonstrated an adverse effect (Oetken et al., 2005). Furthermore, evidence suggests that chemical mixtures can act collectively to cause adverse effects, even when each component is below its individual effect level (Brian et al., 2007). These are examples of an increasing body of scientific knowledge on potential adverse health effects. Significant uncertainty remains regarding the effects of long-term exposure to levels found in environmental settings.

Endocrine disruption is one adverse health effect of concern because it may occur as a result of exposure to very low levels of hormonally active chemicals. One form of endocrine disruption observed in environmental settings affects fish reproductive systems, where fish have been found to be "feminized" by exposure to a range of chemicals that act similar to the natural hormone estrogen. Some ways in which feminization is observed in fish include: (1) elevation in the percent of fish populations that are female, (2) changes in behavioral characteristics, such as nesting behavior, or (3) the presence of male fish with female characteristics, such as the presence of female egg cells in testes or of a female egg protein in their blood. A recent study (Kidd et al., 2007) demonstrated that the addition of ethinylestradiol (one of the active ingredients in birth control pills) at observed environmental concentrations to an experimental lake in Canada caused feminization and near extinction of fathead minnows in the lake.

A wide range of hormonally active chemicals can contribute to endocrine disruption, including actual biogenic hormones, synthetic hormones (pharmaceuticals, such as ethinylestradiol), and other chemicals that mimic or block hormone function (including certain pesticides, detergents, metals, and other industrial chemicals). These chemicals have been found together in waters affected by human and animal wastes and can occur together in various environmental settings. This reinforces why these chemicals must be studied together and not as separate classes of contaminants.

The effects of long-term exposure to the low levels of pharmaceuticals found in the environment on human health are not understood and warrant continued study. The USGS has collected information on the occurrence, concentrations, and mixtures of

pharmaceuticals and other waste-related chemicals in source waters used for drinking water and, to a much more limited extent, in finished (treated) drinking water. Information provided to the Food and Drug Administration (FDA) as part of the drug approval process can be useful in identifying more potent pharmaceuticals as the target of future research on possible effects of pharmaceuticals in the environment. However, whether or not there are adverse human health effects from cumulative lifetime exposures to the low concentrations and complex mixtures of pharmaceuticals found in the environment remains a research priority, particularly the effects on sensitive subpopulations such as children, women of child-bearing years, the elderly, and people with suppressed immune systems.

The USGS has developed the capability to analyze for approximately 70 pharmaceuticals in environmental samples. We have collected and analyzed samples from approximately 1,500 sites across the Nation. About a quarter of these sites were sampled in nationally-designed targeted surveys implemented by the USGS to assess the occurrence of pharmaceuticals across a wide range of environmental settings. The majority of the 1,500 sites were sampled as parts of studies conducted by the USGS in cooperation with State and local governments. These cooperative studies were designed to provide information for local resource managers on conditions in their area, and the findings are available to the public.

California provides an example of an extensive State program. The USGS California Water Science Center, in collaboration with the California Water Boards, has designed and implemented the Groundwater Ambient Monitoring and Assessment (GAMA) Project to assess the quality of California's ground water. The Priority Basin Program, a part of GAMA, will sample approximately 2,500 wells in about 120 ground-water basins in California over an 8-year period (2004-2012). From May 2004 through December 2007, we have sampled about 1,400 wells for a very large suite of constituents, both natural and human-generated, including pharmaceuticals. About 1,000 wells are being evaluated for the presence of pharmaceuticals.

The USGS is continuing to conduct research on pharmaceuticals in the environment. Our research priorities will continue to include assessing:

- Chemical loads of various sources including wastewater treatment plants, Animal Feeding Operations, landfills, and other industrial facilities,
- Ecological effects, including fish endocrine disruption in streams enriched with wastewaters and antimicrobial resistance in settings where antibiotics are released to the environment,
- The occurrence of pharmaceuticals in waters that are the source of drinking water and, to a limited degree, in treated drinking water, and
- The comparative performance of varying water and waste treatment processes to remove pharmaceuticals and other chemicals,

The USGS conducts research on pharmaceuticals in the environment with a number of partner Federal agencies, including the U.S. Environmental Protection Agency (USEPA), Centers for Disease Control and Prevention, ~~the~~ Fish and Wildlife Service, and National Oceanic and Atmospheric Administration. The USGS, USEPA, and FDA co-chair the Federal Interagency Work Group on Pharmaceuticals in the Environment, under the auspices of the Committee on Environment and Natural Resources of the National Science and Technology Council. The Work Group has further increased coordination of Federal research. The USGS is working with our partner Federal agencies, including the FDA, to prioritize pharmaceuticals for environmental study.

Thousands of pharmaceuticals are used for human therapies and veterinary purposes. The USGS is focusing environmental research on chemicals that are more likely to be of environmental concern, to increase the efficiency of research within the existing limited resources available. Similarly, investigations of adverse health effects must consider the actual levels and mixtures of chemicals that organisms are exposed to in the environment. Results of USGS studies of environmental occurrence are used by many scientists to guide both human and ecological health-effects studies to assure that actual environmental conditions are being tested.

We welcome the opportunity to provide any further information or assistance to the Subcommittee. Thank you, Mr. Chairman, for the opportunity to present this testimony, and I will be pleased to answer questions you and other Members might have.

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Vajda, AM, Barber, LB, Gray, JL, Lopez, EM, Woodling, JD, and Norris, DO, 2008, Reproductive disruption in fish downstream of an estrogenic wastewater effluent: *Environmental Science & Technology* (published on-line March 25, 2008).

Questions from Senator Barbara Boxer:

***Question #1** – Please provide:*

A) A list of the United States Geological Survey programs that study or otherwise work on issues related to pharmaceuticals in the environment,

B) A description of the actions that each such program has undertaken related to pharmaceuticals in the environment, and

C) The proposed and enacted funding levels for each such program from fiscal year 2002 to 2009.

D) The specific programs and activities that would have to be cut or reduced, including all available details, if the President's FY 2009 budget cuts were to be enacted.

A) Five USGS programs have investigated issues related to pharmaceuticals in the environment.

1. Toxics Substances Hydrology Program
2. Cooperative Water Program
3. Contaminant Biology Program
4. National Water Quality Assessment Program
5. Fisheries: Aquatic and Endangered Resources Program

B) A description of actions of each Program:

USGS investigations related to pharmaceuticals are conducted by a range of programs that bring varied expertise to bear on a complex set of issues. For example, interdisciplinary investigations of endocrine disruption are conducted jointly by all of the following programs.

Toxic Substances Hydrology (Toxics) Program: As the main USGS Program primarily responsible for water-quality research and methods development, the Toxics Program plays an important role in USGS investigations of pharmaceuticals in the environment. Scientists partially funded by the Toxics Program initiated research in 1998 and published a paper on the occurrence of pharmaceuticals and other emerging contaminants in U.S. streams in 2002. Since this research began in 1998, Program scientists have published over 160 papers on the occurrence of these chemicals in the environment, the relative contributions of different sources (wastewater treatment plants, septic systems, animal feeding operations, landfills, etc.), and potential ecological effects (including endocrine disruption in fish and antimicrobial resistance). Many of these papers were published in cooperation with local projects with state and local governments supported by the Cooperative Water Program. See the following searchable Internet bibliography to access these papers http://toxics.usgs.gov/bib/bib-Emerging.html

Cooperative Water Program (CWP): The Cooperative Water Program provides a means for local water managers to work with USGS, use USGS capabilities, and collect additional information on pharmaceuticals in environmental settings in their area. USGS national studies provide information on local settings and enable local governments to determine their highest priority information needs. Subsequent studies done under cooperative agreements with state and local governments provide additional detail in areas of local concern. Of the 1500 environmental samples analyzed for pharmaceuticals, approximately two thirds were collected under the CWP in collaboration with researchers supported by the Toxics Program. The information that results

from these CWP studies is then integrated with USGS national datasets to provide a significantly improved capability to assess pharmaceuticals in the Nation's water resources.

Contaminant Biology (Contaminants) Program: The Contaminants Program conducts toxicological research on the effects of pharmaceuticals and other wastewater related chemicals on fish in the laboratory and field. The Program collaborates with other USGS programs and with other Federal and State Partners in interdisciplinary studies to identify the cause and extent of intersex and other forms of endocrine disruption. The Program has also developed endocrine biomarker methods for monitoring effects in the field and passive sampling systems to measure short-term pulses of pharmaceuticals and trace organics. Such short-term pulses may represent important exposures to fish during sensitive life stages and would otherwise be difficult to measure.

National Water Quality Assessment (NAWQA) Program: The NAWQA Program does not currently monitor for pharmaceuticals. However, the NAWQA Program includes new contaminants in its assessment activities after assimilating initial information made available by the Toxics and other programs. In the future, this could include pharmaceuticals currently researched in the Toxics program.

Fisheries: Aquatic & Endangered Resources (Fisheries) Program: This Program has conducted fish health studies related to potential endocrine disruption as demonstrated by discovery of intersex fish at a number of sites. USGS researchers have measured the amounts of reproductive hormones in fish and conducted histological analyses to confirm the presence of intersex and functional abnormalities in fish in sensitive environmental settings across the nation. The Program has also started experimental studies to determine the mechanism for the development of endocrine disruptor-induced intersex in largemouth bass.

C) The proposed and enacted funding levels for 2002-2009 are as follows:

USGS Program Funding: Proposed & Enacted, 2002 - 2009 (Dollars in Thousands)

Fiscal	Toxics		NAWQA		CWP		Contaminants		Fisheries	
Year	Proposed	Enacted	Proposed	Enacted	Proposed	Enacted	Proposed	Enacted	Proposed	Enac
2002	3,919*	13,919	44,579	63,986	64,318	64,318	11,900	11,900	16,200	16,2
2003	0	13,437	57,312	63,217	64,339	64,433	12,000	10,800	16,200	27,4
2004	11,065	14,902**	63,818	63,285	64,536	63,995	10,200	10,700	24,900	26,7
2005	12,638	14,476**	62,506	61,645	63,007	62,337	9,800	9,600	25,800	24,9
2006	13,120	14,386**	63,132	62,203	63,770	62,833	10,700	9,700	26,700	24,2
2007	13,215	13,293	62,571	62,818	62,171	64,345	8,900	9,100	21,900	23,8
2008	13,730	13,516	64,925	63,912	62,381	62,849	8,400	8,700	21,700	23,2
2009	10,704***	TBD	54,113	TBD	62,285	TBD	8,500	TBD	22,800	T
Total '02-'08	**67,687**	**97,929**	**418,843**	**441,066**	**444,522**	**445,110**	**71,900**	**70,500**	**153,400**	**166,4**

* 10,000 proposed to be redirected to the National Science Foundation.

** Earmark for Tar Creek contamination site / University of Oklahoma: 2004 - 1,482; 2005 - 1,460; 2006 - 1,213.

*** 2,257 represents a funding shift for Priority Ecosystem Studies from Toxics to Biological Research & Monitoring

D) The Administration's 2009 budget proposal focuses USGS research on issues of societal relevance, including funding for the first National Water Census in 30 years. The Budget reflects the President's commitment to reduce the deficit and balance the Federal budget by 2012. Under the proposed budget, the following programs and activities will be impacted.

Toxic Substances Hydrology (Toxics) Program: The Toxics program provides reliable scientific information and tools that explain the occurrence, behavior, and effects of toxic substances in the Nation's hydrologic environments. The Toxics program works in partnership with other Federal agencies to ensure that priorities for science needs are coordinated, including other Interior bureaus, the Environmental Protection Agency, the United States Department of Agriculture, Department of Defense, Department of Energy, the Nuclear Regulatory Commission, and public health agencies such as the CDC, FDA, and the National Institute for Environmental Health Sciences. A decrease of $2,257,000 shifts funding for research on Priority Ecosystems Studies to the Biological Research and Monitoring subactivity. Increased funds in this subactivity will continue to focus on research in areas such as the Everglades, the Chesapeake Bay, and the San Francisco Bay.

The remainder of the decrease supports the interagency Amphibian Research and Monitoring Initiative (ARMI). These resources provide water-quality information that supports investigations into the causes of declining amphibian populations and the causes of the increasing occurrence of populations with excessive limb deformities. A portion of the decrease not associated with ARMI would reduce some Toxics program research on contamination from hard-rock mining, pesticides, and emerging contaminants. A planned national evaluation of contamination from nutrients, pathogens, pharmaceuticals and other emerging contaminants at animal feeding operations would be postponed.

Cooperative Water Program (CWP): The reduction to the Cooperative Water Program is $564,000 below the 2008 enacted level. This could result in fewer interpretive studies of water resources issues, starting with studies that were scheduled to begin or move into their next phase in 2009. At least 720 studies will continue at the proposed 2009 level. As much as possible, the USGS will avoid ending studies before their planned completion date. Topical areas for these studies could include environmental restoration work such as the Chesapeake Bay study, water availability assessments such as the California Aquifer Storage and Recovery assessment, hazard evaluations such as the Georgia-Carolina flood frequency analysis, and environmental flow studies that provide support to salmon recovery decisions in Washington State and elsewhere.

Contaminant Biology (Contaminants) Program: A number of USGS research and monitoring efforts are funded from both the Fisheries Program and the Contaminant Biology Program. Funds within the contaminant Biology Program for research on endocrine effects were reduced by $400,000 in 2008 to $246,000. A reduction in Contaminant Biology proposed in 2009 of $246,000 related to endocrine disruptors and other reproduction effects will eliminate funds that support research to determine cause-and-effect relationships concerning intersex in the Chesapeake Bay and other research on reproductive effects, research on flame retardants in the Columbia and Willamette Rivers, restoration-related contamination in snail kites in the Florida Everglades, and pesticides effects on endangered sage grouse reproduction.

National Water Quality Assessment (NAWQA) Program: At the funding level proposed for 2009, the NAWQA program would continue the following activities:

- Data collection, assessments, and research for surface-water conditions and trends would continue, including stream-quality monitoring at 113 sites and aquatic biological data-collection at 58 sites.
- Water-quality models for major river basins, (e.g., the Mississippi) would continue to be improved, in cooperation with EPA, leading to the identification of priority watersheds and key point and non-point sources contributing the largest amounts of nitrogen and phosphorus to the northern Gulf of Mexico and Atlantic coastal waters.
- Aquatic-life benchmarks for about 40 pesticides would continue to be developed, in coordination with EPA, to be adopted by the States in their implementation of the Federal Insecticide, Fungicide, and Rodenticide Act.
- Comprehensive national summary reports would be produced on the quality of streams ecosystems across the U.S., on conditions and trends in nutrients in streams and ground water, and on the quality of source water used for drinking water, including in domestic and public-supply wells and at surface-water intakes.
- An aquatic ecological data system for all USGS water program data would be released based on an enhancement of the existing NAWQA Data Warehouse.

Activities that may be deferred, discontinued or reduced include long-term data collection for ground water and source water used for drinking. These reductions could discontinue some assessment of trends in ground water and domestic and public supply wells in principal aquifers under study. These trend evaluations may include topics such as nitrate and MTBE (a gasoline additive) in streams and ground water. The changes are not expected to affect water quality monitoring by EPA that is mandated by law.

***Question #2** – In 1999, the National Academies of Sciences stated, "[T]he use of antibiotics in food animals could contribute to the emergence of antibiotic-resistant microorganisms in animals that could be transmitted to humans and result in infections that could be difficult to treat."*

Researchers have found evidence of antibiotic resistant bacteria in waterways and groundwater near factory farms-so-called Concentrated Animal Feeding Operations. Please describe the benefits of systematically monitoring waterways and groundwater near such facilities, in order to assist the federal government to better understand the potential human health and environmental threats related anti-biotic resistance.

Antimicrobial resistance is more commonly understood in the context of people and animals given doses of antimicrobials for therapeutic purposes or to enhance growth. The environmental release of antimicrobials and the environmental release of antimicrobial resistant microbes from human sources and animal sources, where antibiotics are given to the animals at therapeutic and sub-therapeutic (growth-promotion) doses, also may be a factor in the spread and maintenance of antimicrobial resistance.

A systematic investigation of small watersheds with common types of animal feeding operations (AFOs) where antimicrobials are used including analysis of processes and effects at representative watersheds would: (1) define the levels and mixtures of antimicrobials and antimicrobial resistance microbes that are present in the environment near AFOs; (2) characterize the environmental transport, fate, and effects of antimicrobials; (3) determine whether exposure to environmental levels of antimicrobials would be associated with the presence of antimicrobial resistance in microbes in the environment; (4) determine whether antibiotic resistant pathogens spread from AFO related sources to the environment; and (5) determine whether antibiotic-resistance genes in microbes released from animal feeding operations transfer to other organisms in the environment, further spreading antimicrobial resistance. The results of such an investigation would permit determination of the relative importance of the role of the natural environment in the development and spread of antibiotic resistance. The results would provide the information needed to determine if additional, routine monitoring is needed, and enable that monitoring to be designed in a manner that is focused on the most important issues and in a cost-efficient manner.

***Question #3** – The USGS reports that it can test for approximately 70 pharmaceuticals, and has developed a new laboratory method to measure trace levels of 22 human-health pharmaceuticals in surface and ground water. Please describe whether the USGS's technology can be used to systematically monitor surface waters across the nation.*

USGS conducts systematic national water quality monitoring activities for a range of traditional contaminants, including nutrients, metals, pesticides, and volatile organic chemicals (VOCs). These activities have the benefits of a consistently trained and coordinated team of field specialists highly skilled in efficient collection of accurate and representative environmental data. Furthermore, a consistent set of quality assurance and quality control measures are applied to all the data. The new methods for pharmaceutical and other organic wastewater contaminants can be implemented for systematic monitoring of pharmaceuticals in our Nation's water resources, including ground water, surface water, and drinking water.

***Question #4** – Does the USGS have the technical capabilities to map out the major sources of pharmaceuticals in our nation's waterways and provide the information to EPA in a way that could be used to help develop a monitoring and regulatory program?*

USGS has the capabilities to provide such information to EPA in a manner that would be useful to their management and regulatory decisionmaking. USGS currently analyzes for about 70 pharmaceutical compounds, as well as a significant number of other organic wastewater contaminants that are released to the environment together via the same source pathways (wastewater treatment plant discharges, releases from animal feeding operations, septic systems, combined sewer overflows, etc.). USGS activities to develop additional methods currently are focusing on additional pharmaceuticals and other chemicals that are present in these sources and have the greatest potential for adverse health effects.

USGS has conducted preliminary source characterizations at some sources, such as wastewater treatment plant discharges and animal waste lagoons. Additional characterization would be

needed at these sources for a broader range of chemical and biological contaminants and at other sources that have not as yet been studied.

Question #5 –
A) Has the USGS found that known or suspected endocrine disrupting chemicals can move through the ground and into groundwater aquifers and surface waters, such as lakes and rivers?

B) Please provide copies of studies showing that movement of such contaminants is possible.

Yes. The USGS has studied the environmental distribution of many contaminants that have been found to cause reproductive, developmental, or neurological effects in aquatic organisms and are suspected to be endocrine disruptors. These chemicals include biogenic hormones (made in the bodies of vertebrates), synthetic hormones (such as the active ingredients in birth control pills), and a wide range of chemicals that may mimic the behavior of hormones because of similarities in their molecular form. These hormone mimics include some pesticides, industrial chemicals, household chemicals, and other pharmaceuticals. Using the scientific literature, USGS identified over 75 chemicals (not counting PCBs and furans) that are acknowledged as potential endocrine disruptors, for which USGS analyzes in environmental waters. Our most recent investigations include for biogenic and synthetic hormones, which are measured at extremely low levels in the environment (the part-per-trillion level), levels at which they are active. Our national and local studies of water-quality conditions routinely include information on many of these compounds. National surveys include analyses for approximately 38 compounds that are suspected to mimic hormones. Recent national reports on pesticides (Gilliom and others, 2006) and volatile organic compounds (Zogorski and others, 2006) in the Nation's waters are just 2 recent examples of the results of these studies. Future National assessments will be able to use the newest laboratory methods to add biogenic and synthetic hormones to the list of chemicals they already measure. Research and methods development continually is increasing the number of endocrine disrupting chemicals that USGS is able to measure in environmental samples. These methods development activities are focusing on collecting data on environmental occurrence and effects of potential endocrine disruptors in susceptible settings, such as near wastewater treatment plant discharges to streams and in streams in areas of intensive animal agriculture.

USGS studies of endocrine disruption in fish focus on environmental settings that are susceptible to endocrine disrupting chemicals and are relating evidence of endocrine disruption to environmental occurrence of potential endocrine disrupting chemicals to determine if and when a cause-and-effect relationship exists. Some of these studies include the Minnesota River, MN (Schoenfuss and others, 2008 and Barber and others 2007); Boulder Creek, CO (Vajda and others, 2008; Barber and others, 2006; and Murphy and others, 2003); the Potomac River (Alvarez and others, 2008 and Chambers and others, 2006) and Lake Mead (Rosen and others, 2006).

National Assessments (NAWQA Program):

Gilliom and others, 2006, The Quality of Our Nation's Water—Pesticides in the Nation's Streams and Ground Water, 1992–2001—A Summary. U.S. Geological Survey Circular 1291, 172 p. http://pubs.usgs.gov/circ/2005/1291/

Zogorski, J.S., and others, 2006, The Quality of Our Nation's Water—Volatile organic compounds in the Nation's ground water and drinking-water supply wells. U.S. Geological Survey Circular 1292, 101 p. http://water.usgs.gov/nawqa/vocs/national_assessment/

Site Studies:

Boulder Creek

Murphy, S.F., Verplanck, P.L., and Barber, L.B., 2003, Comprehensive water quality of the Boulder Creek Watershed, Colorado, during high-flow and low-flow conditions, 2000: U.S. Geological Survey Water-Resources Investigations Report 03-4045, 198 p. http://wwwbrr.cr.usgs.gov/projects/SWC_Boulder_Watershed/

Barber, L.B., Murphy, S.F., Verplanck, P.L., Sandstrom, M.W., Taylor, H.E., and Furlong, E.T., 2006, Chemical loading into surface water along a hydrological, biogeochemical, and land use gradient--A holistic watershed approach: Environmental Science and Technology, v. 40, no. 2, p. 475-486. http://pubs.acs.org/cgi-bin/abstract.cgi/esthag/2006/40/i02/abs/es051270q.html

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., and Norris, D.O., 2008, Reproductive disruption in fish downstream from an estrogenic wastewater effluent: Environmental Science and Technology, doi:10.1021/es0720661 (Advanced Web release). http://pubs.acs.org/cgi-bin/abstract.cgi/esthag/2008/42/i09/abs/es0720661.html

Minnesota River

Barber, L.B., Lee, K.E., Swackhamer, D.L., and Schoenfuss, H.L., 2007, Reproductive responses of male fathead minnows exposed to wastewater treatment plant effluent, effluent treated with XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds: Aquatic Toxicology, v. 82, no. 1, p. 36-46. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T4G-4MXJ3RP-1&_user=696292&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000038819&_version=1&_urlVersion=0&_userid=696292&md5=9714792eaedfb2710b6b97c46e5467db

Schoenfuss, H.L., Bartell, S.E., Bistodeau, T.B., Cediel, R.A., Grove, K.J., Zintek, L., Lee, K.E., and Barber, L.B., 2008, Impairment of the reproductive potential of male fathead minnows by environmentally relevant exposures to 4-nonylphenol: Aquatic Toxicology, v. 86, no. 1, p. 91-98. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T4G-4PWF0FX-3&_user=696292&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000038819&_version=1&_urlVersion=0&_userid=696292&md5=938335f5e4d5e14e67d09a1c7dda6108

Potomac River

Alvarez, D.A., Cranor, W.L., Perkins, S.D., Schroeder, V.L., Werner, S.L., Furlong, E.T., Holmes, J., 2008, Investigation of organic chemicals potentially responsible for mortality and intersex in fish of the North Fork of the Shenandoah River, Virginia, during spring of 2007: U.S. Geological Survey Open-File Report 2008–1093, 16 p. http://pubs.usgs.gov/of/2008/1093/

Chambers, Douglas B.; Leiker, Thomas J., 2006, A Reconnaissance for Emerging Contaminants in the South Branch Potomac River, Cacapon River, and Williams River Basins, West Virginia, April-October 2004: U.S. Geological Survey Open-File Report 2006-1393, 23 p. http://pubs.usgs.gov/of/2006/1393/

Lake Mead

Rosen, M.R., Goodbred, S.L., Patiño, R., Leiker, T.J., and Orsak E., 2006, Investigations of the effects of synthetic chemicals on the endocrine system of common carp in Lake Mead, Nevada and Arizona, U.S. Geological Survey Fact Sheet 2006-3131, 4 p. Revised Sept 2007. An overview of existing papers - http://pubs.usgs.gov/fs/2006/3131/

***Question #6** – The Proceedings of the National Academy of Sciences published a study in May, 2007 that found that long-term exposures of certain fish to low concentrations (5-6 parts per trillion) of estrogen steroids "led to the feminization of males... and, ultimately a near extinction of the species in" a lake in which the study occurred. Do these types of studies raise serious questions regarding the impacts of exposures to some chemicals in extremely small amounts?*

It has been found that some endocrine disruptors, specifically biogenic or synthetic hormones, can be active at low levels. This study (Kidd and others, 2007) was particularly persuasive because the synthetic estrogen was added to an experimental lake at low levels and adverse effects on fathead minnows were documented, from early evidence of intersex to eventual population decline. An important factor related to the low-level effects of these compounds is that their effects can be additive. That is, as Vajda and others (2008) demonstrated, the effect of exposure to multiple chemicals with similar modes of action, such as estrogens or chemicals that mimic estrogens is the sum of the effects of the individual chemicals.

Questions from Senator Cardin:

***Question 1** – Given that the USGS has identified pharmaceuticals and other chemical compounds in water, why were the Toxic Substances Hydrology Program and the National Water Quality Assessment Program both scheduled for substantial funding reductions or redirections in fiscal year 2009?*

The Administration's 2009 budget proposal continues to focus USGS research on issues of societal relevance, while reflecting the President's commitment to reduce the deficit and balance the Federal budget by 2012. The budget for the USGS water resources discipline proposes $203.0 million to continue high priority work on issues related to water availability, water quality, and flood and drought hazards. A proposed reduction of $9.8 million to the National Water-Quality Assessment (NAWQA) Program could suspend long-term monitoring of ground-water quality until data analysis and reporting on prior year work is completed. Suspension of this monitoring will allow the USGS to focus its resources on maximizing results from these prior-year investments. Surface water quality monitoring, which makes up a much larger part of NAWQA program activities, will continue at current budgetary levels.

Most of the work associated with the $3.0 million in Toxic Substance Hydrology was in Priority Ecosystems which is receiving an increase under Biological Research and Monitoring. The

remaining $806,000 reduction would terminate research on the hydrologic and water quality factors that affect amphibian populations in support of the Amphibian Research and Monitoring Initiative and would reduce research on emerging environmental contaminants, new and understudied pesticides, and contamination from hard rock mining.

Question 2 – How do these funding reductions and redirections reflect the priorities the USGS has in monitoring of water?

