Critical Review

# Lessons from Endocrine Disruption and Their Application to Other Issues Concerning Trace Organics in the Aquatic Environment

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In the past 10 years, many thousands of research papers covering the many different aspects of endocrine disruption in the environment have been published. What has been learned from all this research? We have tried to reduce this very large volume of research into a relatively small number of "lessons". Hence, this paper is not a typical review, but instead it summarizes our personal opinions on what we consider are the major messages to have come from all this research. We realize that what has been a lesson to us may have been obvious from the outset to someone more knowledgeable on that particular aspect of the burgeoning field of endocrine disruption. In addition, it is inevitable that others will consider that we have "missed" some lessons that they would have expected to find included in our list. If so, we encourage them to submit them as responses to our paper. Our own lessons range widely, from the design and interpretation of data from fieldwork studies, through some key messages to come out of the very many laboratory studies that