These changes are consistent with the Administration's emphasis on high priority water quality and water quantity monitoring activities that are useful on multiple scales. Long-term surface water monitoring would continue at all 113 NAWQA Program sites, and ecological sampling would continue at all 58 NAWQA Program stream sites. Toxics Program research on emerging environmental contaminants, new and understudied pesticides and contamination from hard rock mining would all continue.

Questions from Senator James M. Inhofe:

Question 1 – In your research on affects in fish populations, did your studies decipher which hormones were related to pharmaceuticals and which naturally occurred through normal biological means in human and animal populations?

USGS measures these chemicals separately and can document the levels of different chemicals, including biogenic hormones (those made by vertebrate organisms), synthetic hormones (those manufactured to act like a hormone, such as the active ingredient in birth-control pills), and hormone mimics (other chemicals that can act to different degrees like hormones because of molecular similarities).

Question 2 – Does USGS contemplate actual health affects or only analyze occurrence data?

The USGS conducts extensive studies of environmental occurrence of contaminants (examples provided in answers to previous questions); conducts field assessments of the occurrence of intersex in aquatic organisms (for example, Papoulias and others, 2006; Blazer and others, 2007), conducts laboratory toxicological studies of potential ecological effects (for example Papoulias and others, 2003, effects of DDT; Wright and Tillitt, 1999, effects of mixtures of dioxins, furans and PCBs); and conducts field studies of the links between environmental occurrence of potential endocrine disrupting chemicals and adverse health effects in local aquatic organisms, such as fish intersex (Vajda and others, 2008 and Barber and others, 2007, referenced above), and monitors for indicators of endocrine disruption in fish (for example, Hinck and others 2007).

Blazer, V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith, D.R., Young, J.A., Hedrick, J.D., Foster, S.W., and Reeser, S.J., 2007, Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages: Journal of Aquatic Animal Health, v. 19, no. 4, p. 242-253, doi:10:1577/H07-031.1. http://afs.allenpress.com/perlserv/?request=get-abstract&doi=10%3A1577%2FH07-031.1

Papoulias, D.M., Chapman, D., and Tillitt, D.E., 2006, Reproductive condition and occurrence of intersex in bighead carp and silver carp in the Missouri River. Hydrobiologia, v. 571, no. 1, PP.355–360.
http://www.ingentaconnect.com/content/klu/hydr/2006/00000571/00000001/00000260

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Senator LAUTENBERG. Thank you very much, both of you.

Mr. Grumbles, a recent environmental working group study showed that there are over 140 contaminants in our water supply that are unregulated and that EPA has not set safety standards for. In your comments, I heard you talk about the lack of data about some of these materials that we find in our water. Some of these chemicals, such as MTBE, gasoline additive, we note that that is a matter of great concern and can potentially cause cancer. That is acknowledged by the EPA.

So how do you classify materials like this, chemicals like this, as cancer threats and then not propose any regulation about them?

Mr. GRUMBLES. Mr. Chairman, I think the Committee and your counterparts in the House got it right in 1996, when you laid out a new process, an entirely new process under the Safe Drinking Water Act to use the best available science to focus on occurrence and health effects and if there were meaningful opportunities to reduce risk based on contaminants in the water supplies.

We have been working through that new process. We think it works. It takes time. It takes a lot of time and effort. We are systematically reviewing hundreds and hundreds of chemicals for potential listing under the Safe Drinking Water Act. MTBE and others are good examples of ones where we are required to go through risk assessment, to develop based on the best available peer-reviewed science we have, and then go through a screening process.

I can tell you, for pharmaceutical we are very interested in the potential listing of pharmaceuticals under the contaminant candidate listing process. We have not to date done so with one exception, nitroglycerin, but it is a proposed list and we are seeking additional comment from the public and from the scientific sector on the information, the data, the risks, and if there is a meaningful opportunity to reduce risk. If there is, then we would propose them for listing using the statutory criteria under the Safe Drinking Water Act.

Senator LAUTENBERG. Yes, but if these materials can be identified as cancer-causing in some instances, how do you determine what the pace of review should be? Is there some numerical sequence that just says, OK, we will go down these lists and see what we find and when we get to list item 34, that, oh, yes, this one may be real dangerous. How do you determine what to go to first?

Mr. GRUMBLES. For us, the science should drive the result and the greater the risk, then the greater the likelihood of taking action or regulatory action under the Safe Drinking Water Act. There are a variety of factors that we look at. We also in the new process that we have developed under the contaminant candidate listing, are seeking greater consultation from the science advisory boards in the scientific community.

When there are specific threats or cancer risks, that gets our attention. That gets our attention to put a higher priority on the process.

Senator LAUTENBERG. On the process. How about the product?

Mr. GRUMBLES. Well, the process is——

Senator LAUTENBERG. You say it gets your attention. Well, OK.

Mr. GRUMBLES. It gets our attention to go through and make a decision after we get an appropriate amount of information on the

type of risk to human health, and that involves a risk assessment. We need to also make determinations based on the level of occurrence. Do they occur at a frequency and at a level that is of concern to public health? And then the Administrator is charged with making the determination as to whether or not there is a meaningful opportunity to reduce risk.

Senator LAUTENBERG. If we take something like MTBE, MTBE was said to cause cancer in animals many years ago. What do we way about MTBE's presence in our drinking water?

Mr. GRUMBLES. I think it is important. You are making a good distinction, too, that MTBE is not a pharmaceutical. There are a lot of potential chemicals in water that should receive review and they are receiving review by the agency. We are concerned as well about pharmaceuticals, but our higher focus has been on other chemicals and microbes.

MTBE, our research and development office is carrying out and completing its risk assessment. I am not sure what the timeframe is for that, but they need to gather additional information on the risk so that we can then make a determination.

We do have some standards or guidelines on MTBE, but it is not through the MCL process. It takes into account concerns about odor and taste, other aspects of drinking water.

Senator LAUTENBERG. No, we are talking about MTBE is said to be cancerous in animals. It was done in 1994. So what are we discussing now about an evaluation?

Mr. GRUMBLES. Well, we think MTBE is a pollutant that needs to be kept out of the environment, so we use various regulatory tools to prevent the pollution in the first place; in terms of safe drinking water or potential MCL regulation, setting a maximum contaminant level under the Safe Drinking Water Act. Where we are on MTBE is that the agency is carrying through on the additional research involved in the risk assessment for risk to human health, which is part of the criterion under the Safe Drinking Water Act.

Senator LAUTENBERG. So is it appropriate to say that there is no cause for concern about human health, even though EPA itself said that it could cause cancer in animals? This now is 14 years ago. At what point do you say, wow, this stuff is bad for you?

Mr. GRUMBLES. Well, I say it after I get the complete results from the scientific experts at the agency, in coordination with others. Certainly, Mr. Chairman, we are concerned about potential threats from environmental contamination by MTBE. But in terms of the data and information, I am not qualified to make some judgment about the risk to human health. We are concerned. We do have a risk assessment process underway.

Senator LAUTENBERG. Your concern is not comforting, I can tell you that. Action is what we are trying to get here.

I will call on my colleague, Senator Vitter, now for his questions, and we will come back again.

Senator VITTER. Thanks, Mr. Chairman.

Mr. Grumbles, I take it from your testimony you think the appropriate focus is on a somewhat broader issue than the topic of this hearing. This hearing is called Pharmaceuticals in the Nation's Waters. I assume you think we need to be concerned with a num-

ber of things that may be in the Nation's waters, whether they are pharmaceuticals or other contaminants, figuring out if they are a danger to human health in the amounts in which they may appear in the Nation's waters. Is that fair to say?

Mr. GRUMBLES. Yes.

Senator VITTER. OK. Based on what we know so far, are pharmaceuticals as a class of more concern than all the other stuff that you are also looking at? How would you make that distinction? How do you prioritize the scientific work with regard to pharmaceuticals versus other possible contaminants?

Mr. GRUMBLES. I start by reiterating that America's water supplies are among the safest in the world. One of the greatest buys for Americans today is municipal tap water because of the cost and the safety and cleanliness of it.

We always face challenges and for years and to this day, the biggest challenges continue to be various types of microbial or industrial chemicals of concern. Pharmaceuticals and personal care products are certain types of chemical that are an emerging concern. We are concerned. We are not alarmed. We have known for years as a scientific agency that there are truly tiny trace amounts of pharmaceuticals in water.

We feel it is very important to be aggressive, to be aggressive in stepping up the amount of research and product stewardship. The pharmaceutical industry and municipal water utilities need to do more, just as we are doing more. One of the key messages from the U.S. EPA is pollution prevention and product stewardship.

That is why though the guidance issued in January 2007 that HHS, EPA and the Office of National Drug Control Policy issued is important because it has the basic message of the toilet should not be a trash can. You should not dispose of unwanted or unused pharmaceuticals by flushing them down the toilet. There are a handful of exceptions, controlled substances, that specifically on the label say flush in the toilet because of concerns about security and getting into the hands of those in violation of the Controlled Substances Act. But the general message should be product stewardship, don't flush it down the toilet, and properly dispose of it. Sometimes that means securing it, like in kitty liter or coffee grounds, and putting it in a bag and disposing of it in the trash.

We think it is very important. We look to work with the Committee to encourage stewardship across this Country in terms of voluntary take-back programs that comply with the Controlled Substances Act, but that also get the pharmacies more involved, and encourage the pharmaceutical industry to use a life-cycle analysis and take ownership in helping to return unused or unwanted pharmaceuticals so they don't get into the sewer systems or in the water supplies of this Country.

Senator VITTER. OK. Thank you.

To followup on the Chairman's question, and he was specifically using the MTBE example, I take it that a big issue there, or in a lot of these cases, is in what amounts we find any of this in any water, and whether a true trace amount is a danger to human health. Is that correct? I assume dosage is a huge part of this whole discussion and research.

Mr. GRUMBLES. That is exactly right, Senator. One of the reasons why one of the prongs of our action plan is risk communication, is to help increase public understanding of the context of the risks involved. The dose makes the poison, essentially. The dose is the critical factor. While we should be concerned about the pharmaceuticals in water, we should also remember that these are showing up in truly trace amounts and that we don't have any evidence to date of a risk to human health.

That doesn't mean we should stop looking. It doesn't mean we should stop doing the research, and there is a lot of research that needs to be done, and actions as well. In my office, we are focused on looking at regulatory tools and also increasing public and private stewardship of the products. The dose makes the poison. It is the level and that is one of the key components to keep in mind.

When you are talking about industrial chemicals or microbials, one of the reasons why over the last several years we have issued final rules under the Safe Drinking Water Act is to get at those known, clear risks like protozoa or other types of bacteria or viruses that can show up in surface water or groundwater, or industrial chemicals or disinfection byproducts as a result of chlorination. Those have been our priorities. Pharmaceuticals are an emerging concern and a growing priority for us, but at this point the scientific jury is still out on just how great a risk it presents to public health.

Senator VITTER. OK.

Dr. Hirsch, sort of following up on that, you said in your testimony that the effects of long-term exposure to the low levels of pharmaceuticals found in the environment on human health are not understood and warrant continued study. Looking to USGS in particular, what do you think would be the most productive things for you all to do with regard to that study?

Dr. HIRSCH. I will say two things. One of the things that really we try to focus on is trying to understand the relevant levels that we would expect to find in the environment. We look across a range of environments from those that we would expect to see some of the highest concentrations, and also places where we would expect to see the lowest, to help inform the agencies that have responsibility for testing and regulation in this area, particularly EPA, but also the Food and Drug Administration, to understand what those environmentally relevant levels are so that the testing can be done at those levels.

We also do ecological effects research in our Biological Division, where we do look at the effect of a variety of chemicals, including these, the pharmaceuticals, on a variety of aquatic organisms, not for purposes of understanding human health, although it may be relevant there, and can be extended to that, but directly to understand the effects on certain kinds of species to see whether, for example, there is endocrine disruption and reproductive effects on various species. Those are areas that we are working on.

Senator VITTER. OK. Thank you very much.

Thank you, Mr. Chairman.

Senator LAUTENBERG. Senator Boxer.

Senator BOXER. Thank you.

Mr. Grumbles, you say the toilet shouldn't be a trash can for unused medications. At the same time you are saying that, the White House Office on National Drug Control Policy says the FDA advises the following drugs be flushed down the toilet.

So look, you don't know what your other hand is doing. I understand they are looking at it a different way. Get together. Get together. After this AP story scared everybody, including you, you said you are concerned, don't come up here and say don't use the toilet to flush your drugs down when you have a whole other arm of your very own Administration telling people to flush the drugs down the toilet.

There are a lot of words here between the two of you. Not much is getting done. Let's face it. And conflicting messages are going out.

Mr. Grumbles, you said very nicely that you really are concerned. I appreciate that. I really do. Then why is it that your budget slashed funding for EPA endocrine-disruptive chemical testing program that Dr. Hirsch said is so important? Why did you cut it 35 percent? And why did Mr. Johnson come up here and say he was fine with the cuts across the board, whatever they were for EPA?

Mr. GRUMBLES. Two points or two responses. One, I acknowledged, Madam Chair, that on the guidance that we issued, which is not cast in stone, it can be revised; the guidance we issued we said there are some specific pharmaceuticals that on the label say flush them down the toilet. It is because of the Controlled Substances Act. The Federal agencies did work together and we are committed to working with the pharmaceutical industry to remove some of those from the list so that they don't continue to say flush it down the toilet.

Senator BOXER. OK, sir, sir, sir. I am just making a point in evidence, that while you said, and I have your testimony here, you said it. You didn't couch it. You said don't use the toilet as a trash can. At the same time, another entity is saying flush these down the toilet.

Now, what I am suggesting is, I understand the reasons they are saying it. Believe me, I do. I understand why oxycontin is so dangerous you don't want it lying around. I understand. I get it. But there has to be a way for agencies to get together. When a story like this breaks, why is it necessary for Senator Lautenberg to have to call a meeting of the Subcommittee? Why aren't you working day and night on this? Why aren't you saying to me today, we have had a meeting with the FDA, we are working together, we are coming to you as Chairman and Subcommittee Chair, we are going to work together.

I am with you on a voluntary program. Let's do it. But this is what is going on and why people do get upset with their government.

So if we could get on to the budget cuts, if we could. Because we will work together with you to square this away.

Mr. GRUMBLES. Thank you.

Senator BOXER. But if you could please tell us about these budget cuts in this very important program that Dr. Hirsch said we are doing the work. How does he do the work when you are giving him a 35 percent cut?

Mr. GRUMBLES. In putting together a $7 billion budget, there are tough decisions that have to be made.

Senator BOXER. Obviously.

Mr. GRUMBLES. Another example is there are programs that have worked well, that we initiated funding in, that have matured or have graduated into being self-sustaining programs on the funding such as the work with healthy hospitals.

Senator BOXER. I am talking about a 35 percent cut to the endocrine-disrupter chemical program, just answer this, where you were supposed to start the work in 1999. You haven't listed anything. So I do not understand how you can propose and live with a 35 percent cut in this program that Dr. Hirsch says is important.

Mr. GRUMBLES. What I know is that the agency has proposed listing 73 pesticides and we are moving forward with that program. They did get off to a slow start. What I don't know, Madam Chairman, and I am not in a position to give you the specifics on, but I know that we will followup with specifics describing the 35 percent cut or how it was made.

Senator BOXER. I think it speaks for itself, with open checkbook for Iraq, open checkbook for them, but we can't find a few dollars here to protect the public from what could be a threat to pregnant women and children. That is rather stunning.

Mr. Grumbles, your testimony refers to the White House-convened Pharmaceuticals in the Environment Working Group. This group was supposed to make recommendations last December. Has that happened?

Mr. GRUMBLES. My understanding is that the schedule is to make recommendations to develop a research strategy by the end of this year.

Senator BOXER. Have they made the documents public? It is my understanding they have made no documents public.

Mr. GRUMBLES. I am not sure what the status is.

Senator BOXER. Do you think they should make their documents public, this working group, the Pharmaceuticals in the Environment Working Group? Will you provide this Committee with all EPA records related to the Pharmaceuticals in the Environment Working Group within 10 days?

Mr. GRUMBLES. I can't answer the specifics, Madam Chair, because I don't know what documents we are talking about. What I know is that the group——

Senator BOXER. Any and all documents that this group worked on. We have a job here to do, oversight. Will you make those documents available to us? Because no one else can get them.

Mr. GRUMBLES. Well, I think the right entity to ask that question is the Office of Science Technology Policy because they are the ones overseeing it.

Senator BOXER. No. I am just asking for the EPA records. I am not asking you for others. You are one of the co-chairs of that working group, not you personally.

Mr. GRUMBLES. Not me personally.

Senator BOXER. Not you personally, but EPA.

Mr. GRUMBLES. Madam Chair, what I would commit to you is that EPA in terms of its role and in its involvement in that task force, when I get back from the hearing, I will talk with those who

are, the Research and Development Office and others in the agency that are involved in this, to try to provide whatever we can that reflects our involvement in this.

Senator BOXER. What reason would there be to not make those documents available to Senator Lautenberg, Senator Vitter and myself, Senator Klobuchar? Is there a national security reason here? What is the story?

Mr. GRUMBLES. I don't know that there is a reason. I also know that the work group is committed to making information available, developing that research strategy that will help inform the agencies and benefit the public.

Senator BOXER. OK. My time is up on this round, so let me just say I look forward to receiving those documents, otherwise we will have to consider getting them a different way. I am sorry I went over my time.

Senator LAUTENBERG. We are joined by Senator Klobuchar.

OPENING STATEMENT OF HON. AMY KLOBUCHAR, U.S. SENATOR FROM THE STATE OF MINNESOTA

Senator KLOBUCHAR. Thank you very much, Senator Lautenberg. I appreciate it, because I am going to manage the bill at 4 o'clock, and if I could just have a few minutes here.

I thank our witnesses for being here. Obviously, this is something of great concern to our State, the State of Minnesota, which is the land of 10,000 lakes, and actually have even more lakes than that.

The fact is that small quantities of drugs, including antibiotics, sex hormones, caffeine and anti-seizure medications have been found in drinking water supplied to over 40 million Americans across the Country. My concern is that instead of engaging in action, that the EPA is once again dragging its feet.

Senator KLOBUCHAR. I just have one or two questions before I have to leave, Mr. Grumbles. Right now, my first question would be that local water treatment plants are not required to test for pharmaceuticals. Is that correct?

Mr. GRUMBLES. That is correct, in the sense that if pharmaceuticals are not listed or regulated under the Federal Safe Drinking Water Act, they are not required under the Safe Drinking Water Act to monitor for it, to test for them.

Senator KLOBUCHAR. And do you think that this is a good idea that they are not being tested?

Mr. GRUMBLES. I think that we should look very seriously, very carefully at using our tools under the Safe Drinking Water Act. As we gather the necessary information to make those decisions on whether to list some pharmaceuticals for potential regulation or monitoring, we should do everything we can to encourage water utilities to share with their consumers, their public, any information they have that would be relevant to them.

Senator KLOBUCHAR. Is it true that the testing is only $1,000?

Mr. GRUMBLES. I don't know from a personal standpoint. I have seen that as one of the items. We are encouraging, and I appreciate Chairman Boxer's suggestion. We as an agency, the Water Office, is encouraging water utilities to be more vigilant and to test. I have seen personally that they are increasingly, and they want to gather

more information and know about the potential for emerging contaminants in their product, their water supply.

Senator KLOBUCHAR. I know in your written testimony you focused on the research that EPA is starting to do in this area. One of the things I was thinking as I read that is that it might be better to spend some of these funds to help local communities develop drinking water systems that may eliminate such contaminants.

I will tell you that in Minneapolis, a new ultra-filtration plant opened in 2005, and when a second plant is completed in the next 3 years, Minneapolis will be the largest city in the Country to have all its water processed by ultra-filtration. We like to say we have the best drinking water in the Country. We actually bottle our Minneapolis drinking water.

This is cutting edge, and we are able to meet the standards. I think it is harder for some of our smaller towns in our State and across the Country to meet these kinds of standards.

Mr. GRUMBLES. If I could say that it is important to continue to develop the technology. It can be expensive at times, but that is an important area for further research. The other one that we are focused on, the actions we are taking are providing financial and technical assistance to some communities or institutions to develop better practices for proper disposal, whether that is hospitals or other types of institutions. That, too, is in the name of pollution prevention and will go a long way as we continue to gather information about the degree of risk to aquatic life or even human health.

Senator KLOBUCHAR. I will have to tell you when I read that AP article about how your position is there needs to be more searching and more analysis.

Mr. GRUMBLES. Not paralysis.

Senator KLOBUCHAR. Searching and more analysis.

Mr. GRUMBLES. We understand that there needs to be action. Right now, it is focused on building partnerships for stewardship and looking very closely at potential regulatory tools. We have used some enforcement tools. We just recently reached a supplemental environmental project in an enforcement context with a wastewater treatment facility. As part of a supplemental environmental project, they were putting more effort into proper disposal of pharmaceuticals and increasing public awareness about proper disposal of pharmaceuticals. That was in the context of a Clean Water Act enforcement action.

Senator KLOBUCHAR. What kind of actions were you taking before this story came out? I am just kind of curious because it was news to a lot of us that even though it is small traces——

Mr. GRUMBLES. I know personally 2 years ago I sent around a memorandum to all the EPA offices and the regions to increase our efforts in the National Water Program. I know that the Research and Development Office at EPA has been carrying out extensive research over the years, over the last six or 7 years, on this front.

We have been gathering information not just about the cutting-edge technologies, but also several years ago we started sampling for sewage sludge.

Senator KLOBUCHAR. But still, you have been doing all this, but then suddenly we hear about all these things in the water. What

I am concerned about, have you listed these things? Have you made this public? Because for a lot of us, as I said, and a lot of citizens in this Country, this was news to them. It really shouldn't take a newspaper article to get this story out.

Mr. GRUMBLES. We have shared information, but what we have yet to do is make some conclusion that there is a risk to human health and that the other factors under the Safe Drinking Water Act have been triggered by the presence of these tiny trace amounts.

Senator KLOBUCHAR. When are you going to make this finding?

Mr. GRUMBLES. There are various programs. We are, as I was mentioning earlier, we have a contaminant candidate listing process under the Safe Drinking Water Act. It is open. We proposed candidates for contaminant listing. There was only one pharmaceutical on that list, and it really was not listed as a pharmaceutical per se.

The comment period closes in the coming weeks, on the 21st of May. In the meantime, what we have done is we have written to potentially interested stakeholders or officials, State and local water officials, saying should we add some pharmaceuticals to that list. So that is one example of a potential regulatory listing process.

Senator KLOBUCHAR. Again, I have to go or I will turn into a pumpkin here. My concern here is just that you have talked a lot about potential listing and things you can do. Meanwhile, we know some things that work. I brought up this filtration system. I think you know that works. Shouldn't we be focused more on results to do everything we can to make our water safe? That is what I am urging you to do here because I am concerned there hasn't been enough action.

Thank you.

Mr. GRUMBLES. Thank you.

Senator LAUTENBERG. We have a few questions that members here want to ask, so we will continue for a bit. I will start, Mr. Grumbles, by confirming that the Clean Water State Revolving Fund is the place that problems get cleared up. Is that not correct?

Mr. GRUMBLES. It is an important tool. It is not the only tool. I think the non-point source program is another tool. There are other programs and tools.

Senator LAUTENBERG. This is an important part of the mechanism for cleaning up, is it not, the State revolving fund?

Mr. GRUMBLES. For protecting the environment, it is an important part.

Senator LAUTENBERG. Yes. So how do we justify a cut in the budget proposal of 20 percent for the next budget? How does that strike with the work that remains to be done?

Mr. GRUMBLES. It is entirely consistent with the Administration's vision of the State revolving fund, and that is that eventually over time it would be self-sustaining.

Senator LAUTENBERG. Do you believe that? Do you believe that giving it a good hefty 20 percent cut is going to lead to its self-funding?

Mr. GRUMBLES. I think it needs to be coupled with several things. That is where the four pillars of sustainability come in to play. But also, Mr. Chairman, I think that Congress needs to enact

a water enterprise bond initiative, some innovative financing to bring additional funding.

Senator LAUTENBERG. But for now, there is not enough work to be done that a 20 percent cut would not impair?

Mr. GRUMBLES. We know there are needs. We also know that States and localities need to step up and full-cost pricing needs to be an important part of the equation.

Senator LAUTENBERG. Yes, but does it matter that there is a 20 percent cut?

Mr. GRUMBLES. No, we think that there are other tools that need to be used as well.

Senator LAUTENBERG. I want you to be more precise. Does it matter if there is 20 percent cut in the State Water Revolving Fund?

Mr. GRUMBLES. We think that the cut is——

Senator LAUTENBERG. What do you think? You are a professional. What do you think?

Mr. GRUMBLES. Well, I support the Administration's budget on this.

Senator LAUTENBERG. OK. So whatever the Administration's budget does, you are Johnny OK with that?

Mr. GRUMBLES. Mr. Chairman, the position of the agency and my position as well heading up the National Water Program——

Senator LAUTENBERG. But how do you feel about it? Are you being mechanical about this?

Mr. GRUMBLES. I feel that now is a great opportunity for the Congress to not just focus on the SRF, to continue to support the SRF, and we agree that is important, too, but to use new tools and the broader tools to change the paradigm.

Senator LAUTENBERG. So in your view, the fact that right now at this point in time, with the work that your department has, that a 20 percent cut in resource doesn't really mean a heck of a lot.

Mr. GRUMBLES. No, Mr. Chairman. I would say that it is important to step up and increase efforts in a variety of programs and ways.

Senator LAUTENBERG. If you only had two words you could issue, yes or no, which one would you pick, Mr. Grumbles?

Mr. GRUMBLES. Would you repeat the question?

Senator LAUTENBERG. It is a very serious question. You are a professional. You know what goes on there. You understand what kind of risk there is.

Mr. GRUMBLES. Is a 20 percent cut in the SRF acceptable?

Senator LAUTENBERG. We will let you go with that. I would rather put it my way and say, would you recommend a 20 percent cut? Do you personally, you a professional, Mr. Grumbles, do you professionally think that a 20 percent cut is in order in the State revolving fund, with the work you have ahead of you?

Mr. GRUMBLES. Given the budgetary pressures that we have on our other programs, that it is entirely consistent with our agenda and our priorities, so yes there needs to be an increase in other efforts.

Senator LAUTENBERG. Thank you very much, Mr. Grumbles. I appreciate your illuminating comment.

Dr. Hirsch, the USGS has conducted studies on the amount of contaminants in our water from pesticides to antibiotics to pharmaceuticals. Do you believe that improving our wastewater treatment infrastructure would effectively reduce concentrations of those contaminants?

Dr. HIRSCH. We have been doing some work with State and local agencies in fact, some in New Jersey and some in New York in particular, looking at both wastewater and drinking water facilities and trying to understand how much of these chemicals are removed in either the wastewater process or the drinking water treatment processes. We find that there are significant differences in the effectiveness of these facilities based on the kinds of processes they use.

So clearly, as in the previous questions about Minnesota, comments about Minnesota, that some of these technologies are probably a part of resolution of the potential concerns over pharmaceuticals in our water supply.

Senator LAUTENBERG. So, again being very specific, would improving our wastewater treatment infrastructure, might it substantially reduce the concentration of contaminants?

Dr. HIRSCH. I think it would be correct to say it might. I think there are a tiny handful of studies that are beginning to point in that direction.

Senator LAUTENBERG. OK. Thank you. You are both good landscapers. You caught the hedges.

[Laughter.]

Senator Vitter.

Senator VITTER. Thank you, Mr. Chairman.

I just wanted to very briefly followup on two points. One is just a statement. I think if we look at the record, just to clarify, when Mr. Grumbles was talking about this issue of not using the toilet as a trash can and flushing things down the toilet, he specifically said in his original statement that there were some exceptions to that rule and there were some pharmaceuticals that the current science says should be flushed down the toilet because of other overarching concerns. So I just wanted to clarify that, which I think you will find in the record.

The second point goes to Senator Klobuchar's comments, and I would ask both witnesses to answer in turn. I assume there are tens of thousands or more of pharmaceuticals or other agents that we could mandate testing for today. I assume that there are tens of thousands or more of these agents that we could mandate an absolute zero tolerance policy for in terms of water cleanup.

Would you consider it the right policy to mandate that now with regard to every entity pharmaceutical or other contaminant out there, to mandate testing and to mandate zero tolerance for all of those things based on the knowledge and the science we have?

Dr. HIRSCH. Let me start by just saying I think it is clear that it is highly unrealistic to test for every chemical in our environment and the costs would be enormous. The question of the kinds of stewardship things that Ben Grumbles talked about—I think we have to ask the tradeoffs of those expenditures. Just an analysis of about 70 pharmaceuticals in a single water sample costs about $2,500. To be meaningful, you would have to do it repeatedly

through the course of the year at every single facility in the Nation. Particularly for smaller communities, that is an enormous amount of money and we simply don't know what the meaning of those results would be, to say that there is one part per trillion of this and two parts per trillion of that.

So I think there is a need for prudence about what needs to be mandated testing and what needs to be an action level in a regulatory sense. This is a subject matter that is still very new both in terms of developing of testing for the presence of it and the testing of the effects.

Senator VITTER. It seems to me that before we test, we have to know what the significance of the test result is going to be, what it means to human health or doesn't mean to human health. And therefore, it needs to start with that science. Would you agree with that basic premise?

Dr. HIRSCH. I think there is a need for there to be a feedback in this process. One needs to begin to know something about what is present in the environment in order to help the targeting of the research on effects, and then the effects work can feed back to determining things that ought to be widely tested for. I think there needs to be a flow of information in both directions.

I would comment that the task force that some of you have been asking about, that is precisely the things that are going on in that OSTP task force, which is the trading back and forth of information among the various agencies as to the quantities of various chemicals that are being introduced, and what we are learning about their presence. It is helping all of us to better target our work.

Mr. GRUMBLES. I would just simply add that I agree with everything Bob said. But I also would say, I do worry about the tradeoffs. When you go to mandate testing and monitoring for a risk that may be a much smaller risk, there may be some significant tradeoffs.

A perfect example is coastal recreational waters. Mr. Chairman, I know how committed you are and your leadership in that area. We know that bacteria in coastal waters, different types of pathogens, are real and present danger, a risk to human health. So that is an area that deserves priority—priority testing and continued increase in the criteria and standards under the Clean Water Act. That is a good example of an area for priority. The SRF is an important tool in helping to reduce pollution that ultimately gets to the beach, but it is not the only tool.

We have increased our enforcement efforts, Mr. Chairman, against sewer overflows, particularly in coastal communities, because we see the writing on the wall that that can add risk to human health at the beach. So I agree with the comment that Bob made about the tradeoffs if you mandate testing at this point for all these different types of pharmaceuticals.

Senator VITTER. OK. Thank you.

Senator BOXER. Well, I don't know if a day at the beach has anything to do with all of this. You just throw up straw men. Your job is to protect human health at the beach, at the drinking water tap. It is not one or the other. That is the problem.

I agree with you, Dr. Hirsch, to be prudent is key here. And to be prudent is to protect our children and our pregnant women and

to do the work that is necessary. This Administration has not followed the law, and in your own admission, haven't listed anything.

Now, just for the record, you don't need to know every detail and every answer before you decide to list certain of these unregulated contaminants so that people know what they are drinking. And sadly, you could sit here when the Associated Press did your work, the Associated Press did your work, and your work, and they are telling us what is in the water. And Dr. Hirsch said, well, it is really, we do need to know what is present before we can do anything else. Yes, let's find it out.

Well, you find it. You said it's important in your own testimony. And yes, if you think this is important and you are concerned, then we need to have some of this testing to find out.

Now, has EPA, Mr. Grumbles, required drinking water systems to monitor drinking water for any pharmaceuticals, not 70, not 80, maybe some of the ones we know are more present in the water? Have you required drinking water systems to monitor drinking water for even one pharmaceutical through the agency's unregulated contaminant monitoring rules? Have you done that?

Mr. GRUMBLES. Not yet.

Senator BOXER. You haven't done it. Do you plan to do it?

Mr. GRUMBLES. We may. We are seriously considering that.

Senator BOXER. When are you going to do that?

Mr. GRUMBLES. Well, Madam Chair, I don't know because we need to gather more information on that, but we are taking that very seriously.

Senator BOXER. I am glad you are taking it seriously, but I don't see anything happening. I don't. I don't see it. You are cutting a program 35 percent. Your program was also cut 12 percent. It is unbelievable to me, Mr. Chairman, what is going on.

Mr. Grumbles, your testimony states, "Useful information should be shared with the public in a timely way as it is generated. It is important to communicate with the public so that they can help shape effective public policy in this area and make informed choices." Are you saying with all those words that the public has a right to know when pharmaceuticals have been found in their drinking water?

Mr. GRUMBLES. If you mean "right to know" in terms of a defined term of art under some statute, I don't know what that means.

Senator BOXER. You don't know what I mean by the public's right to know what is in their water? You don't understand?

Mr. GRUMBLES. I don't know if you are meaning to say under EPCRA or some other specific statute.

Senator BOXER. I am asking you, be a person, be a human, throw away the bureaucratic hat that you have had on for a lot of this. As a human being, I don't know whether you are a dad or uncle or whatever your circumstances. I am a grandma and a ma. Do you think our families have a right to know what is in their drinking water?

Mr. GRUMBLES. I agree with that completely. The quarterly reporting requirements under the Safe Drinking Water Act about contaminants is very important.

Senator BOXER. Good. Then get to work under the authorities you have and let's start testing for those pharmaceuticals that the

Associated Press found were found in larger quantities in the water.

Now, would you agree that water utilities and Federal agencies should publicly disclose test results when they know about pharmaceuticals or other contaminants in the drinking water?

Mr. GRUMBLES. I think they should disclose information that is useful to the public. Are you asking that just any raw data they have that they don't know what it means or if it is inaccurate or it hasn't been QA/QC'd I don't know. What I do know is that——

Senator BOXER. I said test results. In other words, what I am getting at it, you see, I believe the public is really smart. And I believe moms and dads really understand what it means to drink safe water and water that makes them sick. They get it.

The White House is keeping their working group secret. You have done nothing under the law. I don't mean you personally, but how long have you been in your position?

Mr. GRUMBLES. Since November, 2004.

Senator BOXER. OK. We haven't moved forward on anything that we were supposed to on the Endocrine Disrupter Act, cutting funding instead.

So I don't want to keep on going here, but I guess I want to say to my Chairman of this very important Subcommittee and to his Ranking Member, it is the saddest of all things for me to see two people sitting here who are really smart, and to know that their work is being done by the Associated Press. There is something wrong in this Country when the Environmental Protection Agency can only sit here and say, well, yes, we made these budget cuts and we support them; we don't think it matters; and gee, these are tough times, so if we are going to protect your beaches, Senator Lautenberg, we can't really do everything we want in other areas.

Unacceptable. That is not what the law requires of you.

So I hope today, after seeing some of us being a little more upset than others here, that you will immediately go back, have a meeting with the FDA, get your act together, try to come up with some voluntary way to dispose of these so we don't see this showing up in the drinking water. Let's work together with this Committee. We are ready, willing and able to work with you on that. Let's start listing some of these unregulated chemicals here so people know what they are drinking.

You can do many, many things, and so far what I hear from you is, I am concerned; I am deeply concerned; it will take time; I will look it over. It is not enough for me. I hope that our Subcommittee Chair will call you back in a few weeks so we can see the progress that you have made. I look forward to getting the documents from the White House.

Thank you.

Senator LAUTENBERG. Thank you.

We are joined by Senator Cardin, who wanted to make a statement.

OPENING STATEMENT OF HON. BENJAMIN L. CARDIN, U.S. SENATOR FROM THE STATE OF MARYLAND

Senator CARDIN. Very briefly, Mr. Chairman, because I know you want to get on to the next panel.

Let me ask unanimous consent that my entire statement can be made part of the record.

Senator LAUTENBERG. Without objection.

[The prepared statement of Senator Cardin follows:]

STATEMENT OF HON. BENJAMIN L. CARDIN, U.S. SENATOR FROM THE STATE OF MARYLAND

Mister Chairman, thank you for holding this hearing today.

While we have made considerable strides in cleaning up our Nation's drinking water by significantly reducing large-scale sources of pollution, technological advances in our ability to monitor the concentrations of contaminants in our Nation's waters have led to the some disturbing findings.

Trace amounts of pharmaceuticals, insecticides, herbicides, cleaning products, as well as chemicals associated with perfume fragrances are being found in the drinking water supplies of at least 41 million Americans, according to an investigation by the Associated Press released last month.

Although these chemicals are found at exceedingly low concentrations, typically less than one part in a billion, the bioaccumulative properties of some of the chemicals, suggest that over time, these chemicals may buildup in the tissue of aquatic wildlife and humans and pose health risks. As a consequence, even though these chemical concentrations may be sufficiently low to label the levels as "trace" amounts, the repercussions of these compounds on long-term human health remain unclear.

Federal officials continue to investigate the effects on human health of the endocrine disruptors found in water. The effects of these pharmaceutical compounds include possible links to neurological problems in children and increased incidence of some cancers. U.S.G.S. scientists are investigating a wide range of fish health problems in Chesapeake Bay and its watershed.

The Potomac River, which serves as the source of drinking water for millions of people who live, work and visit the National Capitol Region, has had serious problems with fish health in recent years. Several studies of the Potomac and Shenandoah rivers, including those by scientists of the USGS, have revealed inter-sex fish, a wide range of "abnormalities in which both male and female characteristics are present within the same fish."

According to an August 2007 E-magazine article, the abnormalities include nine male smallmouth bass from the upstream Potomac River from Washington near Sharpsburg, Maryland that developed female eggs inside their sex organs. Inter-sex bass were also found in a study 3 years earlier, after fish kills about 170 miles upstream in the South Branch of the Potomac in Hardy County, West Virginia. The USGS has recently documented the occurrences of these disorders, but its research is in its infancy.

In addition to the examination of samples of fish physical anomalies and fish tissue condition, the USGS is also sampling water chemistry and sediments within the rivers from which the fish samples have been taken. The chemistry includes evaluating concentrations of hormones, pharmaceuticals, personal care products, and pesticides. The concentrations of various chemicals and chemical mixtures may help explain the fish conditions found in the Potomac. Finding the causes of the fish health conditions and what various species of fish respond to, is a complex problem and will take some years to address adequately.

The urgency of the situation was noted by the EPA's director of America's water programs, Ben Grumbles, who said, "We recognize it is a growing concern, and we're taking it very seriously."

But the Bush administration budget does not reflect that level of seriousness.

Under the President's fiscal year budget request, EPA's budget for Science and Technology faces a cut. In fact, when adjusted for inflation, EPA's R&D funding would fall to the lowest level in more than two decades in real terms. And EPA's budget cuts are not alone.

The U.S. Geological Survey, who has as part of its mission to provide water information that benefits the Nation's citizens, is also facing major budget cuts.

The USGS Programs that are primarily responsible for providing chemical data to help explain fish conditions are the Toxic Substances Hydrology Program, and the National Water Quality Assessment Program. These two water quality programs are both scheduled for substantial funding reductions or redirections in fiscal year 2009.

Specifically, the USGS National Water-Quality Assessment program will be cut by nearly $11 million. (-$10.9 million)—The 2009 budget request for the National

Water-Quality Assessment (NAWQA) program is $54.1 million and 328 FTE, a program decrease of $10.9 million and 72 FTE from 2008 enacted.

The Toxic Substances Hydrology program is slashed by $2.8 million in the President's budget request. That represents a 21 percent reduction in critical funding at a time when our needs are obviously great. My amendment to the Budget Resolution for fiscal year 9, which Madame Chairman co-sponsored, increased funding for this important research work to move forward.

I look forward to learning more about this serious problem. I hope to hear testimony that explains why the President's budget priorities do not match with the apparent urgency of this problem. I further want to learn what studies are necessary to better understand the impact of these trace chemicals on not only aquatic life, but humans as well—more specifically, "Who is at greatest potential risk?" and "Is there a coordinated effort between USGS and EPA with HHS to relate the fish abnormalities and chemical concentrations to human impacts?" Finally I'd like to better understand what are the most effective steps citizens, local water treatment facilities, and the Federal Government can do to ensure that our drinking water is free of potentially harmful "trace" levels of herbicides, insecticides, hormones, and pharmaceuticals.

Thank you Mister Chairman.

Senator CARDIN. Let me make an observation. That observation is following up on Senator Boxer's comments. We have a responsibility to make sure that we do everything to keep people safe. I look at this water that I am about to drink, and I have confidence that it is safe, but I think there are certain questions that should have been answered that have not been answered about how this water has been inspected to make sure that the contaminants that have been reported are not adversely affecting our health. We don't have the answers to these questions and that is what concerns me.

I live in this area. The Chesapeake Bay is very important. The Potomac River serves as the source of drinking water for millions of people in this region. The USGS studies have shown that fish in the Potomac and the Shenandoah suffer from abnormalities that could be linked to the types of pollutants that we are talking about, even though they are in small quantities. We see a single fish that has the attributes of both sexes. We are not exactly sure why that is happening, but it would be good to have more focus on trying to answer these questions.

So Mr. Chairman, I thank you very much for holding this hearing. During the appropriation process, you and I both serve on the Budget Committee, I offered an amendment, and I am proud to have the support of my colleagues here, to increase the funding so that we can provide the money necessary to do the studies as our responsibility.

I just hope we would have a sense of urgency, to be able to answer questions that are being posed by our constituents, reasonable questions as to whether these traces of contaminants that have been discovered from pharmaceutical products and other types of products, whether they have a risk factor and whether we understand that, and whether we have done the right testing to make sure that we have done everything possible to keep them safe. That should be our responsibility and I would urge this Committee to continue its oversight role and insist that the appropriate studies are done. We certainly are making the resources available. Let's make sure the agency provides the answer.

With that, Mr. Chairman, I thank you for your patience.

Senator LAUTENBERG. Thanks very much.

We thank you for your testimony. This record will be kept open for probably a week or two so that we can submit questions and

ask that you respond as promptly as you can. Thank you very much for your testimony.

And now we call the second panel finally to the desk. We apologize for being so long in getting to you, but we had great interest in the subject, and it is obvious by the questions that were asked.

Thank you all for being here. The fact that we are so tardy in getting to you doesn't indicate a lack of interest and the fact that I am the Chairman and only member of the Committee at this moment. We appreciate the work that you have done in preparing your testimony. I would ask that you try to limit it to 5 minutes if you can.

Dr. Sass, you are the first in line. We call on you. Thank you for being here.

STATEMENT OF JENNIFER SASS, SENIOR SCIENTIST, NATURAL RESOURCES DEFENSE COUNCIL

Ms. SASS. Thank you. Good afternoon and thank you for the opportunity to testify on the health concerns and policy proposals addressing pharmaceuticals in the Nation's waterways and drinking water sources.

I am Jennifer Sass, a Senior Scientist in the Health Program at the Natural Resources Defense Council. I have a doctorate degree in molecular and developmental biology and a post-doctoral certificate in environmental toxicology. I have worked at NRDC on a environmental health issues for over 7 years.

As this Committee is aware, the Associated Press recently reported on the presence of pharmaceuticals, including antibiotics, anti-convulsants, anti-depressants, steroids and reproductive hormones in the drinking water serving millions of people. Although the levels reported to contaminate our waterways are much lower than therapeutic doses, it would be naive to think of them as safe, knowing that the agents are chemically reactive in our bodies and that we are exposed daily over a full lifetime to multiple compounds in unknown combinations.

When a medical professional prescribes a drug, they consider the patient's health status, age, gender, nutritional status, and any other drugs that may cross-react. For example, a pregnant woman would not knowingly expose her fetus to chemicals that cause birth defects such as anti-seizure drugs. A doctor would not knowingly prescribe toxic chemotherapy agents to a healthy person. And yet all these things and more are in our Nation's drinking water.

Pharmaceuticals and personal care products end up in the environment through waste from human or animal excretion, improper disposal such as flushing down a toilet, or leaching from municipal landfills. However they get there, they are contaminating our waterways and tap water.

There are two categories of pharmaceuticals that raise particular concern to us: antibiotics and endocrine-or hormone-disrupting chemicals. Large animal feeding operations generate a large amount of antibiotic-contaminated waste that enters waterways and contributes to antibiotic-resistant pathogens. This means that when a person gets sick, the antibiotic that their doctor may reach for may not work.

The traditional toxicology dogma has been the dose makes the poison. But for endocrine-or hormone-disrupting chemicals, the timing of the exposure may be much more important than the dose. Exposures to endocrine-disrupting chemicals during critical windows of development such as infancy and adolescence have been shown to have permanent effects. Some of these effects such as infertility or cancer may not arise until adulthood, even though the exposure occurred during early life.

For example, pre-birth exposure to DES, diethylstilbestrol, increased cancer risk for the daughters born to mothers that took it during pregnancy. Yet since pharmaceuticals that mimic estrogens are excreted as waste by-products from the use of birth control pills, menopause treatments and cancer therapies, they end up in our drinking water.

Endocrine-disrupting steroids used in livestock operations also contribute to widespread environmental contamination. The U.S. Geological Survey found a high incidence of intersex fish in the Potomac watershed, and it was associated with sites of intense farming and high human population density. Male smallmouth bass in those areas had eggs in their testes.

Despite the various safeguards that EPA could have taken to develop a robust picture of the problem, the agency has taken advantage of none. For example, the Safe Drinking Water Act requires EPA every 5 years to publish a list of currently unregulated contaminants that should be considered for potential regulation. For these lists, EPA has identified 130 potential chemicals, and none are pharmaceuticals or personal care products.

Since 1999, EPA has required community water systems to monitor for a list of unregulated contaminants. That list contains no pharmaceuticals. The Safe Drinking Water Act requires community water systems to mail each of their customers an annual report on the level of contaminants in the drinking water that they supply. EPA has not required utilities to inform their customers when pharmaceuticals or personal care products are found.

And Congress mandated EPA to address endocrine-disrupting chemicals in drinking water. It has not been 12 years since this mandate, and the endocrine disrupter screening program has not yet tested its first chemical.

In addition to addressing the above failures, we need to address the unnecessary use of antibiotics and steroid hormones to tackle the problem at its source. We need to invest in our wastewater and drinking water infrastructures and find ways to monitor and treat for chemical contaminants that present the biggest health risks.

We must also continue to reduce the amount of toxic chemicals used in products and promote the development and use of safer alternatives.

Thank you for inviting me to testify before you today. NRDC looks forward to working with the Committee and Subcommittee to address these important issues, and I would be happy to answer any questions.

[The prepared statement of Ms. Sass follows:]

TESTIMONY OF
JENNIFER SASS, Ph. D
SENIOR SCIENTIST
NATURAL RESOURCES DEFENSE COUNCIL

ON BEHALF OF:
NATURAL RESOURCES DEFENSE COUNCIL

BEFORE THE US SENATE
COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS
SUBCOMMITTEE ON TRANSPORTATION SAFETY, INFRASTRUCTURE
SECURITY, AND WATER QUALITY

AT HEARING ENTITLED:
PHARMACEUTICALS IN THE NATION'S WATER:
ASSESSING POTENTIAL RISKS AND ACTIONS TO ADDRESS THE ISSUE

APRIL 15, 2008

Good morning and thank you for this opportunity to testify on the health concerns and policy proposals addressing pharmaceuticals and personal care products in the nation's waterways and drinking water sources. I am Jennifer Sass, PhD., a Senior Scientist in the Health Program at the Natural Resources Defense Council (NRDC). I have a Ph.D. in molecular and developmental biology, and a post-doctoral certificate in environmental toxicology. I have worked at NRDC on environmental health issues for over 7 years. NRDC is a not-for-profit environmental advocacy organization with over 1 million members and activists whose mission is to safeguard the Earth: its people, its plants and animals and the natural systems on which all life depends.

NRDC's Health program focuses on toxic chemical pollutants in air, water, food, and shelter. Over the years, we have focused our particular attention on the "biggest pollutants" in these media, the ones disproportionately responsible for the biggest threats to human health. This has led to successful efforts to substantially reduce diesel air emissions from trucks and buses, for example, and to take a number of dangerous and outdated pesticides off the market. There are more than 70,000 chemicals in commerce, but some are much more toxic than others, and we can make great progress in environmental health protection if we focus on the chemicals pollutants that pose the greatest threat to human and ecological health.

We are very pleased to testify today on the health risks posed to humans and wildlife by pharmaceuticals and personal care products that contaminate our waterways.

Summary of Testimony

Compounds such as nicotine, caffeine, and aspirin that are designed to influence our body's normal chemistry have been identified as environmental contaminants since the 1980s, moving from sewage and human waste into waterways. As our use of pharmaceuticals increases, it is logical to expect them to turn up in our environment. Although the levels reported to contaminate our waterways are much lower than therapeutic doses, it would be naïve to think of this as 'safe', knowing that the agents are chemically reactive in our bodies, and that we are exposed daily over a life-time to multiple compounds in unknown combinations.

When a medical professional prescribes a drug, they are considering the patient's health status, age, gender, nutritional status, and any other drugs that may cross-react. For example, a woman who was at risk for breast cancer would not want to be exposed to high levels of estrogenic compounds. A pregnant woman would never knowingly expose her fetus to chemicals that cause birth defects such as antiepileptic drugs. A doctor would never knowingly prescribe toxic chemotherapy agents to a healthy person. Yet, all these things and more are in our Nation's drinking water. The Associated Press reported that pharmaceutical residues were detected in the drinking water of 24 major metropolitan areas across the country serving 41 million people. Detected drugs included antibiotics, anti-convulsants, and mood stabilizer drugs. These results were supported by findings of the U.S. Geological Survey that found organic wastewater contaminants and pharmaceuticals in 80% of sampled streams- including antibiotics, hypertensive and cholesterol-lowering drugs, antidepressants, analgesics, steroids, caffeine, and reproductive hormones.

Pharmaceuticals and personal care products (PPCPs) may end up in the environment through waste from human or animal excretion, improper disposal such as flushing down a toilet, runoff from animal feeding operations, or leaching from municipal landfills. However they get there, they are contaminating our waterways and tap water systems.

Large animal feeding operations generate a large amount of antibiotic-contaminated waste that contaminates waterways and contributes to antibiotic resistant pathogens. Because many of the same antibiotics are used in both human and veterinary medicine, almost every bacteria that can cause infections in humans has developed resistance to at least one antibiotic, and some are resistant to multiple antibiotics.

Pharmaceuticals that mimic estrogen are excreted as waste by-products from the use of birth-control pills, menopause treatments, and cancer therapy. In addition to human uses, endocrine disrupting steroids used in livestock operations contribute to widespread environmental contamination. Research by the US Geological Survey reported a high incidence of intersex fish in the Potomac watershed associated with sites of intense farming and high human population density; 75% of male smallmouth bass in the most densely populated heavily farmed Potomac basin had eggs in their testicles.

The issue of pharmaceuticals and personal care products in drinking water is not news to EPA, and yet despite the various safeguards and processes that EPA could have taken to develop a robust picture of the scope of the problem, the Agency has taken advantage of none of them. First, the Safe Drinking Water Act requires EPA every five years to publish a list of currently unregulated contaminants that should be considered for potential regulation. For these lists EPA has identified 130 potential chemicals for regulation – none of which are pharmaceuticals or personal care products. Second, in 1999 EPA promulgated an unregulated contaminant monitoring rule that imposed various monitoring requirements on community water systems for a list of unregulated contaminants; there are no pharmaceuticals or personal care products identified as unregulated contaminants for which water systems must monitor. Third, to our knowledge, EPA has never asked water systems to provide voluntarily testing and monitoring data for pharmaceuticals and personal care products. Fourth, the Safe Drinking Water Act requires community water systems to mail to each of their customers an annual report on the level of contaminants in the drinking water that they supply; there are no mandates to inform customers when pharmaceutical or personal care products are identified. Fifth, Congress mandated that EPA must address endocrine disrupting chemicals in drinking water; it has now been 12 years since this mandate and the Endocrine Disruptor Screening Program has failed to even start testing chemical contaminants.

The FY08 funding for the Toxics Program is $13.5 M; for FY09 the request is $10.7 M, reflecting a nearly $3 M cut from the USGS budget. The proposed cuts to the Toxics Program will significantly reduce research capacity on new and understudied environmental contaminants, including pharmaceuticals and personal care products.

Pharmaceuticals and personal care products are contaminating our waterways and sources of drinking water

An investigation by the Associated Press reported that pharmaceutical residues were detected in the drinking water of 24 major metropolitan areas across the country serving 41 million people.[1] Detected drugs included antibiotics, anti-convulsants, and mood stabilizer drugs. These results were supported by findings of the U.S. Geological Survey that sampled 139 streams in 30 states, and found organic wastewater contaminants and pharmaceuticals in 80% of sampled sites- including antibiotics, hypertensive and cholesterol-lowering drugs, antidepressants, analgesics,steroids, caffeine, and reproductive hormones.[2]

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[1] Jeff Donn, Martha Mendoza, Justin Pritchard. Associated Press. March, 2008. http://www.msnbc.msn.com/id/23503485/

[2] Barber, L.B., Murphy, S.F., Verplanck, P.L., Sandstrom, M.W., Taylor, H.E., and Furlong, E.T., 2006, Chemical loading into surface water along a hydrological, biogeochemical, and land use gradient—A holistic watershed approach: Environmental Science and Technology, v. 40, no. 2, p. 475-486, doi: 10.1021/es051270q. (Supporting Information) http://toxics.usgs.gov/highlights/pharm_watershed/

Pharmaceuticals and personal care products (PPCPs) include human and veterinary drugs, both prescription and over-the-counter, medical agents such as chemotherapeutic drugs and x-ray contrast media, antibiotics, anti-inflammatories, blood pressure and cholesterol lowering medications, psychotropic drugs, oral contraceptives, anti-seizure medications, fragrances, sunscreens, 'antibacterial' soaps, lotions, shampoos, and creams. They may end up in the environment through waste from human or animal excretion, improper disposal such as flushing down a toilet, runoff from animal feeding operations, or leaching from municipal landfills.

The problem of unintended movement of toxic and hormone disrupting compounds from pharmaceuticals and personal care products to wastewater effluents and drinking water sources is an international problem that has been documented and publicly reported by government experts and academic researchers for nearly two decades.[3] However, until recently, the public has been in the dark about the presence of these chemicals in our drinking water. As discussed more fully below, pharmaceuticals and personal care products have been excluded from the regulatory safeguards put in place by Congress – and the public's right to know has suffered as a result of that exclusion.

The contaminants come from many sources (medical waste, consumer waste, agriculture and industrial uses, etc.), have diverse toxicology profiles and biological activity, and are likely to have complex and poorly understood toxic interactions (antagonistic, synergistic, additive, etc.). However, among others, these contaminants share one very disturbing characteristic: in general, they are not effectively controlled under U.S. environmental statutes.

Widespread antibiotic contamination poses a serious health threat

Although the human health impacts of these exposures to pharmaceuticals and personal care products are poorly understood, what we do know is troubling. For example, we know that widespread exposure to antibiotics is contributing to the growth of bacterial resistance, and this problem is of grave concern. In the past several decades

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3 Aherne GW, Briggs R. The relevance of the presence of certain synthetic steroids in the aquatic environment. J Pharm Pharmacol 41:735-736 (1989).

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almost every bacteria that can cause infections in humans has developed resistance to at least one antibiotic, and some are resistant to multiple antibiotics. The Center for Disease Control has identified antibiotic resistance as one of the most pressing public health problems to face our nation.[4] Infections caused by bacteria with resistance to at least one antibiotic have been estimated to kill over 60,000 hospitalized patients each year.[5] However, antibiotic resistant bacteria are not limited to our healthcare settings. Methicillin-resistant *Staphylococcus aureus* (MRSA), a skin bacteria resistant to several antibiotics, was once found only in hospitals and nursing homes. MRSA is now commonly found in the community.[6] Infection with MRSA can cause skin and soft tissue infections and pneumonia. Some MRSA infections are now only treatable with one antibiotic, vancomycin; it is extremely worrisome that microbial resistance to even this powerful antibiotic now has been reported.

Antibiotic resistance is caused by a number of factors including repeated and improper use of antibiotics in both humans and animals. Scientists also agree that exposure to low levels of antibiotics actually promotes bacterial resistance by exerting selective pressure for genes that promote resistance. Antibiotics end up in waste water because the body does not completely breakdown all drugs, so both the metabolized and unmetabolized drug are excreted by humans into wastewater. For example, when amoxicillin is ingested, 60-75% of the antibiotic is excreted unchanged into the urine. This antibiotic, now in the environment, may encounter other bacteria and promote resistance. It is unknown how much of an impact current low levels of antibiotics in drinking water are having on the problem of bacterial resistance. However, the potential has been recognized for many years.[7]

Massive quantities of antibiotics are used in agriculture both to treat infections and as food additives to promote growth and to compensate for conditions that contribute to infection. Animals raised in Concentrated Animal Feeding Operations (CAFOs) are at increased risk for infection due to close confinement and stress. In fact, it has been estimated that 70% of the antibiotics used in the US are for animal husbandry.[8] Improper and overuse of antibiotics in livestock and poultry can cause resistance in strains of bacteria that can infect humans. Furthermore, half of the antibiotics used in livestock are in the same classes of drugs that are used in humans. As a result the US Institute of Medicine (IOM) and the World Health Organization (WHO) both stated that the

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[4] http://www.cdc.gov/drugresistance/healthcare/problem.htm

[5] Citation from Health Care Without Harm fact sheet, Antibiotic Resistance and Agricultural Overuse of Antibiotics.. Available at http://www.noharm.org/details.cfm?ID=938&type=document

[6] Centers for Disease Control, Overview of CA-MRSA. http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca.html

[7] USA Today, in an 11/8/00 news article stated, "Experts fear that even low levels of antibiotics fouling the nations water supply may help create super-bugs: micro organisms that have evolved to survive an antibiotic's lethal assault."

[8] Mellon et al. *Hogging It: Estimates of Antimicrobial Abuse in Livestock.* Union of Concerned Scientists: Cambridge MA. 2000.

widespread use of antibiotics in agriculture is contributing to antibiotic resistance in humans.[9]

Large animal feeding operations generate a large amount of waste that can potentially contaminate groundwater and waterways contributing to antibiotic resistance and contamination of waterways with steroid hormones.[10] As occurs in humans, some portion of the antibiotics administered to livestock will pass unchanged through their bodies and will be excreted in their waste. It has been estimated that between 25-75% of antibiotics are excreted unchanged in feces and can persist in the soil after land application.[11] Manure is applied in large quantities as fertilizer in farm fields. In addition to potentially contaminating the food supply with antibiotic resistant bacteria, antibiotics in manure can persist in soil promoting the development of more antibiotic resistant bacteria. Animal waste and its associated contaminants can enter waterways through groundwater contamination, overflow of waste lagoons into surface water or by over-application of manure as fertilizer in farm fields. A recently published study found evidence of fecal contamination and increased levels of antibiotic resistant bacteria downstream of a swine concentrated feeding operation.[12] Other studies have found antibiotic resistance in groundwater underlying a swine waste lagoon.[13]

Widespread contamination of waterways with steroid hormones and endocrine disrupting chemicals may pose a serious health threat

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[9] Quotes from the Healthcare Without Harm factsheet. Antibiotic Resistance and Agricultural Overuse of Antibiotics. Novermber 8, 2005. Available at http://www.noharm.org/us/food/issue

U.S. Institute of Medicine/National Academy of Science: "Clearly, a decrease in antimicrobial use in human medicine alone will have little effect on the current [antibiotic-resistant] situation. Substantial efforts must be made to decrease inappropriate overuse in animals and agriculture as well."

World Health Organization: "There is clear evidence of the human health consequences due to resistant organisms resulting from non-human usage of antimicrobials. These consequences include infections that would not have otherwise occurred, increased frequency of treatment failures (in some cases death) and increased severity of infections."

[10] Gilchrist MJ, Greko C, Wallinga DB, Beran GW, Riley DG, Thorne PS. The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. Environ Health Perspect. 2007 Feb;115(2):313-6. Epub 2006 Nov 14.

Wallinga D, Mellon M, Roach S. Antibiotic use in swine farms in Alberta. Can Vet J. 2006 Dec;47(12):1153; author reply 1153-4.

Wallinga D. Public health advocate. Prev Vet Med. 2006 Feb 24;73(2-3):221-8. Epub 2005 Oct 28.

[11] Chee-Sanford, J.C., et al. Occurrence and Diversity of Tetracycline Resistance Genes in Lagoons and Groundwater Underlying Two Swine Production Facilities. *Applied and Environmental Microbiology*, April 2001. Vol. 6 no. 4, pp. 1494-1502.

[12] Sapkota AR, et al. Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated Swine feeding operation. Environ Health Perspect. 2007 Jul;115(7):1040-5.

[13] Chee-Sanford, J.C., et al. Occurrence and Diversity of Tetracycline Resistance Genes in Lagoons and Groundwater Underlying Two Swine Production Facilities. *Applied and Environmental Microbiology*, April 2001. Vol. 6 no. 4, pp. 1494-1502.

Congress and the EPA have recognized that some environmental contaminants are able to interfere with the actions of normal hormones.[14] These contaminants are called endocrine disrupting chemicals and have been demonstrated to interfere not just with sex hormones but other hormonal systems including the thyroid gland. Pharmaceuticals that mimic estrogen are excreted as waste by-products from the use of birth-control pills, menopause treatments, and cancer therapy. In addition to human uses, endocrine disrupting steroids used in livestock operations contribute to widespread environmental contamination.

Beef cattle raised in large feedlots are also treated with anabolic steroids to promote the growth of muscle. One of the most common steroids used is the androgen mimic, trebolone acetate. The breakdown products of this steroid are stable in water and bind with high affinity to the androgen receptor in fish. [15] Exposure to trebolone metabolites causes masculinization of female fish and cause reduced fertility at concentrations in the parts per trillion range. [16] Although we are much larger than fish, our bodies do not require larger doses of hormones to have effects. Sex hormones in all vertebrate species work in the parts per billion to parts per trillion range.

A recent study was done at an Ohio-based CAFO with a capacity for 9,800 cattle.[17] This study found detectable concentrations of trebolone in the discharge from the facility at levels that were sufficient to induce gene expression associated with exposure to androgens.

Recognition of the effects of endocrine disrupting chemicals in laboratory animal models has resulted in questions about whether problems in wildlife could be caused by environmental contaminants that are endocrine disrupting chemicals. For example, there is increasing public scrutiny and mounting evidence of the environmental effects of chemical contamination of waterways resulting in intersex fish in our nation's rivers and drinking water sources. Research by the US Geological Survey (USGS) reported a high incidence of intersex fish in the Potomac watershed at sites of intense farming and high human population density.[18] The USGS found 75% of male smallmouth bass in the most densely populated heavily farmed Potomac basin had eggs in their testicles. Other research has found environmental androgens associated with masculinization in female

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[14] http://www.epa.gov/endo/

[15] Durhan EJ, et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect. 2006 Apr;114 Suppl 1:65-8.

[16] Durhan EJ, et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect. 2006 Apr;114 Suppl 1:65-8.

[17] Durhan EJ, et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect. 2006 Apr;114 Suppl 1:65-8.

[18] US Geological Survey (2008, February 11). Intersex Fish Linked To Population And Agriculture In Potomac River Watershed. *ScienceDaily*. Retrieved March 24, 2008, from http://www.sciencedaily.com /releases/2008/02/080208115302.htm

fish living downstream of pulp mills and concentrated animal feeding operations.[19] These organisms serve as sentinels for environmental contamination by endocrine disrupting chemicals and also raise concerns about potential impacts on human health, since humans share many of the same metabolic systems as fish.

There is concern that exposure to endocrine disrupting chemical contaminants could promote the growth of estrogen dependent cancers in some exposed people.[20] We must achieve greater understanding of which chemicals are causing these effects and conduct further laboratory testing to understand the potential human health effects.

Low dose exposures to endocrine disrupting chemicals pose health risks

The traditional toxicology dogma has been "the dose makes the poison" but when considering the toxicity from exposures to endocrine disrupting chemicals, the timing of exposure maybe more important than the dose. Exposures to endocrine disrupting chemicals during critical windows of development have been shown to have permanent effects.[21] Some of these effects, such as infertility or cancer, are not manifest until adulthood even though the exposure occurred during fetal or neonatal life. In animal studies, prenatal exposures to low levels (1 part per billion) of the synthetic estrogen, DES, has been shown to cause infertility, cancers of the reproductive tract and obesity.[22] Other laboratory studies have demonstrated that exposures to endocrine disrupting chemicals found in the environment, sometimes at low doses, are associated with reproductive harm or the development of reproductive tract cancers.[23] In humans, there is a noted decrease in the age at puberty, increases in infertility, increases in birth defects of male genitalia, increases in testicular cancer and continued high rates of breast cancer.

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[19] Hotchkiss AK, et al. 2008 Fifteen years after "Wingspread" – Environmental Endocrine Disruptors and human and wildlife health: Where are we today and where we need to go. Toxicological Science Advance Access published Feb 16, 2008.

Durhan EJ, et al. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect. 2006 Apr;114 Suppl 1:65-8.

[20] *State of the Evidence 2008*: The Connection Between Breast Cancer and the Environment Edited by Janet Gray, Ph.D., published by the Breast Cancer Fund and available at: http://www.breastcancerfund.org/site/pp.asp?c=kwKXLdPaE&b=206137

[21] Grandjean P, et al., The Faroes Statement: Human Health Effects Of Developmental Exposure To Chemicals In Our Environment. Basic Clin Pharmacol Toxicol. 2008 Feb;102(2):73-5.

[22] Newbold RR.Lessons learned from perinatal exposure to diethylstilbestrol. Toxicol Appl Pharmacol. 2004 Sep 1;199(2):142-50.

Newbold RR, et al. Perinatal exposure to environmental estrogens and the development of obesity. Mol Nutr Food Res. 2007 Jul;51(7):912-7.

[23] Vandenbergh JG. Animal models and studies of in utero endocrine disruptor effects. ILAR J. 2004;45(4):438-42.

Gray LE Jr, et al. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl. 2006 Feb;29(1):96-108.

Each of these conditions has been shown in laboratory experiments to occur associated with exposures to endocrine disrupting chemicals during critical periods of development. We do not yet know whether similar exposures to endocrine disrupting chemicals in our environment could be causing the increased incidence of disease.

Despite the fact that low level contamination by pharmaceuticals and personal care products has been documented in the technical literature for some time, we have but an extremely rudimentary understanding of the hazards that these chemicals might pose to human health and the environment, or even the patterns of their occurrence in our nation's drinking water. We do know that many of these chemicals are designed to interact with our body's normal functioning, even at low doses, and that at therapeutic doses, drug interactions occur that can be harmful or even fatal. For example, we have no idea how low level exposure to psychotropic drugs might affect behavior of people and/or wildlife when occurring as a water contaminant. Therapeutic-level exposure to the anti-seizure medication valproic acid has been associated with increased birth defect risk,[24] but we do not know if exposures at lower levels will confer a similar risk. The long-term potential harm from the interactions of all these compounds, even at low exposures, is unknown, particularly for vulnerable populations such as infants, the elderly, and people with health ailments.

The Endocrine Disruptor Screening Program has failed to even start testing after 12 years

It is unknown how many of the pharmaceuticals and personal care products which contaminate our nation's drinking water are endocrine disrupting chemicals. We do know that some of the pharmaceuticals found in waterways are intentionally made to mimic our body's hormones. These include medications such as thyroid hormone replacement, estrogen replacement therapy, or birth control pills. Other pharmaceuticals are synthesized to block the action of our body's hormones, for example breast or prostate cancer therapies. We also know that certain chemicals found in personal care products are endocrine disrupting chemicals. For example, some chemicals used to carry fragrance, called phthalates, are known to inhibit the production of the male sex hormone, testosterone. However, for the vast majority of chemicals found as contaminants in the environment, there is no information to tell us whether or not they are endocrine disrupting chemicals.

In 1996, the Food Quality Protection Act (FQPA) required EPA to develop a screening program that would ascertain whether certain substances may have estrogenic (or other endocrine) effects on humans. The Safe Drinking Water Act extended this mandate to include substances that may be found in sources of drinking water. The Endocrine Disruptor Screening Program (EDSP) was intended to help define which

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[24] Duncan S. Teratogenesis of sodium valproate. Curr Opin Neurol. 2007 Apr;20(2):175-80. Review.

James L, Barnes TR, Lelliott P, Taylor D, Paton C. Informing patients of the teratogenic potential of mood stabilizing drugs: a case note review of the practice of psychiatrists. J Psychopharmacol. 2007 Nov;21(8):815-9. Epub 2007 Sep 19.

chemicals could be capable of causing these effects and ultimately, provide information to be used to protect the public's health.

The Food Quality Protection Act mandated that EPA develop a screening program by 1998 and implement it by 1999. According to the original EPA timeline for implementation, the priority setting database was to be complete by June 2000 and was to be used for priority setting in November 2000. Despite the established deadlines, EPA failed to adhere to the original timeline for priority setting, which was the subject of prior litigation between NRDC and EPA. The Agency settled that lawsuit by agreeing to publish a proposed initial list of chemicals for screening by December 31, 2002 and to validate and begin requiring chemicals to be tested by December 2003. EPA missed these deadlines as well. In fact, it has been twelve years since Congress mandated EPA to create this screening program, but the Agency has yet to begin actual testing of any chemicals under this program.

EPA has failed to protect the public's health not only by delaying implementation of the testing program but also by refusing to regulate any chemicals that have been shown to be endocrine disruptors by other research studies. In addition to failing to reduce exposures to known endocrine disrupting chemicals, EPA has failed to begin testing the tens of thousands of other chemicals where we have no information about their potential endocrine disrupting effects.

A decade ago, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) devised clear recommendations for a prioritization process for selecting chemicals for screening. These recommendations were adopted by EPA in 1998. For the first round of screening, EPA has proposed to test 73 chemicals that are in pesticides or are considered High Production Volume (HPV) pesticide inerts. EPA is under a mandate from Congress to begin testing by August 2008.

Although EPA finally appears to be making progress on this program, we are concerned that EPA has diverged from the EDSTAC's recommendations about how to select the chemicals for this initial list. In particular, and in light of concerns regarding pharmaceuticals and personal care products and other endocrine disrupting chemicals in water, EPA should include drinking water contaminants and not just pesticides in future testing protocols. Although EDSTAC recommended that EPA should test drinking water contaminants and mixtures of chemicals found in drinking water, EPA has not indicated that it intends to screen drinking water contaminants in the EDSP. In fact, EPA has the authority to do this under the Safe Drinking Water Act Amendments of 1996. These amendments authorized EPA to screen drinking water contaminants for endocrine disrupting effects. Specifically, Section 136 of the SDWA Amendments states, "In addition to the substances referred to in [the Food Quality Protection Act], the Administrator may provide for testing under the screening program authorized by [the FQPA] for any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed to such substance."

EPA's failure to regulate pharmaceuticals and personal care products as drinking water contaminants

The issue of pharmaceuticals and personal care products in drinking water is not news to EPA. The Agency has been aware that these chemicals were contaminating our drinking water; studies on these chemicals ending up in our drinking water date back well over a decade. Yet despite this, EPA has failed to take the steps that would provide a fully, informed snapshot of the scope of the problem.

The Safe Drinking Water Act (SDWA) governs the regulation of contaminants in our drinking water supplies. Pursuant to the SDWA, EPA sets health-based standards for certain contaminants that may appear in drinking water. In addition to these regulated contaminants, the SDWA also created a system that would push EPA to determine whether other contaminants should also be regulated. Despite the various safeguards and processes that EPA could have taken to develop a robust picture of the scope of the problem of pharmaceuticals and personal care products in drinking water, the Agency has taken advantage of none of them.

First, the SDWA requires EPA every five years to publish a list of currently unregulated contaminants that should be considered for potential regulation. EPA is then required to make a final determination about whether or not to regulate at least five of the contaminants identified on the Candidate Contaminant List (CCL).

To date, the Candidate Contaminant List listing process has gone through 3 iterations, beginning in 1998 with the publication of CCL1 and then CCL2 in 2005. CCL1 contained 50 chemical contaminants, including industrial organic chemicals, pesticides, and inorganic chemicals; in July 2003, EPA decided not to regulate any of the nine chemicals it evaluated on the CCL1. CCL2 consisted of a subset of the chemical contaminants listed on CCL1; and in May 2007, EPA again decided not to regulate any of the 11 chemicals it considered from the CCL2. In February 2008, EPA published the draft CCL3. According to EPA, the 104 candidates for the draft CCL3 emerged from their evaluation of approximately 6,000 chemicals, 287 of which were pharmaceuticals.[25] EPA narrowed down the universe of 6,000 chemicals to a preliminary CCL3, which consisted of over 500 chemicals. In our review of the preliminary CCL3, aspirin was the only PPCP identified for further evaluation. According to the Associated Press, only one chemical on the draft CCL3 – nitroglycerine – is considered a pharmaceutical; however EPA indicated that it chose to include it on the draft CCL3 because of its use in explosives, not because of its pharmaceutical uses. All told, between the three CCLs, EPA has identified 130 potential chemicals for regulation – none of which are pharmaceuticals or personal care products.

Second, the Safe Drinking Water Act includes a process to inform both the determination about whether to regulate a contaminant on the Candidate Contaminant

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[25] http://www.epa.gov/OGWDW/ccl/ccl3.html, last visited 11 April 2008; 73 Fed. Reg. 9627, 9652 (February 21, 2008).

List and whether even to list a contaminant on the Candidate Contaminant List. Specifically, public water systems are tasked with collecting monitoring data on unregulated contaminants, and that data help EPA to decide whether or not to regulate a given contaminant. In 1999 EPA promulgated an unregulated contaminant monitoring rule (UCMR) that imposed various monitoring requirements on community water systems for a list of unregulated contaminants. The first round of the UCMR consisted of 26 unregulated contaminants that required some amount of monitoring data. The second UCMR identified an additional 24 unregulated contaminants not identified by the first UCMR. Just as with the three rounds of CCLs – there are no pharmaceuticals or personal care products identified as unregulated contaminants for which water systems must monitor.

Third, to our knowledge, EPA has never asked water systems to provide voluntarily testing and monitoring data for pharmaceuticals and personal care products.

Fourth, in recognition of the public's right to know the Safe Drinking Water Act requires community water systems to mail to each of their customers an annual report on the level of contaminants in the drinking water that they supply. These consumer confidence reports (CCRs) must contain, among other things, information on the source of the water, detections of regulated contaminants in the water, and levels of unregulated contaminants found in the water (those unregulated contaminants identified by the UCMR). Because none of these chemicals appear on any of the Candidate Contaminant List or Unregulated Contaminant Monitoring Rule lists, there are no mandates requiring water systems to inform their customers of the presence of these chemicals. As a result, the public's right to know about contaminants in their drinking water has been harmed by EPA's inaction.

Fifth, Congress mandated that EPA must address endocrine disrupting chemicals in drinking water. As discussed earlier, EPA has dragged its feet in implementing the endocrine disruptor screening program. Since so many pharmaceuticals and personal care products are potential endocrine disruptors, the Agency's failure to begin timely testing of chemicals continues to put all Americans at potential risk, simply from drinking water. Despite the recommendations of its advisory committee (EDSTAC) to include drinking water contaminants on its list of chemicals intended to be screened in the endocrine disruptor screening program as required by the SDWA, EPA's list of 73 chemicals does not include any pharmaceuticals or personal care products.

Sixth, EPA has yet to conduct a risk assessment of pharmaceuticals and personal care products in drinking water or assessed the health effects of the presence of these chemicals in our drinking water. EPA has failed in this respect, despite the fact that it claims it "established a leadership role [on the issue of pharmaceuticals and personal care products in drinking water] beginning in 1999 with publication of a critical review article that attempted to bring together the many different aspects of this complex issue."[26] Despite almost 20 years of evidence that these categories of chemicals were appearing in

[26] http://www.epa.gov/ppcp/faq.html#Whatwasepa, last visited April 11, 2008.

drinking water, EPA still has yet to conduct a risk assessment for pharmaceuticals and personal care products in drinking water.

Finally, as identified by the Associated Press report on pharmaceuticals in drinking water, not all water providers will test for these chemicals in their water. Those that do, rarely disclose this information to their customers. Until EPA chooses to include these chemicals on the UCMR – action EPA has so far refused to take – no disclosure is required under the SDWA and the public remains unaware of the presence of these chemicals in our drinking water.

USGS budget cuts will severely impair critical water monitoring programs

We look to the federal government to protect the public's health. The public has reasonable and serious concerns that exposures to medications and other synthetic chemicals found in drinking water could be impacting human health. However, with limited data, it is impossible to gauge whether or not pharmaceuticals and personal care products in drinking water could be contributing to the development or progression of human health ailments. A commitment to rigorous long-term monitoring of our nation's waterways is absolutely essential for identifying contaminants, assessing risk, characterizing and localizing contamination patterns, identifying sources of contamination where possible, and measuring the effectiveness of mitigation measures. Resources to undertake this task are inadequate and falling, which will severely undermine our nation's ability to address this issue. Congress should allocate resources to undertake this task as a top priority.

The US Geological Survey (USGS) is responsible for the two main water-quality monitoring programs for the Nation's waterways: the National Water Quality Assessment Program (NAWQA) and the Toxic Substances Hydrology Program. Both water quality programs will suffer devastating blows if the proposed FY09 budget cuts are enacted. Adequate funding for these two programs is necessary to understanding many crucial aspects of water quality, including the impacts of new and under-studied contaminants such as pharmaceuticals and personal care products, and the efficacy of water quality policy-decisions. In short, the proposed deep cuts to these programs will strip the regulatory agencies of their ability to make wise decisions about how to allocate their limited resources to protect the Nation's waterways effectively.

The NAWQA is the larger of the two USGS water-quality monitoring programs; it monitors for environmental contaminants using established measurement methodologies for measuring (pesticides, volatile organic compounds, metals, etc.). Budget constraints over the last eight years has forced NAWQA to cut back from 496 surface-water fixed station water-quality monitoring sites in 2000, to only 113 sites in 2008. The FY09 proposed President's budget would reduce current funding by $9.8M, or 15% from the FY08 enacted funding. To adjust to this severe reduction, the USGS will

be forced to cut monitoring at half of the existing ground water-quality sites so that it can continue to conduct research and analysis of existing data.[27]

The Toxic Substances Hydrology (aka Toxics Program) is the smaller of the two programs. It is a water quality research and methods development program that examines new and understudied environmental contaminants, such as the hormones, pharmaceuticals, and personal care products at issue in this hearing. The Toxics Program develops new capabilities, new methodologies, and new information that enable state water quality programs and NAWQA to address new issues effectively. FY08 funding for the Toxics Program is $13.5 M; for FY09 the request is $10.7 M, reflecting a nearly $3 M cut from the USGS budget. The proposed cuts to the Toxics Program will significantly reduce research capacity on new and understudied environmental contaminants, including pharmaceuticals and personal care products, as well as methyl mercury, arsenic, and nanomaterials.

EPA and Congress should act to address the problem of pharmaceuticals and personal care products contaminating the public's drinking water

Under the Safe Drinking Water Act and the Food Quality Protection Act, EPA has the authority and obligation to ensure the safety of our drinking water. As discussed elsewhere in this testimony, EPA should:

- Include pharmaceuticals and personal care products in the unregulated contaminant monitoring rule to require public water systems to monitor for their presence in our drinking water and to identify in the consumer confidence reports the levels found in the drinking water; in the meantime, water systems should test for pharmaceuticals and personal care products and report the results to their customers;
- Add pharmaceuticals and personal care products to the candidate contaminant list 3 (CCL3) and evaluate the need to regulate the presence of these chemicals in drinking water;
- Immediately finalize and implement testing under the endocrine disruptor screening program and add drinking water contaminants, including mixtures of pharmaceuticals and personal care products to the list of chemicals that must be screened under that program;
- Evaluate and identify wastewater and drinking water treatment practices for removing pharmaceuticals and personal care products;
- In consultation with FDA and other federal research bodies, conduct studies to understand the health effects of discarded pharmaceuticals and personal care products on the nation's waterways and drinking water supplies; and
- Work with other federal agencies and states to prevent or limit the overuse of antibiotics in agriculture, particularly those that are critical for human use.

Congress needs to take additional steps to help address this issue, including:

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[27] USGS briefing sheet: Impacts of proposed FY09 budget cuts on National Water-Quality Assessment (NAWQA) program. February, 2008

- Establish take back programs for pharmaceuticals;
- Increase funding for wastewater and drinking water infrastructure; and
- Reform the Toxic Substances Control Act to reduce the number and amount of persistent, bioaccumulative and toxic chemicals that are released into the environment.

Thank you for inviting me to testify before you today. NRDC looks forward to working with the Subcommittee and Full Committee to address these important issues. I would be happy to answer any questions from the Committee.

RESPONSE TO Questions from: Senator Barbara Boxer

Question #1
Dr. Sass, can you give me an idea of how sensitive the human endocrine system is during periods of particular vulnerability, such as pregnancy or when infants are rapidly developing in the first few years after birth?

The endocrine system is comprised of multiple organs and glands in the body that secrete hormones into the bloodstream. The endocrine system regulates growth and development, metabolism, and tissue function as well as sexual function and reproductive processes. In a fetus or infant, organs are rapidly growing and undergoing differentiation, many under the control of hormones secreted by the endocrine system. We know that exposures to endocrine disruptors during these critical periods of development can have permanent and irreversible effects.

For example, in the 1960-70's, pregnant women were given the synthetic estrogen pharmaceutical, DES, to promote a "healthy pregnancy". Instead of preventing harm to the fetus however, exposure to this hormone during fetal development resulted in malformations in female reproductive organs such as the fallopian tubes, uterus, cervix and vagina. As a result, when these girls reached reproductive age, many of them developed an otherwise-rare cancer of the reproductive tract or experienced infertility.[1]

Likewise, thyroid hormone in precise amounts during fetal and infant life stages is critical for normal development of the nervous system. The serious health risks of low thyroid hormone levels are so well accepted and recognized that all newborns are immediately screened for thyroid hormone levels at birth. If low levels are left untreated, mental retardation will develop. Exposure to a number of thyroid disrupting chemicals has been associated with impairment of the developing nervous system resulting in problems with behavior, memory and IQ. Some of these thyroid disruptors include PCBs, dioxins and furans, flame retardants (PBDEs) and perchlorate.[2]

Question #2
Dr. Sass, please provide copies of peer-reviewed studies that you have that demonstrates that biological systems, including the endocrine and reproductive systems, can be affected by exposure to chemical substances in the range of parts per trillion or lower.

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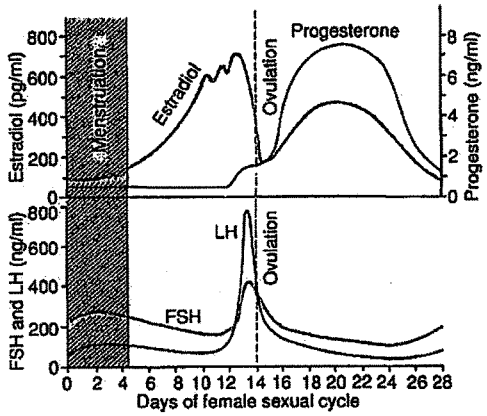
[1] Rubin MM. Antenatal exposure to DES: lessons learned...future concerns. 2007 Obstet Gynecol Surv. 62(8):548-55.

[2] Crofton KM. Thyroid disrupting chemicals: mechanisms and mixtures. 2008. Int J Androl. 31(2):209-23.

Normally, physiological concentrations of sex hormones are in the parts per billion (ppb; ng/ml) to parts per trillion (ppt; pg/ml) range as demonstrated in the graph below which depicts circulating levels of the hormones, estrogen and progesterone, during the human female menstrual cycle.



Therefore, it is not surprising that endocrine disrupting chemicals can interfere with the normal action of hormones at low doses. This phenomenon has been explained more thoroughly by Welshons et al.[3] in an attached manuscript.

In animal studies, the estrogenic chemical, bisphenol A, has been shown to cause alterations in mammary (breast) gland development at 25 ppt[4]. More recently, in studies of human cells, low levels of exposure to BPA were shown to cause gene expression changes consistent with those seen in aggressive breast cancer.[5] Other estrogenic chemicals such as DES have been shown to interfere with development of the prostate

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[3] Welshons, WV, KA Thayer, BM Judy, JA Taylor, EM Curran and FS vom Saal. 2003. Large effects from small exposures. I. Mechanisms for endocrine disrupting chemicals with estrogenic activity. Environmental Health Perspectives 111:994-1006.

[4] Muñoz-de-Toro, M, C Markey, PR Wadia, EH Luque, BS Rubin, C Sonnenschein and AM Soto 2005. Perinatal Exposure to Bisphenol A alters peripubertal mammary gland development in mice. Endocrinology 146: 4138-4147.

[5] Dairkee SH, Seok J, Champion S, Sayeed A, Mindrinos M, Xiao W, Davis RW, Goodson WH. Bisphenol A induces a profile of tumor aggressiveness in high-risk cells from breast cancer patients. 2008. Cancer Res. 68(7):2076-80.

gland in the ppt range.[6] Low dose exposures to another estrogenic herbicide, atrazine, have been associated with hermaphrodism in frogs.[7]

Endocrine disruption at low doses is not limited to the sex hormones, however. In studies of workers, exposure to the dioxin, 2,3,7,8-TCDD, in the ppt range has been associated with changes in thyroid hormone levels[8] and the development of diabetes[9] (full manuscripts attached).

Question #3
Dr. Sass, please describe the following:

A. The importance, in terms of protecting public health, of taking into consideration human exposures to multiple pharmaceuticals that are designed to affect very delicate biological systems, like the human endocrine system?

B. The need to consider these types of exposures occurring during windows of vulnerability, such as pregnancy?

As described above, hormones naturally function in the ppb-ppt range. Many pharmaceuticals are synthesized and intended to exert their biological effects on endocrine organs in this concentration range. For example, the typical dose of an oral contraceptive contains 20 micrograms of ethinyl estradiol. For a typical woman weighing 60 kg, this is about 300 ng/kg or 300 ppt of synthetic estrogen with each dose. This dose of estrogen, when combined with a low dose of synthetic progesterone and taken on a daily basis, will prevent pregnancy by inhibiting ovulation.

Research done in the United Kingdom has found synthetic estrogens in sewage effluent in the ppt range (1 – 80 ng/L).[10] These levels of synthetic estrogens have been associated with the development of female characteristics in male fish.[10] Likewise, levels of androgenic (anabolic) hormones from confined animal feed operations (CAFOs) are in

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[6] Timms BG, Howdeshell KL, Barton L, Bradley S, Richter CA, vom Saal FS. 2005, Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Proceedings of the National Academy of Sciences (US) 102(19):7014-9

[7] Hayes, TB, A Collins, M Lee, M Mendoza, N Noriega, AA Stuart, and A Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide, atrazine, at low ecologically relevant doses. Proceedings of the National Academy of Sciences (US) 99:5476-5480.

[8] Johnson E, Shorter C, Bestervelt L, Patterson D, Needham L, Piper W, Lucier G, Nolan C. 2001. Serum hormone levels in humans with low serum concentrations of 2,3,7,8-TCDD. Toxicol Ind Health. 17(4):105-12.

[9] Henriksen GL, Ketchum NS, Michalek JE, Swaby JA. 1997 Serum dioxin and diabetes mellitus in veterans of Operation Ranch Hand. Epidemiology. 8(3):252-8.

[10] Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol 32:1549–1558.

the ppt (ng/L) range and have been shown to cause masculinization of female fish and reductions in fertility.[11]

We do not know what, if any, effect these levels of contamination are having on human health. However, in vulnerable populations, such as those people with hormone dependent cancers, it is the standard of care in clinical medicine to limit exposure to hormones that promote cancer growth. For example, some breast cancer cells are stimulated to grow after exposure to estrogen and therefore, women with estrogen-receptor positive cancer are treated with the anti-estrogen, tamoxifen, and are not given synthetic estrogens for birth control or post-menopausal replacement therapy. It is conceivable that low levels of exposure to synthetic estrogenic contaminants in water could promote the growth of estrogen responsive tumors. In men with prostate cancer, exposure to androgens promotes the growth of this tumor and therefore, prostate cancer is routinely treated with anti-androgen therapy. Likewise, it is conceivable that exposure to low levels of androgenic contaminants, such as those released from CAFOs, could promote the growth of prostate cancer.

The paucity of information on the endocrine disrupting effects of synthetic chemicals points to an urgent need for our federal agencies to investigate the scope of this problem and for EPA to immediately initiate screening of chemicals under the Endocrine Disruptor Screening Program (EDSP). It is even more concerning that for the little information we have on individual chemicals, we have almost no information on the potential effects of exposures to chemical mixtures.

Emerging independent research has indicated synthetic chemicals are able to act in an additive manner. For example, laboratory studies on mixtures of estrogenic chemicals, including pharmaceuticals and synthetic chemicals, have found exposure to a combination of low dose estrogens causes feminization of male fish, whereas when these chemicals were given individually at the same low levels, no effect was observed.[12] Likewise, exposure to a mixture of anti-androgenic phthalates and pesticides causes male reproductive harm in an additive manner.[13] Because none of us are exposed to just one chemical at a time, we desperately need to have a better understanding of how exposures to mixtures of endocrine disrupting chemicals are impacting human health. Indeed, EPA should be testing mixtures of chemicals for the endocrine disrupting effects, especially the chemical mixtures that are commonly found in drinking water.

Question #4
Dr. Sass, please describe your view as to whether EPA has adequately implemented the

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[11] Durhan EJ, et al. 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ Health Perspect. 114 Suppl 1:65-8.

[12] Brian JV, Harris CA, Scholze M, Backhaus T, Booy P, Lamoree M, Pojana G, Jonkers N, Runnalls T, Bonfà A, Marcomini A, Sumpter JP. 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ Health Perspect. 113(6):721-8.

[13] Rider, C. V., Furr, J., Wilson, V. S., and Gray, L. E. (2008). A mixture of seven antiandrogens induces reproductive malformations in rats. International Journal of Andrology 31, 249-262

Endocrine Disruptor Screening and Testing Program, including the requirements under both the Food Quality Protection Act and under section 1457 of the Safe Drinking Water Act.

EPA's implementation of the Endocrine Disruptor Screening Program (EDSP) has been a decade-long tale of delays, setbacks, and more delays. Despite congressional mandates and judicial decrees, EPA continues its woefully inadequate implementation of a screening program that is a crucial first step to protecting public health from a dangerous family of chemicals.

In 1996, through the Food Quality Protection Act (FQPA), Congress required EPA to develop a screening program that would ascertain whether certain substances may have estrogenic (or other endocrine) effects on humans. Congress, including a unanimous Senate, extended EPA's authority to include substances that may be found in sources of drinking water through the Safe Drinking Water Act (SDWA) Amendments of 1996. The EDSP was intended to help define which chemicals could be capable of causing these effects and ultimately, provide information to be used to protect the public's health.

The FQPA mandated that EPA develop a screening program by 1998 and implement it by 1999. According to the original EPA timeline for implementation, the priority setting database was to be complete by June 2000 and was to be used for priority setting in November 2000. Despite these deadlines, EPA failed to adhere to any of them for priority setting. As a result, NRDC sued EPA for missing the statutorily mandated deadline. The Agency settled that lawsuit by agreeing to publish a proposed initial list of chemicals for screening by December 31, 2002 and to validate and begin requiring chemicals to be tested by December 2003. EPA missed these deadlines as well.

So far, EPA has only published a *draft* List of Chemicals for Initial Tier 1 Screening in July 2007 and has not yet even finalized all the assays for Tier 1 screening. To date, no testing has begun. In fact, it has been ten years since Congress mandated EPA to create this screening program, but the Agency has yet to begin actual testing of any chemicals under this program. Again, Congress has had to push the Agency to act – most recently directing EPA to begin testing by August 2008.

NRDC remains extremely disappointed with EPA's failure to meet the agreed upon deadlines for chemical screening and implementation of the EDSP. In fact, the dramatically scaled-down process that EPA used to produce the initial screening list should not have taken so long to devise. To date, the initial screening list has not been finalized and EPA has not made any announcements of when the list will be finalized.

EPA has failed to protect the public's health not only by delaying implementation of the testing program but also by refusing to regulate any chemicals that have been shown to be endocrine disruptors by other research studies. In addition to failing to take meaningful action to reduce exposures to known endocrine disrupting chemicals, EPA has failed to begin testing the tens of thousands of other chemicals where we have no information about their potential endocrine disrupting effects.

RESPONSE TO Questions from: Senator Benjamin L. Cardin

1) Do you have any information that would indicate how much of the problem of pharmaceuticals in water is caused by the disposal of unused and/or out-of-date prescription medications?

Unfortunately, this question cannot be addressed because of the lack of available data. It is an extremely important question, and we hope that the appropriate Agencies will be tasked with collecting and compiling these data.

It is clear that there are multiple significant sources of pharmaceuticals that contribute to environmental water contamination, including human and animal excretion, disposal of expired or unwanted pharmaceuticals into toilets and drains, runoff from livestock operations, and application of animal waste to fields as fertilizer. However, how much of a contributor to the overall problem each of these and other sources may be is not well understood.

Despite a dearth in data, experts are in agreement that the portion of pharmaceuticals in the environment originating from disposal is likely to be very significant.

2) By almost any measure, abuse of prescription drugs - especially opioids - has become an enormous public health problem. In March of 2007, the Office of National Drug Control Policy (ONDCP) reported that abuse of prescription drugs is considered to be America's second most abused illegal drug, with marijuana ranking first. ONDCP also said that nearly 60% of these drugs are received free from family and friends. What efforts are you aware of to encourage the safe and proper disposal of left-over prescriptions so they aren't available to teenagers and young adults?

Regulators have indicated that the public and the healthcare community routinely request guidance on how to properly dispose of medications. Unfortunately, while there are recommendations and guidelines advising consumers on how to dispose of left-over prescriptions, there is no federal policy requiring pharmaceutical companies to operate take-back programs, or other mandatory measures.

The Federal Office of National Drug Control Policy provides guidance on its website and has a printable fact sheet asking people to discard unused or expired prescription drugs in the trash after mixing them with something like coffee grounds or kitty litter to further ensure that they are not consumed (www.whitehousedrugpolicy.gov/drugfact/factsht/proper_disposal.html). The guidelines specifically say that drugs should be flushed down a toilet only if the label or accompanying instructions specifically recommend this. Importantly, the guidelines also direct consumers to determine if their community has take-back programs that provide a central repository for these products. This webpage has a direct link from the EPA webpage on Pharmaceuticals and personal care products (www.epa.gov/ppcp/).

RESPONSE TO Questions from: Senator James M. Inhofe

1. You seem fairly critical of the way EPA develops the Candidate Contaminant List or CCL. In your opinion, should EPA place every chemical on that list and set a MCL for each contaminant? Do you think EPA should prioritize contaminants to reflect known potential adverse health affects and prioritize their resources accordingly?

The Candidate Contaminant List (CCL) consists of contaminants which are currently unregulated by EPA, but are known or anticipated to occur in public water systems, and which may require regulation under the Safe Drinking Water Act (SDWA). EPA is required to make a determination about whether to regulate at least 5 of the contaminants on the CCL every 5 years. A determination to regulate a contaminant must be based on a finding by EPA that a contaminant on the CCL (a) may have an adverse human health effect, (b) is known to occur or is substantially likely to occur in public water system "with a frequency and at levels of public health concern," and (c) regulation of that contaminant "presents a meaningful opportunity for health risk reduction for persons served by public water systems."

Every contaminant that is known to occur in public water systems should be placed on the CCL. NRDC does not believe that this necessitates that every contaminant must be regulated with a Maximum Contaminant Level (MCL). Rather, contaminants that meet the criteria set forth by the SDWA for regulation should be regulated. If exposure to a contaminant does not have any adverse human health effects, or if it does not occur or it is not substantially likely to occur in public water systems, or if regulating it will not present a meaningful opportunity for health risk reduction, then such contaminant need not be regulated. In making these judgments, where there is uncertainty, EPA should err on the side of protecting public health.

Since 1996, when Congress first created the CCL, EPA has not determined to regulate one single contaminant on the CCL. This is despite the fact that the list contains contaminants like perchlorate, acetochlor, and alachlor, which have proven adverse health effects and are widespread pollutants. Perchlorate is highly mobile in water and can persist for decades under typical ground and surface water conditions.[14] It can concentrate in crops such as wheat, lettuce, alfalfa, and cucumbers, resulting in much greater exposures than might be predicted by water or fertilizer concentrations.[15] Newer data have shown perchlorate contamination to be widespread in store-bought fruit, vegetables, cow's milk, beer and wine.[16] Perchlorate has been reported in human breast

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[14] U.S. EPA Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization Based on Emerging Information (External Review Draft). Office of Research and Development, Washington, D.C. NCEA-1-0503, 1998.

[15] Jackson WA, P Joseph, P Laxman, K Tan, PN Smith, L Yu, and TA Anderson. 2005. Perchlorate accumulation in forage and edible vegetation. J Agric Food Chem. 53(2):369-73.

[16] El Aribi, H, YJC Le Blanc, S Antonsen, and T Sakuma. 2006. Analysis of perchlorate in foods and beverages by ion chromatography coupled with tandem mass spectrometry (IC-ESI-MS/MS).

milk and it was found in every one of 2820 urine samples the Centers for Disease Control and Prevention (CDC) recently tested.[17]

Perchlorate interferes with the normal uptake of iodide into the thyroid gland, as well as normal transport of iodide across the placenta and into the lactating mammary gland, which can result in decreased capacity to synthesize thyroid hormones. In the developing fetus and infant, adequate thyroid hormones are necessary for normal brain development. Therefore, subtle alterations of thyroid hormone during pregnancy – even within the normal range – have been associated with decreased intellectual and learning capacity in childhood.[18]

Despite this evidence of adverse human health effects and widespread contamination by perchlorate, EPA has not chosen to regulate perchlorate levels in drinking water, despite having perchlorate on the CCL since 1996. Instead, EPA chose not to regulate 9 contaminants on the first CCL[19] and has preliminarily decided not to regulate 11 contaminants on the second CCL.[20] In effect, EPA has abdicated its duty to protect public health by ignoring the presence of known contaminants in drinking water that are known to be causing adverse human health effects. In the case of perchlorate, states such as California and Massachusetts have had to promulgate their own drinking water standard in the absence of action by EPA.

Yes, EPA should prioritize its work to ensure that the chemical contaminants in our drinking water that pose human health risks are regulated to protect public health. In fact, taking such basic actions to fulfill the agency's mission, as well as its obligations under the Safe Drinking Water Act is long overdue. In 1996, Congress created the CCL requirement to replace the previous statutory mandate established in 1986 that required EPA to set health standards for 25 contaminants every 3 years. That previous mandate was a result of Congress' dissatisfaction with EPA's inaction between the original

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Analytica Chimica Acta. 567(1): 39-47; Food and Drug Administration. 2004. Exploratory Data on Perchlorate in Food. Available at http://www.cfsan.fda.gov/~dms/clo4data.html

[17] Kirk AB, PK Martinelango, K Tian, A Dutta, EE Smith, and PK Dasgupta. 2005. Perchlorate and iodide in dairy and breast milk. Environ Sci Technol. 39(7):2011-2017; Blount BC, L Valentin-Blasini, JD Osterloh, JP Mauldin, and JL Pirkle. 2006. Perchlorate exposure of the US population, 2001-2002. J Expo Sci Environ Epidemiol. Oct 18, 2006 [Epub head of print].

[18] Haddow JE, GE Palomaki, WC Allan, et al. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. New Eng J Med 341:549-555, 1999;

Pop VJ, JL Kuijpens, AL van Baar, et al. Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in early infancy. Clin Endocrinol. 50:149-155, 1999.

[19] Acanthamoeba, Aldrin, Dieldrin, Hexachlorobutadiene, Manganese, Metribuzin, Naphthalene, Sodium, and Sulfate

[20] boron, the dacthal mono- and di-acid degradates, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), 1,3-dichloropropene (Telone), 2,4-dinitrotoluene, 2,6-dinitrotoluene, s-ethyl propylthiocarbamate (EPTC), fonofos, terbacil, or 1,1,2,2-tetrachloroethane.

enactment of the Safe Drinking Water Act in 1974 and its reauthorization in 1986. Ultimately, some parties argued that the 1986 requirement for EPA to regulate 25 contaminants every three years was too onerous. As part of the compromise that yielded the 1996 amendments, the 25 contaminant requirement was replaced with the significantly less onerous CCL process. That process was intended to reduce the workload of EPA, and allow the agency to use its expertise and focus on setting health-protective standards for the drinking water contaminants of greatest public health concern. Unfortunately, the intent of Congress has not been fulfilled in this regard.

NRDC finds it troubling that with this greatly reduced burden, EPA still has done essentially nothing, only enacting health standards for those contaminants which had specific deadlines included in the 1996 amendments. By all appearances, there are numerous contaminants that are likely to affect human health and are found in drinking water (e.g. perchlorate, trichloroethylene, perfluorinated chemicals, methyl tertiary butyl ether) and yet EPA continues to list and monitor for chemicals that are not as important to protecting public health. The problem with EPA and the SDWA is not that the Agency has set too many standards for drinking water contaminants, but rather that it has set too few. Indeed, EPA's miserable performance in implementing the CCL process has led to greater momentum for legislation requiring EPA to set health-protective standards for individual contaminants by a date certain.

2. Is there anything in the Safe Drinking Water Act that precludes a municipality from monitoring for pharmaceuticals and disclosing the monitoring results to the public? Shouldn't local governments and their individual constituents decide as to whether or not they want to pay for additional monitoring and reporting?

Since 1974, it has been the policy of the Congress as established in the Safe Drinking Water Act (reaffirmed via successive reauthorizations), that there is a national interest in maintaining safe drinking water supplies throughout the country, rather than relying upon a patchwork system of state-by-state or local-by-local government standards. The Safe Drinking Water Act sets national standards for the maximum level of specific contaminants that can be present in drinking water. States may choose to set standards that are more stringent than those set by the federal government.

All Americans deserve to know that when they turn on the taps in their homes, they are getting clean, safe drinking water. Recognizing the importance of the public's right to know, in the 1996 Amendments to the Safe Drinking Water Act, Congress (including a unanimous Senate) required all water systems either to mail to or to make available to customers annual reports that identify the detections of regulated and unregulated contaminants in their drinking water. 42 U.S.C. § 300g-3(c)(4). These reports play a critical role in providing information to the public about the quality of their drinking water. As explained by EPA in promulgating the rules for these reports:

> These reports will provide valuable information to customers of community water systems and allow them to

> make personal health-based decisions regarding their drinking water consumption.
> These reports are the centerpiece of public right-to-know in SDWA. The information contained in consumer confidence reports can raise consumers' awareness of where their water comes from, help them understand the process by which safe drinking water is delivered to their homes, and educate them about the importance of preventative measures, such as source water protection, that ensure a safe drinking water supply. Consumer confidence reports can promote dialogue between consumers and their drinking water utilities, and can encourage consumers to become more involved in decisions which may affect their health. The information in the reports can be used by consumers, especially those with special health needs, to make informed decisions regarding their drinking water. Finally, consumer confidence reports are a key that can unlock more drinking water information. They will provide access through references and telephone numbers to source water assessments, health effects data, and additional information about the water system.

63 Fed. Reg. 44511 (August 19, 1998).

These reports are meant to allow the public to make informed decisions about their drinking water.

The SDWA creates a minimum standard for monitoring and reporting contaminants in drinking water for public water systems. It also includes a process for EPA to select for monitoring and reporting those chemicals that raise the greatest concern for health effects and occurrence (as discussed more fully in the previous answer). As such, public water systems cannot opt out of complying with the federal law – and the SDWA was not written to give a public water system carte blanche to pick and choose which contaminants found in its water system to report on to their customers. EPA should exercise its authority under the SDWA to ensure that the public knows about the presence of contaminants in our drinking water supply.

Senator LAUTENBERG. Thank you, Dr. Sass.

Dr. Goldhammer, we are pleased to have you here, the Deputy Vice President of Regulatory Affairs for the Pharmaceutical Industry Association, also known as PhRMA. Welcome.

STATEMENT OF ALAN GOLDHAMMER, DEPUTY VICE PRESIDENT, REGULATORY AFFAIRS, PHARMACEUTICAL RESEARCH AND MANUFACTURERS ASSOCIATION

Mr. GOLDHAMMER. Thank you very much, Mr. Chairman.

Today, my focus will be on four key areas: where do these trace amount of pharmaceuticals come from; is there any impact on human health; do they have any impact on aquatic life; and the activities or pharmaceuticals in the environment, or as we call it, the PIE Task Force.

Pharmaceuticals are found in the environment primarily because trace amounts of medicines pass through the human body without being completely metabolized. They make their way through to surface waters, through municipal wastewater treatment systems. These concentrations of pharmaceuticals in the environment are extremely low. In fact, we probably would not be here today were it not for the development of improved analytical testing permitting the detection of these trace amounts in surface waters. The concentrations of pharmaceuticals in drinking water are generally at trace levels in the parts-per-trillion range. To put this in context, one part-per-trillion is about one penny in $10 billion. On average, the pharmaceuticals detected in U.S. drinking water are present at only 18 parts-per-trillion.

It is currently not possible to prevent medicines from entering the environment. Wastewater treatment plants were designed to mimic natural biodegradation process and to reduce, not eliminate, pollutants present in domestic wastewater. Compounds including pharmaceuticals, consumer products and household cleaning agents are expected to be present at trace amount levels in discharges from wastewater treatment plants.

Pharmaceuticals are rigorously tested prior to their approval by regulatory agencies. Numerous years are spent studying their efficacy and safety in animal studies and directly in human clinical trials. As part of this effort, the time course of absorption into the body, metabolism, and ultimately excretion are measured. Unmetabolized medicine and their byproducts that are excreted find their way into the environment.

Scientific studies from several countries, including the United States, conducted to date suggest that these small quantities of pharmaceuticals are unlikely to be harmful to human health. For example, dietary exposure to hormones such as the estrogen that naturally occurs in milk and soy products is much higher than the exposure to residues of any estrogen-like pharmaceutical in water. Trace levels of antibiotics found in surface waters are far below concentrations necessary to develop antibiotic resistance in microbes.

Even though much research has been done over the past decade, there are still understandable questions raised by the public about possible impacts from the presence of these molecules. PhRMA and its member companies are committed to working with experts to

openly consider and help answer these questions. We believe that environmental impacts are already addressed through current regulations. An environmental assessment or EA is part of the drug registration process to evaluate the potential for environmental impacts of a human pharmaceutical. Substances entering the environment at less than one part-per-billion are typically excluded, although an EA for these substances based on extraordinary circumstances may be required. Data on environmental fate, transport, and potential effects may be developed and submitted as part of this regulatory process.

While there is speculation that the potential might exist for pharmaceuticals to impact wildlife, the scientific community believes that pharmaceuticals will not result in short-term toxicity. This consensus is based on the documented low concentrations of pharmaceuticals in the environment and a substantial quantity of acute toxicity data. PhRMA supports this view.

The mere presence of a substance in water does not mean harm will result. The critical factors are the concentration present in the water and whether that concentration is a high enough level to cause an effect. If the concentration is not high enough, that compound is simply likely to be a part of the vast background of chemicals both natural and synthetic present in natural environments such as soil and water.

PhRMA is committed to understand the environmental significance of trace concentration of pharmaceuticals in the environment. We developed the PhATE model to predict concentration of pharmaceuticals in a variety of water sources. This model is now being used by researchers in Korea, Japan and Canada to predict environmental concentrations of pharmaceuticals in the surface water of those countries. Representatives from our PIE Task Force have published dozens of articles in the peer-reviewed scientific literature evaluating the fate and effects of pharmaceuticals in the environment.

We developed the PhACT data base to summarize all published English-language peer-reviewed literature about the effects of pharmaceuticals on aquatic life, as well as treatment data for pharmaceuticals in wastewater and drinking water. Our representatives have participated in and led numerous scientific conferences over the past 5 years.

Although a minor contributor to the environment, unused medicines that are flushed down toilets or poured down sinks can find their way into the environment. In March, 2008, PhRMA joined the American Pharmacist Association and the U.S. Fish and Wildlife Service in launching the SMARxT Disposal Program aimed at educating the public about not flushing or pouring any unused medicines down the drain. This program is designed to raise public awareness and promote the proper disposal of unused medicines.

PhRMA remains committed to the ongoing study of trace amounts of prescription pharmaceuticals in the environment. Our PIE Task Force will continue to work with interested stakeholders to explore the scientific issues associated with pharmaceuticals in the environment, and we will also partner with interested parties to better communicate the message about responsible disposal of unused medicines.

As the available science demonstrates and PhRMA concurs with, trace amounts of pharmaceuticals in the environment are unlikely to pose any human health or environmental risks.

[The prepared statement of Mr. Goldhammer follows:]

Statement PhRMA

Statement at the Senate Environment and Public Works Committee
Subcommittee on Transportation Safety, Infrastructure Security, and Water Quality
Pharmaceuticals in the Nation's Drinking Water:
assessing potential risks and actions to address the issue

April 15, 2008

Alan Goldhammer, PhD
Deputy Vice President, Regulatory Affairs
Pharmaceutical Research and Manufacturers of America

Thank you Mr. Chairman and members of the Committee. My name is Alan Goldhammer, Ph.D., and I am the Deputy Vice President for Regulatory Affairs at the Pharmaceutical Research and Manufacturers of America (PhRMA), a trade association representing the leading research-based pharmaceutical and biotechnology companies. PhRMA member companies invested an estimated $44.5 billion in 2007 for innovative biomedical research to discover and develop new medicines that meet medical needs.

It is not a new development that drinking water may contain trace levels of pharmaceuticals as well as other household chemicals. What is new is that we now have more sensitive analytical methods to detect these substances at very low levels.

Reports of the presence of trace levels of pharmaceuticals in the water sparked the creation of a Pharmaceuticals in the Environment (PIE) Task Force within PhRMA in the late 1990s. The PIE Task Force is familiar with essentially all of the reports published worldwide of pharmaceuticals in water sources, including testing conducted by the U.S. Geological Survey (USGS), routine municipal water testing, testing by scientists from Europe to Asia, and even high school science students whose projects were on display in school science fairs.

PhRMA and its member companies advocate the safe and effective use of medicines and support the principles of product stewardship. As such, we are committed to applying the same level of scientific rigor to studying pharmaceuticals in the environment (PIE) as we apply to other areas of our business. PhRMA and its member companies have conducted research on a variety of PIE-related issues, including: 1) evaluating if detectable levels of pharmaceuticals in drinking water pose a risk to human health, 2) evaluating methods for the effective disposal of human medicines, and 3) determining the potential effects of human pharmaceuticals and their metabolites in surface waters on aquatic life. This research led to the publication of several research reports. We also continue to have an open dialogue with federal and state government officials and other stakeholders about the scientific findings. My testimony today will focus on the detection of trace amounts of pharmaceuticals, the potential human and environmental impacts, and how collectively we can minimize pharmaceuticals in the environment by proper disposal of unused medicines.

Where do these compounds come from?

Pharmaceuticals are found in the environment primarily because trace amounts of medicines

pass through the human body without being metabolized completely and make their way to surface waters through the municipal wastewater treatment system. These pharmaceuticals along with many other consumer products and household cleaning agents find their way into the wastewater from all households where there are family members who take medicines.

It is important for all of us to recognize that the concentrations of pharmaceuticals in the environment are extremely low. In fact, we probably would not be here today were it not for the development of improved analytical testing technology that has made it possible to detect trace amounts of consumer chemicals, including pharmaceuticals, in surface waters. The concentrations of pharmaceuticals reported in U.S. drinking waters are generally at trace levels of nanograms per liter (ng/l) or part-per-trillion (ppt). To put this in context, one ppt is about one second in 32,000 years or 1 penny in $10 billion. On average, those pharmaceuticals detected in U.S. drinking water are present at only 18 ppt.

It is not currently possible to prevent medicines from entering sewage. Wastewater treatment plants are designed to mimic the natural biodegradation processes that occur when organic compounds enter the environment. These systems are designed to reduce, but not eliminate, pollutants present in domestic wastewater. Therefore, a majority of the compounds used in households are expected to be present at trace levels in the discharges from wastewater treatment plants.

There are other minor sources for pharmaceuticals in the environment. Unused medicines can contribute to pharmaceuticals found in surface waters if they are flushed down toilets or poured down sinks. Recent Federal guidance on disposal recommends that medicines be disposed of in household trash or alternatively taken to local collection programs that accept unused medicine.

Is there an effect on human health?

Many technical experts have contributed to the on-going scientific discussions about pharmaceuticals in the environment. The studies conducted to date suggest that it is highly unlikely that the very small quantities of even potent pharmaceuticals detected in the environment would be harmful to human health. Dietary exposure to hormones such as the estrogen that naturally occurs in milk and soy products is much higher than exposure to residues of any estrogen-like pharmaceutical in water. Trace levels of antibiotics found in surface waters are far below the concentrations necessary to develop antibiotic resistance in microbes. In summary, there appears to be no demonstrable risk to human health from detected concentrations of pharmaceuticals in surface waters.

Active pharmaceutical ingredients (APIs) are the most thoroughly studied substances in the world for their effects on human health. Billions of dollars are spent every year to discover and evaluate the efficacy and safety of new pharmaceuticals that can combat the effects of human diseases. Government agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have rigorous regulations that guide the collection and evaluation of information required to support the safety and efficacy of new pharmaceutical compounds. The FDA creates summaries of its evaluations of new APIs and makes them available to the public when the molecule is approved for use as a new medicine in the U.S.

Acceptable use of an API in patients is judged by comparing the benefits from therapy with the risk of potential side effects. Approval of a new API routinely requires testing the efficacy and safety of the molecule in animal studies and directly in human clinical studies. Depending on the targeted use of an API, this testing normally includes evaluation of the molecule for properties of therapeutic efficacy, of acute, chronic and reproductive toxicity, and of genotoxicity and carcinogenicity. Additional evaluations of therapeutic efficacy and side effects are determined for patients in clinical settings. In order to understand systemic exposure from administration of an API, animal and human studies are conducted to measure the time course of absorption into the body, distribution into the blood and throughout the body, metabolism by organs and tissues, and excretion of the molecule and its metabolites via the liver and kidney into feces and urine. APIs and the metabolites eliminated from humans are piped to sewage treatment systems and then to surface waters. Detection of these chemicals has raised questions among some scientists about their safety to humans who might be exposed through drinking water.

Questions about the safety of drinking water have been addressed over several years by authors from the pharmaceutical industry, academia, government and the non-government sector. To date, no published investigation has found that exposure to these detectable residues creates a demonstrable risk to human health. A report for the Drinking Water Inspectorate of the United Kingdom concluded that even worst-case exposure for most major pharmaceuticals provided a high margin of safety.[1] Similarly, a study of human health risk assessments for 26 pharmaceuticals representing 14 general classes found in U.S. waters by the USGS was carried out.[2,3] The safety of drinking water and fish consumption was conducted using methods equivalent to those used by the U.S. Environmental Protection Agency (U.S. EPA) for drinking water under worst-case exposure conditions. The evaluation demonstrated that those detectable residues of pharmaceuticals in surface waters, and concentrations modeled under worst-case conditions, were safe and presented no demonstrable risk to human health.

An evaluation of the safety of drinking water in Germany concluded that risk is likely to be low from exposure to trace levels of pharmaceutical residues.[4] Another study evaluating the safety of potential residues of representative anti-cancer, lipid-regulating, anti-inflammatory, or analgesic drugs in drinking water found no demonstrable risk to human health.[5] A scientific paper published about a decade ago concluded that there was negligible human health risk from environmental exposure to an antibiotic that can be an allergen (phenoxymethylpenicillin), a potent estrogen agonist (17-alpha ethinylestradiol) or an anti-cancer drug (cyclophosphamide).[6]

For some perspective, researchers calculated that more than 90% of the 64 pharmaceuticals they evaluated would have a safety margin of at least 150,000 to reach a single therapeutic dose from water.[7] Put another way, over a 70-year lifetime, less than 20 percent of a single therapeutic dose would be ingested in drinking water. All of the APIs that they evaluated had a safety margin of 1000 or more to reach a single therapeutic dose from water. These safety margins should not be a surprise even for potent pharmaceuticals, since total use and excretion by patients are lower with higher potency pharmaceuticals.

Even though questions about the safety of detectable residues of pharmaceuticals in surface waters to human health have been evaluated in published articles for at least a decade, there are still understandable questions raised by the public about the possible impacts from the

presence of these molecules. PhRMA and its member companies are committed to working with experts to openly consider and help answer these questions.

What is the Potential Impact on Aquatic Life?

PhRMA believes that environmental impacts are already addressed through current regulations. The FDA requires an environmental assessment (EA) as part of the drug registration process in order to evaluate the potential for impacts to the environment (wildlife) as a result of patient use of a pharmaceutical. Substances that may enter the environment at less than 1 part per billion are typically excluded, although the FDA can require an EA for these substances based on extraordinary circumstances. Data on environmental fate (i.e., biodegradation, photolysis), transport (i.e., sorption, solubility), and potential effects (i.e., algae, invertebrates, fish) may be developed and submitted as part of the EA or as part of the drug registration process in other countries. The core finding of an EA is the relationship between the Predicted Exposure Concentration (PEC) and the Predicted No Effect Concentration (PNEC).

The presence of pharmaceuticals in surface waters has captured the attention of scientists, some of whom have speculated that the potential might exist for pharmaceuticals to impact wildlife. However, the scientific community and published literature believe that pharmaceuticals will not result in short term (acute) toxicity. This consensus is based on the documented low concentration of pharmaceuticals in the environment and a substantial quantity of acute toxicity data. PhRMA supports this view.

The results of acute and chronic toxicity testing demonstrate that a significant majority of pharmaceuticals do not present a significant risk from chronic exposure to the concentrations that exist in the environment (i.e., the PEC is well below the PNEC). Some compounds may, however, present extraordinary circumstances based on their potency or exposure scenarios. Compounds such as hormones are being investigated to determine whether extraordinary circumstances are warranted.

In a recent report the Associated Press (AP) claimed that "Drugs in Water Hurt Fish and Wildlife." This claim pointed to the potential for some of these situations to be considered extraordinary. Although the AP did not reference specific scientific reports that were the basis of these claims, most of the evidence cited by AP appears to be already known to scientists. In some cases, AP mentions preliminary results from ongoing studies. Without peer review or the ability to examine the methodology used and data produced from these studies, it is impossible to evaluate the science and validity of the AP claims.

The mere presence of a substance in water does not mean harm will result. The crucial factors determining whether a risk to wildlife exists are the concentration present in the water and to which an organism is exposed, as well as whether that concentration is at a high enough level to cause an effect. If the concentration is not high enough, that compound is likely to simply be part of the vast background of inorganic and organic chemicals, both natural and synthetic, present in natural environments such as soil and water.

What is PhRMA Doing?

When this issue first arose, PhRMA committed to understand the environmental significance of

trace concentrations of pharmaceuticals in the environment and since that time has worked to assess the range of concentrations of pharmaceuticals that could be in the environment due to patients taking medicines and their potential effects on human health and aquatic life.

PhRMA developed the P*h*ATE® model to predict the concentrations of human health pharmaceuticals in wastewater treatment plant effluent, surface water, and drinking water throughout twelve watersheds in the U.S.[8] The model was recently upgraded to predict concentrations of APIs in biosolids that are removed from wastewater treatment systems. The P*h*ATE model is being used by researchers in Korea, Japan and more recently in Canada to predict environmental concentrations of APIs in the surface water of those countries.

PhRMA published an assessment of the human health effects of the APIs that were investigated by USGS during the national reconnaissance survey. In addition, PhRMA member companies have published dozens of articles in peer reviewed scientific journals evaluating the fate and effects of APIs in the environment.

PhRMA has developed the P*h*ACT® database to summarize all published English language peer reviewed literature about the effects of pharmaceuticals on aquatic life as well as treatment data for pharmaceuticals in wastewater and drinking water. Over 1,200 scientific papers have been entered into the P*h*ACT database through the end of 2007.

PhRMA representatives have participated in and led numerous scientific conferences over the past five years. PhRMA scientists also have chaired sessions on the PIE issue at several technical conferences, including a recent Water Environment Federation Symposium in Providence and the Society of Toxicology 47th Annual Meeting in Seattle.

With respect to unused medicines, PhRMA scholarship leads to the conclusion that the best action for the patient and the environment is for patients to take all of their medication as prescribed. Although the contribution of unused medicine disposal to the presence of pharmaceuticals in water is small, PhRMA encourages patients not to flush unused medicine and to dispose of them in an environmentally acceptable manner. In March 2008, PhRMA joined the American Pharmacists Association and the U.S. Fish & Wildlife Service in launching the SMARxT DISPOSAL™ program aimed at educating the public about not flushing or pouring any unused medicines down the drain. The SMARxT Disposal program is designed to raise public awareness and promote the use of household trash disposal or local collection programs as alternatives to drain disposal. Following these guidelines will also reduce the potential for abusing unused medicines where this may be a concern.

PhRMA has determined that both household trash disposal and incineration of unused medicines are environmentally acceptable ways to dispose of unused medicines. PhRMA is working with the University of Michigan's Graham Environmental Sustainability Institute to study the environmental impacts of take back for incineration and household trash disposal in order to better understand the overall environmental impacts of both these alternatives.

PhRMA is in the process of publishing a scientific study that finds if all unused medicines were placed in household trash and disposed of in municipal landfills, less than 0.1% of the total amount of medicine found in the environment would be contributed from landfills. In order to verify the conclusion of the landfill study, PhRMA is seeking the opportunity to partner with a researcher that is conducting landfill leachate sampling.

PhRMA is working with U.S. EPA, excelleRx, Inc. and other stakeholders to address the unique disposal issues associated with long-term care facilities (LTCFs). These facilities are required to witness destruction of unused medicines and which have typically relied on flushing down the toilet as the primary disposal method. PhRMA believes that approximately one-third of all unused medicines are generated by LTCFs.

Conclusion

PhRMA and its member companies remain committed to the ongoing study of trace amounts of prescription pharmaceuticals in the environment. Our PIE Task Force will continue to work with interested stakeholders to explore the scientific issues associated with pharmaceuticals in the environment. PhRMA will also partner with interested parties to better communicate the message about responsible disposal of unused medicines. As the available science demonstrates, and PhRMA concurs with, trace amounts of pharmaceuticals in the environment do not pose any human health or environmental risks.

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RESPONSE TO QUESTIONS FROM SENATOR CARDIN

Question 1: In your testimony on page two, you note that unused medicines can contribute to pharmaceuticals found in surface waters if they are flushed down toilets or poured down sinks. You suggest this process as a "minor [source] for pharmaceuticals in the environment." What leads you to believe this is minor?

In 2004, PhRMA conducted a study to determine the answer to this question and found that there is very limited available data. Four published studies determined that between 2% to 3% of medicines prescribed for the general public and 7% to 13% of medicines prescribed for patients in nursing homes and other long term care facilities are unused. This data when summarized indicates that patients consume approximately 90% of the medicines sold. In addition, PhRMA investigated patient disposal practices for unused medicines. Published studies show that approximately 54% of patients dispose of medicines in the trash, 35% flush unused medicines and 9% either store medicines or take all of the medicines they purchase.[1] [2] [3] [4] [5]

PhRMA also evaluated the fate of pharmaceuticals disposed of with household trash and landfilled. The study indicates that if all unused medicines were disposed of in the household trash, less than 1% of the pharmaceuticals in surface water would result from disposal in landfills. [6]

Question 2: When discussing unused medicines, you encourage patients not to flush medicines, but to dispose of them in an environmentally-acceptable manner. What are patients supposed to do with opioids and other dangerous drugs?

PhRMA is very concerned by the potential for inappropriate uses of prescription drugs. We have worked extensively with law enforcement and national advocacy groups like DARE America, Community Antidrug Coalition of America, DEA Museum Foundation, and Partnership for Drug Free America to prevent the abuse of prescription and over-the-counter drugs and reduce drug diversion.

In February 2007, the White House Office of National Drug Control Policy and the Environmental Protection Agency (EPA) issued a document entitled "Proper Disposal of Prescription Drugs." They recommended that medicines be disposed of in household trash or through participation in community waste collection programs. The document also included a list of medicines whose labels instruct patients to flush any unused portions of those medicines to prevent abuse of those prescription drugs. PhRMA believes that patients should follow these guidelines when disposing of unused medicines.

Question 3: Dr. Goldhammer, "A Prescription for Cleaner Water" is a voluntary public/private partnership committed to reducing the risks from improper disposal of unwanted or expired medications. By joining A Prescription for Cleaner Water, organizations pledge to provide citizens with environmentally sound disposal centers that will safely dispose of these potentially hazardous medications. Partners will be recognized for their commitment to a clean environment by obtaining a mark through meeting the certification requirements in the Prescription for Cleaner Water Program. Are you familiar with this program?

We are familiar with "A Prescription for Cleaner Water" and their efforts to establish take back programs for unused medicines. PhRMA believes that other approaches also exist to dispose of unused medicines in an environmentally friendly manner. The data that we currently have indicates that both take back for incineration and household trash disposal are environmentally sound disposal options in the United States. The United States has invested in building landfills for municipal waste that meet the comprehensive standards defined in regulations adopted under Subtitle D of the Resource Conservation and Recovery Act. PhRMA has evaluated the effectiveness of landfills for disposal of unused medicines and that evaluation indicated that household trash disposal is an environmentally protective way to dispose of unused medicines. Our assessment of a group of 24 pharmaceutical compounds indicated that if 10% of all of those medicines sold was disposed of in landfills, disposal would be responsible for less than 1% of their total mass in the environment. [7]

Through a grant to the Graham Institute of Sustainability at the University of Michigan, we are evaluating the overall environmental footprints of medicines take back for incineration programs and household trash disposal. We will be happy to share the results of this study with the Committee when it is complete.

RESPONSE TO QUESTIONS FROM SENATOR INHOFE

Question 1: Is PhRMA working with government agencies to address the pharmaceutical presence in drinking water? How do you think those partnerships are working?

PhRMA's PIE Task Force has actively engaged government agencies on the issue of pharmaceuticals in the environment (PIE) at both the federal and state level since approximately 2000. On the federal level, the PIE Task Force has met with the Food and Drug Administration (FDA) and the EPA on a regular basis to provide updates on various PhRMA projects related to the PIE issue. In addition, PhRMA met with the U.S. Geological Survey (USGS) on several occasions to discuss analytical methodologies and the results of their reconnaissance survey. These meetings have focused on scientific studies conducted by both PhRMA and the agencies to better understand the presence of pharmaceutical compounds in water and potential impacts on human health and aquatic organisms.

As an example of these partnerships, PhRMA is working with EPA and FDA to evaluate the potential effects of pharmaceuticals in drinking water. EPA has a well developed scientific and regulatory approach for evaluating safe levels of chemicals in drinking water; and PhRMA companies have extensive knowledge and data regarding the human health effects of pharmaceutical compounds. As another example, PhRMA recently joined the U.S. Fish &

Wildlife Service and the American Pharmacists Association in launching the SMARxT DISPOSAL™ program aimed at educating the public about appropriate disposal of medicines. PhRMA is committed to continuing these cooperative working relationships to further develop our understanding of the PIE issue using sound science.

Question 2: What percent of the estrogen compounds in the environment are due to prescribed medicines?

Prescribed medicines account for a very small percentage of estrogenic compounds in the environment. To put this question into the proper context, we estimated the combined environmental and dietary exposure to the three types of estrogens: those naturally produced by humans and animals, those produced by plants, and those from prescribed products. This indicates potential drinking water exposures to estrogens in the United States are small compared to dietary intake. Comparison of the relative contribution of different sources of estrogens clearly indicates that on a mass basis diet comprises greater than 99% of total estrogen exposure and exposure to prescribed estrogens through drinking water is a very small fraction (less than 0.0001%) of total exposure.[8]

Further analysis considering relative potency of the various types of estrogens demonstrates exposure to prescribed estrogens via drinking water compared to total estrogen exposure is so small (between 700 to 18,000 times lower than dietary exposures) that adverse effects to human health from prescribed estrogens in drinking water are not likely to occur[9].

Question 3: Pharmaceuticals have been detected but does that mean they are harmful? Do they pose a danger to human and aquatic life at the observed concentrations?

The mere presence of pharmaceuticals in the environment does not imply that they are harmful. Measured levels of pharmaceuticals in surface water usually falls well below the equivalent of a teaspoon of sugar in an Olympic-sized swimming pool. The risk of harm from pharmaceuticals depends on comparing the level of exposure that actually occurs and the exposure level that is known to result in a harmful effect. If the actual exposure level is far below a level known to result in a harmful effect, the risk is not considered to be significant. The evaluations published to date demonstrate that it is highly unlikely that the very small quantities of pharmaceuticals in the environment would be harmful to human health, no matter what their potency may be in therapeutic doses.

Similarly, there has not been a causal link established between the concentrations of pharmaceuticals present in water and aquatic life impacts. In environmental monitoring studies, a number of pharmaceuticals have been identified in surface waters around the globe at low, but measurable, concentrations (e.g., aspirin, ibuprofen, acetaminophen, naproxen, carbamazepine). The potential environmental effects of those pharmaceuticals sampled for by the USGS in their survey of surface water contaminants are being studied by PhRMA. The pain medications - aspirin, ibuprofen, and acetaminophen - have been extensively studied because they are present at higher concentrations in surface water samples than most other pharmaceuticals. The aquatic toxicity data for these substances show low potential for effects on organisms at existing environmental exposure levels of these substances.

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Senator LAUTENBERG. Thank you very much.

We are fascinated by the volume of important data that the two of you have put into a relatively short timeframe, so we appreciate that.

Dr. Snyder, the Research and Development Project Manager at the South Nevada Water Authority. We welcome you, sir.

STATEMENT OF SHANE SNYDER, R&D PROJECT MANAGER, APPLIED RESEARCH AND DEVELOPMENT CENTER, SOUTHERN NEVADA WATER AUTHORITY

Mr. SNYDER. Good afternoon. My name is Dr. Shane Snyder, and I am the Research and Development Project Manager for the Southern Nevada Water Authority.

I am appearing today on behalf of the American Water Works Association, the AWWA, whose membership provides drinking water to more than 80 percent of the American people. We commend this Committee for its concern over our Nation's drinking water, and I appreciate the opening comments. Clearly, we share a common objective, which is to ensure that Americans continue to have safe and sustainable drinking water.

Personally, I have conducted research related to trace pharmaceuticals in water for more than a decade, and have served as the principal investigator for numerous research projects related to pharmaceuticals in U.S. water supplies. My work in this field has been transparent, and I have authored more than 50 peer-reviewed scientific publications related to trace contaminants in water during my career. I presented the findings of my study at more than 20 venues in the past year alone.

I would like to make it perfectly clear that I am a scientists. I am not a policymaker. My intent today is to provide you with an objective scientific perspective and offer my professional insight regarding pharmaceuticals in water.

Contrary to recent stories that characterize pharmaceuticals in water as an entirely new issue, pharmaceuticals were first reported in U.S. waters by the EPA more than 30 years ago. Since that pioneering effort, pharmaceuticals have been detected at diminishingly minute concentrations. This is not simply due to greater contamination of our Nation's water supplies. This is a reflection of improved sensitivity and modern analytical technology.

My research team has analyzed hundreds of water samples from across the United States only through the voluntary effort of this Nation's water utilities. While it is true that we detected some pharmaceuticals in U.S. drinking waters, it is important that we place these concentrations into perceivable contexts. Consider that the highest concentration of any pharmaceutical my team detected in U.S. drinking water and placed in terms of time would be equivalent to 1 second in 750 years, in distance, a half-inch from the Earth's surface to the moon. That is the highest concentration we detected.

Now, I am not providing this analogy to dilute the importance of this issue, not at all. Nor am I implying that these levels are safe or unsafe. But it is difficult for all of us, myself included, to perceive nanograms per liter. To illustrate this point further, consider this: If my studies had been constrained by the ability to find phar-

maceuticals in drinking water at parts-per-billion levels instead of parts-per-trillion, we would not have detected one pharmaceutical in any of our studies.

Given the powerful analytical tools today, it is certain that all water on Earth will have detectable levels of some unregulated contaminants. This raises a critical question: Are we going to make decisions about monitoring and treatment based on our ability to find contaminants, or based upon the protection of public health? Again, I am not a policymaker. However, I can tell you with absolute certainty that if we regulate contaminants solely upon detection, we are embarking on a futile journey without end.

The reason is simple. Over the past several decades, analytical methodology has evolved from detection limits at parts-per-million to today's method detection limits in parts-per-trillion, to tomorrow's parts-per-quadrillion. We already have laboratories that can measure things in parts-per-quadrillion. That is about 1 second in several million years.

Utilities must have meaningful numerical targets for monitoring and treatment that are based upon the protection of human health. The detection of pharmaceuticals alone does not imply risk. Just as one cannot assume safety if they are not detected. It is of paramount importance that we consider the topic of pharmaceuticals in drinking water holistically. From the limited evaluation that my team has made, it does not appear that any of the trace pharmaceuticals we have detected in drinking water pose a risk to human health. However, our study is simply the beginning.

My team has also evaluated conventional and advanced water treatment technologies in relation to removing pharmaceuticals from water. Clearly, some technologies are better than others. However, I urge you to consider all the costs, including environmental impacts from increased energy consumption, greenhouse gas emissions, and waste and byproduct issues that follow these advanced processes.

If we should embark into treatment, it is clear that the treatment before release into the environment makes the most sense to protect both the environment and the public health. We encourage more studies related to health effects and further studies toward the development and implementation of sustainable technology to treat water.

Again, I commend this Committee for your sincere concern regarding pharmaceuticals in U.S. drinking waters. I look forward to your questions and comments, and I invite you to contact myself and the AWWA for more information. We are certain that only by working together in a cohesive and collaborative manner can we continue to lead the world in providing our citizens with safe and sustainable drinking water.

Thank you.

[The prepared statement of Mr. Snyder follows:]

American Water Works Association

The Authoritative Resource on Safe Water™

Government Affairs Office
1300 Eye Street NW
Suite 701W
Washington, DC 20005-3314
T 202.628.8303
F 202.628.2846

Headquarters Office
6666 West Quincy Avenue
Denver, CO 80235-3098
T 303.794.7711
F 303.347.0804
www.awwa.org

Statement
of Dr. Shane Snyder, Southern Nevada Water Authority
before the
Senate Subcommittee on Transportation Safety, Infrastructure Security, and Water Quality
on
Pharmaceuticals in the Nation's Water:
Assessing Potential Risks and Actions to Address the Issue

April 15, 2008

Good afternoon. My name is Dr. Shane Snyder and I am the Research and Development Project Manager for the Southern Nevada Water Authority. I have conducted research related to trace contaminants in water, including pharmaceuticals, for nearly 15 years. I have served as principal investigator for numerous research projects related to the trace-level detection, removal, and toxicology of pharmaceuticals in water supplies, and have published approximately 50 peer-reviewed articles and book chapters on this topic. I would like to make it perfectly clear that I am a scientist, not a policy maker. While I am honored to share some of my findings with you today, please keep in mind that I do not establish, suggest, or enforce policy decisions. I am appearing today on behalf of the American Water Works Association (AWWA). AWWA is the world's oldest and largest association dedicated to safe water. Our utility members serve safe and affordable drinking water to over 80 percent of the American people.

Contrary to recent reports that characterize pharmaceuticals in water as an entirely new issue, pharmaceuticals were first reported in US waters by the EPA in 1975. The fact that more pharmaceuticals are detected today is not due to greater contamination of our nation's water, but a reflection of the increasingly sensitive analytical technology that allows us to identify and quantify diminishingly minute concentrations of these chemicals in water.

My research related to trace pharmaceuticals in drinking water has been conducted entirely without federal mandate through the volunteer efforts of our nation's water utilities. The fact is, the cities that participated in my current study by submitting water samples for analysis, did so in the absence of any regulatory requirement, going well above and beyond the regulations in the interest of furthering understanding of this issue.

My previous studies have been transparent, and have been published in open literature and frequently presented in public forums. I will do that again when my current research is complete. However, as a scientist, I would strongly caution against presenting preliminary findings of partially completed studies. In order to provide meaningful information on pharmaceutical compounds and other substances in water, scientists

need both occurrence data and human health effects information. It is scientifically inadequate to communicate solely on what we can measure at any level without a frame of reference for what that means.

I have frequently been asked about the sources of these products in our waters. I will not go into it here in detail, but will note that both nonpoint source runoff and sewage effluent from properly operated waste treatment plants may contain minute traces of these compounds. Some minute quantities of these products will pass through animals and humans who use them, and enter the waste stream. They are typically not completely destroyed or removed by waste water treatment processes.

A more central point about our studies is that the few pharmaceuticals we did detect in US drinking waters occurred at unfathomably low concentrations. To illustrate that point, consider this: If our study had been constrained by the ability to find these compounds at parts-per-billion levels instead of delving into the parts-per-trillion range, none of them—not a single one—would have been found.

This raises a critical question. Are we going to make decisions based upon our ability to find contaminants, or based upon protection of public health? I am not a policy-maker; I am a scientist. However, I can tell you with absolute certainty that, if we regulate contaminants based upon detection rather than health effects, we are embarking on a futile journey without end. The reason is simple: Decades ago, we could only detect contaminants at parts per million levels. Years ago, we advanced to parts per billion. We are now able to detect compounds at the parts-per-trillion level, and are breaching the parts-per-quadrillion boundary in some cases. The truth is that the concentrations of pharmaceuticals found in water supplies are millions of times lower than a medical dose. Consider that the highest concentration of any pharmaceutical we detected in US drinking waters is approximately 5,000,000 times lower than the therapeutic dose. This concentration is difficult to perceive, so consider these analogies. This concentration is roughly equivalent to ½ of an inch in the distance between the earth and the moon, or in terms of time, this concentration would be equivalent to approximately one second in approximately 750 years. Based upon our four-year study of the health relevance of trace pharmaceuticals, using the highest concentrations found and the most conservative safety factors to protect susceptible populations such as infants and pregnant women, our report will demonstrate that one could safely consume more than 50,000 eight-ounce glasses of this water per day without any health effects. While the report will not be published until later this year, I can tell you that the bottom-line conclusion is that the concentrations of pharmaceuticals we studied are orders of magnitude lower than would pose a public health threat. I am not suggesting that this is the final, definitive study on this issue; in fact, I urge you to support further health effects research.

That said, the Safe Drinking Water Act already has established processes for identifying and regulating drinking water contaminants to protect human health. The Candidate Contaminant List and the Unregulated Contaminant Monitoring Rule are appropriate processes that entail great scientific rigor. As a scientist, I would caution against regulating pharmaceuticals any differently than the scores of contaminants currently covered by the Safe Drinking Water Act, because in reality they are no different. Our decision as humans to improve and extend our lives by using pharmaceuticals dictates that some infinitely small amount of these products can and will make their way into the environment. The fact that we can detect trace contaminants does not alone imply risk.

With regard to removing these compounds through treatment, my team has tested the effectiveness of a diverse array of water treatment technologies on removal of pharmaceutical compounds, and to be certain, some technologies are more effective than others. However, the pinnacle question is whether the use of these treatment technologies is warranted to protect public health, because there are environmental and societal costs associated with using them. In an age where we are concerned about greenhouse gas emissions and minimizing our nation's energy demands, is it wise to dictate energy-intensive water treatment systems when there is no evidence of public health benefits? Additionally, there is a looming crisis related to aging water infrastructure that will require a vast financial investment by utilities. Should that be set aside so they can chase down the last nanogram of a compound?

So what should we do? A couple of things make sense. This issue does highlight the need to better protect America's sources of drinking water from various sources of contamination. And clearly there is a pressing need for additional research on this issue. As a scientist, I recommend we focus on research related to health effects from trace pharmaceuticals with a lesser emphasis on occurrence, in order to determine whether there is in fact a problem to solve. The critical question we must address is not "Do they exist?" but rather, "At what concentration are these compounds harmful to human health?" Only then can we make intelligent, rational decisions that protect the health of this country's municipal water customers.

Our recommendations are spelled out in more detail below:

1. EPA should work with states, water and wastewater utilities, and the agricultural community to minimize contamination of source waters by pharmaceutical products as well as other contaminants.

It is imperative that the nation do a better job of protecting its waters, and especially sources of drinking water, from contamination. We have said previously that there is an imbalance between the enforceable controls on point sources, such as Publicly Owned Treatment Works, and the less rigorous programs used to limit nonpoint sources of pollution, such as agricultural runoff. Congress may wish to evaluate this issue to assure that all sources of pollution are equitably contributing to the protection of the nation's waters.

2. We urge support for proper pharmaceutical disposal programs to reduce the flushing of pharmaceutical products into sewage systems to the greatest degree possible, while recognizing that this addresses only a small part of the problem.

Although more research would be needed to accurately characterize this issue, we believe it is likely that more pharmaceuticals end up in the environment after passing through humans than after flushing unused products. However, some unused pharmaceutical products are undeniably flushed into waste streams, contributing to the problem but also offering an opportunity to make reductions in the pollutant loading through a "pollution prevention" approach. We urge support for pharmacy "take back" programs that make doing the right thing obvious and convenient for consumers.

3. Elevate EPA's drinking water health effects research budget at least equivalent to the air pollution health effects research budget. Even though this Subcommittee does not appropriate funds, we ask you to support this increase.

To date, no peer reviewed published research has found ill effects on humans from pharmaceuticals in the environment at the trace levels we have seen in drinking water. However, drinking water providers would like to see more research on this matter, so that we can either take appropriate action to address an actual health risk if there is one, or reassure the public that there is not one. Treatment to completely remove all traces of pharmaceuticals from drinking water will be very expensive, and our customers have a right to expect that we will only undertake the investment necessary to do this – and increase their utility bills to pay this expense - if doing so addresses an actual health risk.

We also specifically support 1) a dedicated authorization in the Research Title of the Agriculture Reauthorization bill for collaborative research between the drinking water community and the agriculture industry on ways to limit contaminants from entering water supplies; and 2) a dedicated research authorization to support decisions on contaminant listing and rulemaking by EPA's Office of Ground Water and Drinking Water. These funds should be used to focus research on priority drinking water areas of concern.

4. We should continue to rely upon EPA's science-driven Contaminant Candidate List (CCL) process to identify candidates for new drinking water standards.

Though at times this process appears to move slowly, a methodical, science-based process is necessary for determining which contaminants need to be regulated, so that we focus on actual risk and on the higher risks first. The standard setting process detailed in the Safe Drinking Water Act is sound, and setting standards through a science-driven process gives the public confidence that the regulations they pay for are necessary, reasonable, and protect public health. An increase in human health effects research, as mentioned in Item 3 above, would improve this process.

5. We should continue to rely upon the Unregulated Contaminant Monitoring Rule (UCMR) for decisions concerning testing and reporting to customers about contaminants that are not currently regulated.

EPA employs a comprehensive and science-based approach to determining which unregulated contaminants utilities should monitor for, and what utilities should say about these contaminants (if detected) to their customers. It is appropriate to use this kind of science-based process to determine which, if any, additional currently unregulated contaminants utilities should investigate.

Thank you for your time. I would be happy to answer any questions you may have.

Senator Barbara Boxer

Q: What are the most important steps that you think EPA should take to reduce such pollution in our nation's waterways?

A: First and foremost, I must emphasize again that the majority of compounds detected in source waters are not the result of improper disposal, but rather are attributable to human excretion of non-metabolized medications. Therefore, reduction strategies aimed at disposal will address only a very small fraction of the compounds entering source waters. Ultimately, the only solution with the potential to significantly reduce (although not completely eliminate, because that may be scientifically infeasible) the amount of these compounds entering America's source waters would involve changes to wastewater treatment facilities. My research has quantified the degree to which various water treatment technologies can remove these compounds, and we know that some processes are markedly more effective than others. However, given the capital costs and electric power needs—and therefore carbon footprint ramifications—linked to currently available advanced treatment technologies, I believe it is essential that the human and environmental health implications of these trace compounds become better understood before moving forward. Otherwise, we risk incurring tremendous financial and environmental costs without any appreciable health benefit. Consequently, I believe the EPA's focus should be on advancing health effects research associated with these compounds. Only as those questions are answered will we know whether there is in fact a problem to solve.

Senator Benjamin L. Cardin

Q: Are there existing technologies to reduce these already small concentrations by (perhaps) a few orders of magnitude? What would be the cost of deploying these technologies?

A: While technologies exist that would allow wastewater treatment agencies to reduce the concentrations of these compounds, it is of paramount importance to understand that the degree of removal will be largely a function of analytical detection limits. It is critical to note that none of the technologies evaluated in our studies, including the most advanced membrane and oxidation processes, were capable of removing all trace contaminants to less than detection. This means that zero is a non-viable treatment goal, which again dictates that meaningful goals based upon human and environmental health be established. In terms of cost, that would vary by agency based upon the treatment capacity volume, general water quality, and energy costs. Collectively, financial investments would certainly range in the tens of billions of dollars or greater, along with potential increases in operational costs. Of greater concern is that in trying to

reduce concentrations of compounds that may not actually represent a health risk at their existing levels, we would dramatically increase energy use because of the power-intensive nature of these alternative treatment technologies. This has both energy and environmental implications that may far outweigh the potential risks from trace levels of pharmaceuticals in drinking water. In short, before we strive to take a compound from 5 parts per trillion to 1 part per trillion and accept the financial/environmental costs of applying the necessary technology associated with doing so, it would be beneficial to know whether 5 parts per trillion is even harmful. If not, the cure is worse than the ailment.

Senator James M. Inhofe

Q: Can you give the Committee an idea what it might cost for a municipality to test and treat for pharmaceuticals in their drinking water?

A: This is a particularly important question, because soon after the Associated Press articles were published, a number of utilities engaged private laboratories to conduct an analysis of their drinking water for pharmaceuticals. The costs routinely exceed one thousand dollars per sample. More daunting is that no standardized tests for pharmaceuticals exist; therefore, utilities can only hope that a particular laboratory has the experience and capability to produce accurate and precise test results, which is challenging considering the 0.000000001 g/L concentrations at which these compounds exist. Furthermore, there is no "blanket" test for trace pharmaceuticals; each compound must be analyzed individually. There are literally thousands of compounds for which a utility could analyze. Our study, which was in part undertaken to guide such efforts, screened a multitude of compounds and ultimately selected specific compounds based either upon their toxicity and/or their likely occurrence in source waters. Only by screening potential compounds of interest in this manner were we able to refine the list to a manageable number. Mandatory monitoring requirements, such as those required under the Safe Drinking Water Act and through the Unregulated Contaminant Monitoring Rule, would be costly and pointless in advance of health effects research. The fact that pharmaceuticals can and do exist at ultra-trace levels in US water ways is indisputable, and additional monitoring efforts will yield sparse useful data. We know these compounds exist; the question is one of toxicological relevance.

Q: How accurate and precise are the methods to measure these compounds at trace levels? Are these analyses expensive? Are there standard methods available? If not, could that have dissuaded municipalities from monitoring?

A: There are no standard methods to detect pharmaceuticals in drinking water. Therefore, each laboratory builds their own methods and compound lists based on availability of standards and analytical capabilities. This approach can lead to markedly different detection limits, and therefore inconsistent occurrence information. My research team literally created the detection methodology for identifying pharmaceutical compounds at these almost incomprehensibly small

concentrations, and they are generally accurate to within approximately 20 percent of the indicated value. Even in the case of the national reconnaissance study by the USGS, some hormone data were later retracted due to analytical errors. Trace analytical methods are quite challenging and the quality control measures associated with sample collection and processing are exceptionally rigorous. There is no question that the expense associated with these analyses may have dissuaded some utilities from testing. However, that is only a minor point. The key issue is that none of these compounds are regulated, or even on the Contaminant Candidate List. The question to answer is not why some utilities did not analyze for pharmaceuticals, but rather why any chose to do so. The reason is that these proactive utilities wanted to further our scientific understanding of this issue and determine whether in fact trace pharmaceuticals represent a legitimate threat to public health. To compel others to do likewise would not further that goal and would consume ratepayer dollars.

Q. Why did you undertake this research in the absence of federal mandates? Should there be mandates in place?

A: I work for a particularly progressive water agency, the Southern Nevada Water Authority. As the organization's Research and Development Project Manager, my role is to scan the horizon for potential water quality issues that may affect our agency and others in the water industry. I have been conducting work related to endocrine disrupting compounds and pharmaceuticals for over 15 years. My early research sought to identify potential contaminants in wastewater effluents that may have result in changes to fish reproductive biomarkers. In this case, there was a documented impact to fish and the question was one of identifying causality. During initial screening in the late 1990's we discovered a spectrum of pharmaceuticals in wastewater effluents at extremely low levels. The next question was to determine the fate of these contaminants through various conventional and advanced water treatment processes, which we completed and published in 2005. My most recent research efforts have focused on the human health relevance of these compounds in drinking water. So, my research has been a natural progression of answering questions related to the occurrence, fate, and toxicology of these trace water contaminants. This research was conducted to expand the global knowledge of this topic and to advance the science in terms of water reuse and sustainability. In terms of the second question, I must preface my answer by reiterating that I am a scientist, not a policy-maker. My concern—which unfortunately may yet come to pass—is that public sentiment, rather than science, may become the basis for drinking water regulation. Modern instrumentation and innovative analytical methods are allowing us to see compounds at concentrations that were previously undetectable. Consider the analogy of stars in the night sky. Over the centuries, we have developed increasingly powerful telescopes that have been used to identify new stars and planets that were not detectable using older technology. With the launch of the Hubble telescope in 1990, scientists have identified thousands of new stars and planets, and now predict that there are 10 stars for every grain of sand on earth. The James Webb Space Telescope slated to launch in 2013 will undoubtedly continue to see further and identify more stars and planets. The same is true for analytical technology. Each decade sees the development of more sophisticated and

sensitive instruments now capable of routine measurements of contaminants at sub-parts per trillion levels. A part per trillion equates to one second in nearly 32,000 years. For as long as humans have been consuming medications, there have been pharmaceutical compounds in water supplies. We simply never noticed before. Now that it has come to our attention, are we to react by passing regulations that have no basis in human health protection, but are rather based upon the public perception that the mere trace-level presence of pharmaceutical compounds is worrisome? If so, let us explore that path and assume for a moment that we regulate a compound at the current detection limit of 1 part per trillion, despite the fact that there is no evidence whatsoever that it is harmful to humans at that concentration. Utilities are compelled to comply and, at great financial and environmental (energy-related) cost, they are successful in "removing" this compound to below the 1 part per trillion threshold. The "problem" is presumed to be solved. Soon thereafter, a scientist like me discovers a way to enhance our detection ability, allowing us to find this compound at 1 part per quadrillion. Utilities around the country discover that they didn't eliminate the compound entirely, but rather just reduced it to 50 parts per quadrillion. Now that the compound has "appeared" again—at a level 50 times greater than the new detection limit—do utilities now have to expend even greater resources to reduce it below 1 part per quadrillion? The idea of regulating compounds based upon a presence/absence litmus test is futile, because detection limits continue to improve. The only rational way to approach this issue is by conducting the health effects research necessary to determine at what concentration a given compound poses a health threat to the most susceptible members of society, such as infants and pregnant women. There is a process already in place designed to accomplish exactly that. It is the process by which every contaminant currently regulated under the Safe Drinking Water Act is evaluated. Treating pharmaceutical compounds differently solely because of public sentiment is unwarranted and has troubling ramifications.

Q: Where do you believe research funding for this topic would be best spent?

A: Unquestionably, the focus must be on health effects research. There are three critical questions with regard to pharmaceuticals in drinking water: 1) How do you detect them in such minute concentrations? 2) How do you remove/reduce them from drinking water? 3) At what concentration are these compounds harmful? My research team has already answered the first two questions, and we have taken the first small steps toward answering the third and most critical question. However, we strongly encourage others to build upon our work and conduct their own independent research. I have no desire to be parochial about this field of research; to the contrary, I have shared my work freely with scientists around the world. I believe the EPA can serve a key role in spearheading and coordinating research efforts related to health effects. There is no way to draft scientifically legitimate regulation related to this topic without first understanding the public health issues that underpin it. This is a daunting amount of work, considering that the Acceptable Daily Intake, which should serve as the basis for any regulation, will differ for every compound. I strongly believe that additional research funding should be spent to continue to develop and refine water treatment technologies that produce clean, safe, and sustainable drinking water. The key is to drive the technology into more energy efficient

disinfection and contaminant removal processes. Water utilities need research funding in order to support programs that allow the evaluation of modern treatment technologies and to characterize their own watersheds for a variety of emerging water quality challenges. Many of our nation's water utilities would conduct additional testing and treatment evaluations for pharmaceuticals if appropriate funding were made available. Continued investigations into water treatment technologies and monitoring of emerging water quality issues is especially critical considering the decaying infrastructure in the United States. Research funding on health effects is clearly the most important aspect of addressing the relevance of trace pharmaceuticals in US drinking waters; however, funding also is needed to continue the advancement of efficient and sustainable water treatment and conveyance technologies to keep America's drinking water safe and secure.

Q: Should data related to pharmaceuticals in water be released to the public as soon as results are in?

A: I appreciate your noting that this is a communications issue and I will attempt to provide a purely scientific perspective. The research for which we collected data from proactive utilities around the country is of the cutting-edge variety. It was not driven by regulation, but rather by a genuine desire on the part of participants to advance our understanding of this issue. My studies were designed to address the removal of trace contaminants through conventional and advanced water treatment technologies and to begin to address the human health relevance of trace pharmaceuticals in drinking water. The sites selected for these studies were based upon treatment technologies employed and source water quality. The specific site names and location were not pertinent to the scientific nature of our study and would likely have diluted the impact of our findings by distracting readers with specific locations. In our most comprehensive study, our research is still undergoing rigorous peer review and has not been finalized. For our team to publicize raw data without context or explanation would have been utterly irresponsible. Much of the data we provided to our participating utilities was preliminary and had not been subjected to thorough review. I believe that utilities must be transparent with the public regarding all emerging water quality issues. However, I do not make policy decisions for utilities. As discussed previously, there also are data quality issues that must be addressed before data should be released. In terms of advancing the science regarding pharmaceuticals in drinking water, I believe that utilities of the US should be commended for participating in studies that were not required under any regulatory framework. Consider that if none of the US utilities had voluntarily participated in monitoring programs, there would have been no story to tell. I fear that if US utilities are penalized for being proactive, future participation in research studies will be thwarted. Utilities should continue to be proactive and to communicate openly with the public; however, it should be understood that when armed only with incomplete and/or preliminary findings, utilities may be reluctant to release these raw data before thorough review and evaluation.

LAUTENBERG. Thank you very much, Dr. Snyder.

Now, we have an opportunity to hear from a fellow New Jerseyan who is here as a witness. David Pringle has been very active on environmental issues in New Jersey, and helped my State lead the way on protecting the environment. We welcome you here, Mr. Pringle, as I use the formality here, but welcome David. It is nice to see you.

STATEMENT OF DAVID PRINGLE, CAMPAIGN DIRECTOR, NEW JERSEY ENVIRONMENTAL FEDERATION

Mr. PRINGLE. Thank you. I am David Pringle, Campaign Director for the Garden State Chapter of Clean Water Action. Our primary focus nationally is on water policy and has been since our founding. I am also the State Assembly Speaker's appointee to the Drinking Water Quality Institute in New Jersey that sets drinking water standards, and I chair the Public Health Committee.

New Jerseyans, more than most because of our population density, literally live on top of and right next to our drinking water. As a result, we face more risks in terms of supply shortages, even though we have the kind of wet weather that parts out west do not, and we have contamination issues.

You have my written testimony, and to avoid repetition I really want to focus on the parts of my testimony that have been less addressed today and that frankly I think are the most important, which is that the Nation's current regulatory framework is not really set up to address this problem. I will also make some recommendations on how this Committee can help fix that situation.

Common sense dictates it is not a good idea to drink somebody else's medicine, but that is what we are doing today because of agricultural runoff, human waste, industrial discharge, and using manure as fertilizer. As a result, as we have heard, hundreds of different kinds of organic compounds are getting out into our waters. This is not just a drinking water issue. It is a Clean Water Act issue as well. Pharmaceuticals, while it is only part of the problem, is a big part of the problem because they are designed to be biologically active. And this isn't just about human medication. A big part of this situation is the veterinary and agricultural aspects of it.

New Jersey has been on the forefront of these issues. Because or our population density, we faced the problem sooner, but we have had a lot of great work done. USGS, the New Jersey office there, has led some of the work in documenting the occurrences there, in collaboration with our own State DEP, the Centers for Disease Control, and several of our State universities have done a lot of the documenting of the occurrence and improving the technology so that we can find these issues and study the health effects.

One study alone documented 600 unregulated contaminants in the State's water supplies. While the levels are relatively low, again, current conventional treatment doesn't remove them. They are designed to be biologically active. We know very little about their health and ecological effects, yet field studies are already starting to document ecological impacts on a Noah's ark of wildlife.

The Nation's regulatory framework is not set up to address this problem. It is too costly. It takes too long, and it looks at too nar-

row of the problems. This is going to get worse as we rightfully reuse water more, given the water wars we are seeing and as medical breakthroughs hopefully continue. So we really have to get on top of it.

As Barker Hamill, the Director of the New Jersey Bureau of Safe Drinking Water said, there are thousands upon thousands of chemicals out there. Even adding one more substance to the regulatory list can be a lengthy, costly and frustrating process.

I will give you one example. In Toms River, New Jersey, there is a probable cancer cluster there. The leading suspect is a contaminant that was found in the 1980's at a Superfund site, a few feet from a drinking water supply. It wasn't a priority pollutant. It wasn't looked at or addressed under Superfund. Ten years later, they find it in the drinking water. Ten years after that, we are still reviewing it. We have spent over $5 million on this one contaminant. There is still no standard, yet we are re-treating the water anyway. That is just one contaminant, when there are hundreds out there. How much are we going to spend continuing this chemical by chemical, only looking at a very narrow field of health impacts without any coordination between various agencies?

The FDA is regulating pharmaceuticals, but they are looking at it from an acute basis, not a chronic one. EPA isn't even looking at that. The other programs have a variety of flaws in them. They are not looking at cumulative or synergistic effects. They are too focused on just carcinogenic effects. They are not looking enough at the most vulnerable populations, the sick, the elderly, the young, women of childbearing age.

And our wastewater systems and our drinking water treatment plants are not set up to address this new-age type of contamination. The sewer systems were basically set up in the Victorian era, and the drinking water systems are more a function from the last 70 years. We are not ready to handle the problem.

So I implore the Committee to take this issue very seriously. I am very happy this hearing is happening today. We need to restore the $10 million in cuts from the Bush administration for NAWQA. There is a series of other smaller cuts on monitoring that are critical. The State revolving fund needs to be refinanced this year. This is a bipartisan issue. On this one, the House Democrats cut $250 million that hope this Committee will restore.

I would like to just close by emphasizing one point. We really need to be preventive here. We need to take a precautionary approach because these chemicals are designed to be biologically active. I hope that we look to fund aggressively programs like we are doing in New Jersey. There are pilot projects where they are looking at activated carbon to reduce this, and also looking at when is the appropriate time to trigger this, to move away from chemical to chemical, and look more at a treatment technique-kind of situation where if you know you have a significant level of treated wastewater in your water supply or if you have an organic problem, that is a good indicator that you have other problems and we should be aggressive in treating them.

Thank you.

[The prepared statement of Mr. Pringle follows:]

PHARMACEUTICALS IN DRINKING WATER

Testimony of David Pringle
Campaign Director
New Jersey Environmental Federation
On Behalf of:
New Jersey Environmental Federation and Clean Water Action

Before the U.S. Senate Environment and Public Works Committee
Subcommittee on Transportation Safety, Infrastructure Security and Water Quality

"Pharmaceuticals in the Nation's Water: Assessing Potential Risks and Actions to Address the Issue"

April 15, 2008

New Jersey Environmental Federation
1 Lower Ferry Road
Trenton NJ 08628

Clean Water Action National Office
4455 Connecticut Ave. NW; Suite A-300
Washington DC 20008

Pharmaceuticals in Drinking Water
Testimony of David Pringle of the NJ Environmental Federation / Clean Water Action
April 15, 2008

Introduction -- Thank you, Mr. Chairman and Members of the Committee, for the opportunity to testify before you today at your hearing entitled: "Pharmaceuticals in the Nation's Water: Assessing Potential Risks and Actions to Address the Issue."

My name is David Pringle. I am the Campaign Director for the New Jersey Environmental Federation (NJEF), the Garden State Chapter of Clean Water Action (CWA). NJEF has over 100,000 individual members and an additional 100 member groups in the Garden State. CWA has offices in seventeen states and one million members across the nation including in many of the committee members' home states. Since CWA's founding in the early 1970's, NJEF's a decade later, and the launch of my own professional career in the late 1980's, CWA's, NJEF's, and my primary focus have been advancing water protection policies at the local, state and federal level.

While I come before you today representing CWA, NJEF and myself, for the past 5 years I have also served as an appointee of the Speaker of the New Jersey State Assembly to the Drinking Water Quality Institute (DWQI) and currently serve as chair of its Health Subcommittee. Created by New Jersey statute over 20 years ago, DWQI is a professional body of scientists, engineers, government officials and public health experts appointed by the Governor, State Senate President and State Assembly Speaker that must recommend drinking water standards to the NJ Department of Environmental Protection (NJDEP) before NJDEP acts on such standards.

As the most densely populated state, New Jerseyans live most literally on top of and right next to their drinking water, which accordingly is too often in short supply and threatened with contamination. In response, NJDEP, DWQI, CWA, NJEF, et al. have sought to ensure that New Jerseyans, whether tapping their own private individual well or a public water system, benefit from some of the strongest drinking water protections in the nation.

Summary – The presence of hundreds of unregulated pharmaceuticals and other manmade chemicals in the nation's surface, ground, waste and drinking water is becoming increasingly well documented due to increased monitoring, better testing techniques and greater use. While the data to date reveals concentrations at relatively low levels, current conventional treatment does not effectively remove them. This is cause for concern, albeit not panic, and cause for timely action. More research and other common sense measures are needed, and some are well beyond the current regulatory framework process and timeframes.

The recent Associated Press investigation ("AP Probe Finds Drugs in Drinking Water," March 9, 2008) brought to greater light what the scientific literature has been documenting for a decade -- a potential toxic stew of organic pollutants: human and veterinary medicine (steroids, antibiotics, anti-depressants, hormones, et al.), personal care products, and various industrial and commercial products. The primary sources of these pollutants include wastewater due to pharmaceuticals excreted unchanged by the body, industrial discharge, disposal of unused drugs, biosolids and manure used as fertilizer and agricultural runoff.

There are no federal or state standards or monitoring requirements for the vast majority of these pharmaceuticals in drinking water or waste water. While the health effects of these contaminants

at medical doses are relatively well-known, their ecological and public health impacts, especially their side, cumulative, and synergistic effects at lower doses are largely unknown and cannot be dismissed. Pharmaceuticals by their very nature are designed to be biologically active and scientific studies indicate that these chemicals are already harming a wide array of wildlife.

Further, the nation's current regulatory framework is so slow, narrowly focused and costly that it is unfit to address this problem, especially if it grows as anticipated with greater water re-use and new medical breakthroughs, of which this problem is in part an unintended consequence.

Accordingly, while we don't know enough and need to learn more, we do know enough to be concerned and take precautionary action – e.g., more research on health and ecological impacts and occurrence, upgraded treatment for wastewater and drinking water and most importantly pollution prevention through pharmaceutical, agricultural and water industry reforms.

Occurrence of Pharmaceuticals and Other Unregulated Contaminants in the Nation's Waters – As noted above, the presence of hundreds of unregulated pharmaceuticals and other manmade chemicals in the nation's water is increasingly well documente. The primary sources of these pollutants are wastewater excreted unchanged by the body, industrial discharge, disposal of unused drugs, biosolids and manure used as fertilizer and agricultural runoff.

While the data show concentrations at relatively low levels, current conventional treatment does not effectively remove them. More than 100 different pharmaceuticals have been detected in lakes, rivers, reservoirs and streams throughout the world (*Damming the Flow of Drugs in Drinking Water*, Environment Health Perspectives, Volume 113, Number 10, October 2005). It can be anticipated that the concentrations and numbers of pharmaceuticals in the nation's waters will increase given ongoing medical advances and increased reliance on water reuse as demands for water grows and the supply diminishes. Some of the early and most important scientific studies documenting this problem were conducted in New Jersey including:

- NJDEP-Environmental and Occupational Health Sciences Institute, *The Characterization of Tentatively Identified Compounds (TICs) in water samples collected from public water systems in New Jersey*, March 2003 – 600 non-volatile and semi-volatile compounds were detected in samples from 20 sites, primarily community systems using groundwater with known historic organic contamination and near known contaminated sites. 51 compounds were detected in raw and finished water;
- NJDEP-United States Geological Survey (USGS), *Occurrence, Distribution, and Concentration of Pharmaceutical and Other Organic Wastewater-Related Compounds in New Jersey's Surface Water Supplies*, February 2003 – 30 stream sampling locations in New Jersey sites with a range of 0 to 51 municipal wastewater treatment facilities upstream and estimated wastewater contributing to stream flow ranging from 0-70%. Over 90% of samples contained at least 1 and as many as 32 of the 95 targeted compounds with a median of 11. Total concentrations of these compounds ranged from non-detect to 81 ppb (parts per billion) with a median of 1.7 ppb and the most commonly detected compounds included caffeine, the pharmaceuticals carbamazepine and cotinine, flame retardants and plasticizers, a fragrance, steroids and the pesticides prometon, diazinon, and metolachlor; and
- USGS-Centers for Disease Control-NJDEP, *Fate of Organic Wastewater Contaminants in a Drinking Water Treatment Facility*, February 2003 -- 11 organic wastewater contaminants were tracked through the 4 stages of "traditional" treatment (pre-disinfection with chlorine, flocculation/sedimentation, filtration, and post-disinfection with chlorine) at the Passaic Valley Water Supply Commission's Little Falls, NJ plant. All 11 contaminants were detected in raw, settled, filtered and finished water samples with concentrations for each contaminant ranging

from .1 to .4 ppb and reductions not very significant (most reductions ranged from 10-30% and for one contaminant there was actually a 10% increase.)

- USGS, *Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminant in U.S. Streams:1999-2000: A National Reconnaissance* – at least one chemical was detected in 80% of the 139 streams sampled in 30 states, 75% had more than one, 50% 7 or more, and 34% 10 more, while 82 of 95 chemicals sampled for (including steroids, insect repellant, caffeine, triclosan [the anti-microbial disinfectant often used in liquid hand soap], detergent metabolites, and plasticizers) were detected in at least one stream.

Human Health and Ecological Risks – Common sense dictates it's not a good idea to drink somebody else's medicine: "We know we are being exposed to other people's drugs through our drinking water, and that can't be good," says Dr. David Carpenter, who directs the Institute for Health and the Environment of the State University of New York (SUNY) at Albany (AP, 2008).

Pharmaceuticals are designed to be biologically active. Studies indicate that these chemicals are already harming wildlife at levels found in water. While the effects of many of these contaminants at medical doses are relatively well-known, their ecological and human health impacts at lower doses are largely unknown, especially their side, cumulative and synergistic effects.

"These are chemicals that are designed to have very specific effects at very low concentrations. That's what pharmaceuticals do. So when they get out to the environment, it should not be a shock to people that they have effects," says zoologist John Sumpter at Brunel University in London (AP, 2008).

Further, standards, guidelines or criteria are in place for only a fraction of the pharmaceuticals being detected in drinking water. For example, a 2001 USGS study found only 29% of over 50 semi-volatile organic pesticides and 34% of volatile organics compounds (VOC's) detected in New Jersey ground and surface water had drinking water standards or guidelines. Of the 600 TICs from the NJDEP, March 2003 study, "the majority have no standards or guidelines associated with them [and] in fact, there is scant information at all on health effects from most of the [them]" (Mark Robson, et al., NJ School of Public Health, University of Medicine and Dentistry of New Jersey, March 2003).

Ecotoxicity data is even less available -- for less than 1% of human pharmaceuticals according to estimates published in the April 2004 issue of Regulatory Toxicology and Pharmacology. However, 'while researchers do not yet understand the exact risks from decades of persistent exposure to random combinations of low levels of pharmaceuticals, recent studies … have found alarming effects on human cells and wildlife. … "[w]e recognize it is a growing concern and we're taking it very seriously," said Benjamin H. Grumbles, U.S. Environmental Protection Agency (USEPA) Assistant Administrator for Water' (AP, 2008).

Studies that taken together represent a modern Noah's Ark- from algae, zooplankton and earthworms to vultures, mussels and fish - suggest a host of species can be impacted by this contamination. Perhaps the most documented is the feminization of male fish living downstream from wastewater treatment plants. Two prime examples of this phenomenon come from Colorado streams (e.g., *Comparative Biochemistry and Physiology C, Toxicology and Pharmacology* 144: 10-15, 2006) and from striped bass and winter flounder in Jamaica Bay, New York (McElroy, et al., Sea Grant/SUNY-Stony Brook, May, 2007).

"It doesn't take a lot of estrogen to feminize male fish. If you can measure the estrogen in the water, then that's enough to cause an effect, and we can measure it at very low parts per trillion," said Karen A. Kidd, a biology professor at the Canadian Rivers Institute, University of New Brunswick in the February 25, 2008 cover story, "Side Effects", in *Chemical & Engineering News*, the weekly magazine of the far from radical American Chemical Society.

We also know little about how these compounds degrade and react in the environment, during treatment and inside our bodies, as well as long-term exposure to multiple contaminants at low levels. "Sometimes … the biodegradation product is more toxic than the parent," said Diana Aga, an analytical chemist at SUNY, Buffalo (*Chemical & Engineering News*, 2008).

Even some in the pharmaceutical industry are expressing concern: 'At a conference last summer, Mary Buzby - director of environmental technology for drug maker Merck & Co. Inc. - said: "There's no doubt about it, pharmaceuticals are being detected in the environment and there is genuine concern that these compounds, in the small concentrations that they're at, could be causing impacts to human health or to aquatic organisms".' (AP, 2008)

Current Regulatory Framework Flaws – The presence of pharmaceuticals in the nation's waters highlights how severely flawed the nation's current regulatory framework for water protection is and the challenge we face. '"There are thousands upon thousands of chemicals out there" … even adding one more substance to the regulated list can be a "lengthy, costly and frustrating process"' Barker Hamill, NJDEP's Bureau of Safe Drinking Water Chief, told (Camden, NJ) *Courier Post* (Removal of drugs in water gets test in S.J., Richard Pearsall, March 16, 2008).

That framework is a series of laws and agencies (Resource Conservation and Recovery Act, Superfund, Clean Water Act, Safe Drinking Water Act, Food and Drug Administration and U.S. EPA, et al.) that add up to a process that is costly, time consuming, and unprotective -- each looking at too narrow of a set of contaminants (e.g., Superfund and the Safe Drinking Water Act (SDWA) focus on about 100 each but not the same 100) in too narrow of a setting (chemical by chemical, limited health effects) without enough coordination between programs (clean water staff and don't communicate enough with drinking water staff even though one might be regulating a discharge just upstream of an intake).

The Toms River NJ, cancer cluster investigation drives this point home. In an effort to explain and address why leukemia, brain and nervous system cancers were elevated among the area's children, millions of dollars have been spent to identify, develop health data on and treat the local water supply for an organic TIC. The TIC had been picked up in test results from a Superfund site literally feet away from the area's drinking water supply in the 1980's but was ignored as it was not a priority pollutant. Ten years ago it was found to have leached into the areas's water supply. The water is is currently being treated using air stripping and a carbon filter, but the health data is too lacking to develop a drinking water standard.

FDA regulates pharmaceuticals for acute health effects but much less for chronic effects: "It takes a lot of 17a-ethinylestradiol [a synthetic estrogen] to kill an aquatic organism, so by current testing standards, the compound would appear to have a very low potential risk. But feminization of male fish – something those short-term tests would have never detected – occurs at very low concentrations of the drug" says Bryan Brooks, an environmental science professor at Baylor University" (Chemical & Engineering News, 2008).

The other programs similarly ignore important health effects. Most of these contaminants occur in concert with other contaminants yet cumulative and synergistic effects are not evaluated. Standard

setting is too often focused soley on carcinogenic effects and sometimes teratogenic and mutagenic effects and based on an average adult male at the expense of more vulnerable populations – the young, women of child-bearing age, seniors, and the already sick.

"It could be that the chemical stress that's put on any organism is the result of minute stresses of a multitude of chemicals," says Christian G. Daughton, chief of the environmental chemistry branch at the EPA's National Exposure Research Laboratory (Chemical & Engineering News, 2008).

Finally, traditional wastewater systems are designed to treat microorganisms and nutrients, not pharmaceuticals and other organic compounds found in the studies referenced in this testimony. Making matters worse, loopholes in the Clean Water Act permit industrial discharge into sewers in greater amounts than directly into surface or groundwater even though the sewer plants are not designed to manage such waste.

Advanced treatments such as ozonation, granulated activated carbon, reverse osomosis and nanofiltration membranes can remove significant amounts of pharmaceutical but are expensive (Stackelberg, Gibs, et al., USGS, 2004, 2006, 2007; Black and Veatch Consultants -- NJDEP, January 2007). Black and Veactch, NJDEP and several water utilities are working together on several pilot projects to explore treatment options. More research, as well as federal leadership from U.S.EPA, are needed to build on this and other projects in states and on the information base that USGS and others have begun.

Recommended Actions – As described above, the occurrence of pharmaceuticals in the nation's waters is a complex problem and requires a comprehensive multi-faceted response by policy makers, industry, scientists and consumers:

- Monitoring of both raw and finished water should be expanded – for starters, Congress needs to restore President Bush's FY09 $10 million budget cut for USGS's National Water Quality Assessment (NAWQA) and other programs which includes monitoring, analysis and research activities that are critical to further understanding of this issue.
- Research is needed into treatment technology upgrades that industrial dischargers, wastewater systems and drinking water utilities can use to remove drugs from water intended for consumption or other use – priority should be placed on pilot projects like New Jersey's and the consideration of precautionary approach treatment techniques (requiring treatment in some instances even when presence and/or health impacts are not confirmed).
- Programs to discourage flushing of unused drugs into public wastewater systems should be encouraged – it must be emphasized that this is just a piece of a much larger plan.
- Pollution prevention and toxic use reduction must be vigorously pursued, especially in the reformulation of human and veterinary medicines at each stage of their life cycle.
- Waters with evidence of deformed fish and other ecological impacts possibly due to pharmaceutical pollution should be targeted for cleanup activities.

Finally, bottled water is not a solution, because it is less regulated than tap water, more expensive (200-10,000%) and is drawn largely from the same sources as public tap water supplies. Activity should be focused on pollution prevention and on ensuring healthy drinking water, a task which is well within our capacity.

I appreciate the opportunity to provide testimony here today and welcome questions now or in the future from the Committee.

Senator LAUTENBERG. I thank all of you for your comments and your contribution.

One of the things that I find perplexing is the fact that I listened to Dr. Goldhammer with respect, and I wonder whether or not there is any risk because these are infinitesimally small amounts of trace chemicals. I think Dr. Snyder, you also said something similar. Do we dismiss it?

Dr. Sass, I ask you, do you think the fact that there is—and I didn't mean to start with you, Dr. Goldhammer, and leave you—but I want to execute this in a particular style, not any of you.

[Laughter.]

Senator LAUTENBERG. Dr. Sass, do you think that the fact that the quantities are so minute that worry can be put aside and say that it really doesn't matter what is in the water, because there is so little of it—millions, billions. There was a quadri-that I heard introduced for the first time. What do you think, Dr. Sass?

Ms. SASS. Well, we don't have to guess at this. We know that many biological particles in the body normally act at those levels. The endocrine or hormone system is an example of one that is designed to act in these smaller than parts-per-trillion levels; immune system molecules and molecules that direct neural or brain development are active at these low levels.

So what we need to do is understand what a fine and sensitive balance these systems are and make sure that we are not interfering with it. So we don't have to guess whether compounds, even at those levels, could have an effect on human health. We know they could have an effect on human health.

Your question is to understand what we know with certainty, and I think what we need to do is take a look at the animal data, take a look at the laboratory data and take a look at the wildlife data, and then use our intelligence to allocate our resources wisely, to prevent problems before they happen.

We have a saying in science that if we wait for the epidemiology or we wait for the human evidence, then we are waiting for something to occur literally at epidemic proportions, and that is a failure of public health.

Senator LAUTENBERG. Dr. Goldhammer, do we dismiss risk altogether because the fact that these quantities are trace amounts? Is it possible they don't affect human health or animal health in any way to be concerned about?

Mr. GOLDHAMMER. I don't think we can ever say risk gets down to zero. I think we would be fooling ourselves to say that everything out there is with zero risk. I think in this case, though, we have with respect to human pharmaceuticals, a significant amount of data that gets submitted to the FDA with regard to what the dosage is that the doctor prescribes for the patient. That dose is orders of magnitude greater than what these trace amounts are that are found in the environment.

We have a lot of toxicity data on these compounds from that work as well. The best available evidence today suggests that it is unlikely that these pose a human health risk and even an environmental risk.

I think as my statement made clear, though, that we do need to continue to work and study these issues. PhRMA stands ready to cooperate with our PIE Task Force in this regard.

Senator LAUTENBERG. Let me ask you this, how about a fetus, embryonic stage. Is there more risk there in the development of a child, where suddenly—not suddenly becoming, that these things have reached a different proportionality in their occurrence—but is it better understanding of childhood auto-immune diseases, et cetera? Or are we to believe, for instance, that in the State of New Jersey, David Pringle, where it is now said that one in 94 male children will be born autistic. Now, that number has continued to increase. I watch these things very carefully. Is there danger to the health of a pregnant woman, the mother who is carrying that embryo? Do we say that all people are equal and it doesn't matter what the amounts of pharmaceutical material, even including more than pharmaceuticals or was described as other chemicals? What about that?

Mr. GOLDHAMMER. I think these are all good areas for further study. I think that work needs to be done across the board. It is not just a question of the burden of these very trace amounts of pharmaceuticals, but I think, as was noted by Dr. Snyder, if we want to go out with current technology and look for a given chemical, I think chances are very good it will be found in the environment at these parts-per-trillion or even lower amounts.

The question we have to ask ourselves is——

Senator LAUTENBERG. But putting that aside, you know, accepting that as a condition of existence, Dr. Snyder you are referred to, what do you think about that? Could it affect, these minuscule amounts, could that have a different effect on an embryo or a mother carrying a child? Yes?

Mr. SNYDER. Absolutely. We know that some of our population is more susceptible than others. This isn't the field of which my study is involved. What I can say is that my concern would be that pharmaceuticals become more of a priority than chemicals we know have documented impacts to humans such as disinfection byproducts, which are in parts-per-billion.

I concur that we need more research, and the utilities of this Country demand it.

Senator LAUTENBERG. Well, I don't want to make judgments on what you intended, Dr. Goldhammer, but is there any concern by the industry at all about these discharges into the water, however they get there? I mean, the fact is that we know that there are trace amounts of all kinds of things that are detectable in the water. Does the industry, even though they report regularly on the materials that they produce and that they put into the market, does the industry have any concern about the volume of these things that we might see, or the presence of these things that we might see in drinking water?

Mr. GOLDHAMMER. No, I think it is fair to say we do have a concern. Our PIE Task Force was established over 10 years ago to exactly address that very point. Our data base of published papers in this area now runs over 1,200 papers. Our group meets on a very regular basis to look at the science. We are collaborating with

a number of external groups to try to advance the science in this area. We take our product stewardship extremely seriously.

I think what we can say is from the available toxicity data that we generate during the course of all of our studies indicates at this point in time that there is unlikely to be a human health effect. Can I say it is zero? No, I can't say it is zero.

Senator LAUTENBERG. It is somewhere between unlikely and zero? Because it is obviously a matter of growing concern. As we know from other research that is being done, that there is an ever-increasing presence of these materials in the water through whatever stream.

David Pringle, we have New Jersey studies with utility companies who are looking at new treatment options. Have any of these pilot projects been so effective because even if here we don't hear the alarm that many us feel, and I am one of those. I have 10 grandchildren and I want them to be healthy and well. As a consequence, I want everybody's grandchildren to be healthy and well because I just can't select out mine. So I worry about these things.

What do you see, Mr. Pringle?

Mr. PRINGLE. We know that Victorian-era treatment technologies that we currently employ are not set up to address this problem. And so, it depends on how you look at the question. New Jersey is putting forward several different pilot projects. There are two that are in the design stages now that will be online this summer, one in Fair Lawn and the other in Pennsauken-Merchantville primary groundwater systems to put in granulated activated carbon to start looking at how effective it is at removing these kinds of compounds. Those two systems were picked because they have organic problems. We are just starting to work out some of the details—how often do you have to replace the filter, and what combination of treatment systems work, and those kind of things.

The scientific literature is out there and we know that that kind of technology is much better than what we currently have on the ground. How effective, how expensive, when to use it, those are some of the questions we need to employ.

But we also need to look at this as an opportunity to move away from yesterday's generation and move to alternatives like fulfilling the vision of the Clean Water Act, which was ultimately a zero-percent discharge. Why do we use water as a vehicle to dispose of waste? Why aren't we using more closed-loop systems?

Senator LAUTENBERG. Well, I hate to answer that question, but convenience has overtaken us. I grew up in an industrial city in New Jersey, Patterson, New Jersey. Companies were invited to come there, establish their operations there so they could discharge their effluent into the river. Well, we have one big toxic river that we are now trying to clean up that runs through the city.

I would ask any of you to respond. Can you think of any advantage that we obtain by cutting money for either State revolving funds or other research that EPA does? No one? What a surprise.

Thank you all very much for your testimony. We will keep the record open and supply questions if necessary and ask that you respond quickly.

With that, this hearing is concluded. Thank you.

[Whereupon at 4:55 p.m., the subcommittee was adjourned.]

STATEMENT OF HON. JAMES M. INHOFE, U.S. SENATOR
FROM THE STATE OF OKLAHOMA

Mr. Chairman, thank you for calling this hearing today on Pharmaceuticals in the Environment. I'm sure you would agree that Americans enjoy one of the safest drinking water supplies in the world, as well as reliable pharmaceutical drug supplies. Over the years, science has helped answer many questions and provide remarkable cures for common viruses to complex diseases. At the same time, science often creates many new and challenging questions. It has moved us to a point where we can now detect contaminants in our water all the way down to the parts per trillion. Those emerging contaminants have caused the public to rightly question, is our drinking water safe? I believe the answer is yes, as we will hear in testimony today.

A few weeks ago, the Associated Press reported on emerging traces of pharmaceuticals in several municipal drinking water systems, spurring public concern and this hearing. Although, we should note that this is not a new issue. In fact, this subject has been studied for nearly 40 years, even before the Safe Drinking Water Act was signed into law. However, that doesn't discount the public concern created over the media report.

Mr. Chairman, I sent a letter to EPA requesting that they first respond to the public, ensuring their health and safety is not immediately at risk. I also asked that the Administrator convene an advisory committee or working group comprised of all relevant Federal agencies, interested public and industry to review the emerging scientific data and identify possible mitigation practices to reduce overall disposal of pharmaceuticals. I appreciate EPA's timely response on both requests and am happy to know there is no immediate health risk. I am also happy to hear that the administration is currently reviewing cross jurisdictional guidelines to find a better way for drug disposal. I look forward to hearing from our government panel.

We will also hear testimony today from Dr. Alan Goldhammer from Pharmaceutical Research and Manufacturers of America, or PhRMA, who has done extensive research on pharmaceuticals in the environment. PhRMA has developed a watershed-based model to estimate concentrations of active pharmaceutical ingredients discharged into surface waters through everyday consumption of medicines. Through that base model, industry, in cooperation with USGS, has further developed human health risk data on 26 active pharmaceutical ingredients. A significant amount of time and money between the Federal Government and private industry has produced favorable studies suggesting that the public is indeed safe. I appreciate the time and effort by all in this area.

I'm also pleased to have Dr. Shane Snyder from Southern Nevada Water Authority here to discuss his research on both the concerns that were raised by the media, as well as whether current scientific findings warrant expensive treatment mandates. Dr. Snyder has published several manuscripts and book chapters on endocrine disrupters and pharmaceuticals in water and we are happy to have him here today.

Before we get started, anytime we discuss issues surrounding drinking water, I must take the opportunity to remind the committee that we need to improve our nation's drinking water facilities by reauthorizing the States Revolving Loan Fund programs, both drinking and waste water. This committee has the responsibility to ensure clean, safe, and affordable water for our country by providing the necessary resources to our states and local governments.

I hope this hearing provides clarity to the status of public health and safety, while recognizing that current treatment facilities are already under enormous compliance pressure.

IBWA
International Bottled Water Association

April 25, 2008

Senator James Inhofe
U.S. Senate
Washington, DC

Dear Senator Inhofe:

The International Bottled Water Association[1] respectfully requests that the following clarifying comments be submitted for the record for the April 15, 2008 Subcommittee on Transportation Safety, Infrastructure Security, and Water Quality hearing entitled, "Pharmaceuticals in the Nation's Water: Assessing Potential Risks and Actions to Address the Issue."

In his opening statement, Chairman Frank Lautenberg made reference to bottled water by stating that "40 percent of bottled water simply comes from the tap." IBWA respectfully requests that the record be clarified on this point.

The statement made by Chairman Lautenberg suggests that bottled water companies are taking water directly from a community water source and putting it in a bottle without any additional treatment or processing. That is simply not the case. Virtually all bottled water companies that use community water systems as their source purify, filter and disinfect the water before it is placed in a bottle and sold to consumers as a packaged food product regulated by the United States Food and Drug Administration (FDA). Reverse osmosis, one-micron filtration, ozonation, ultraviolet light, and deinonozation are among the processes that are used by bottled water companies to treat the municipal source water. As a result, the composition of the bottled water is substantially different from the community water system drinking water. If a bottled water company

[1] The IBWA is a trade association representing all segments of the bottled water industry. Founded in 1958, IBWA member companies include U.S. and international bottlers, distributors and suppliers. IBWA works closely with the U.S. Food and Drug Administration (FDA), which regulates bottled water as a packaged food product, and with state governments to set stringent standards for safe, high quality bottled water products. IBWA also has a Code of Practice that sets strict standards for bottled water. As a condition of membership, IBWA bottlers must submit to an annual, unannounced inspection for compliance with the Model Code by an independent third party.

International Bottled Water Association
1700 Diagonal Road, Suite 650
Alexandria, VA 22314
(703) 683-5213

uses a municipal source and does not further treat it, FDA requires the label to state that it comes from a community water system.[2]

IBWA shares the concerns enumerated during the hearing about pharmaceuticals in municipal water supplies. IBWA members use a multi barrier approach to processing bottled water, which includes source protection, source monitoring, reverse osmosis, distillation, filtration and other purification techniques, ozonation or ultraviolet (UV) light. The combination of FDA and state regulations, along with a multi-barrier approach and other protective measures, means that consumers can remain confident in choosing bottled water.

Stephen C. Edberg, Ph.D., ABMM, Yale University School of Medicine commented on the recent Associated Press article that reported finding trace levels of pharmaceuticals in certain municipal water systems. He stated that consumers should be confident in bottled water as a safe beverage choice and noted that "The technical and safety measures used to produce and process bottled water are extremely effective in protecting the product from these and other substances that were reported in the article, should they be present in source water to begin with. This report raises no concern for the safety of bottled water."

Thank you for your assistance in providing our comments for the hearing record. If you have any questions or need more information, please do not hesitate to contact IBWA.

Sincerely,

Patrick Donoho

Patrick Donoho
Vice President, Government Relations

[2] 21 C.F.R. § 165.110 (a) (3)

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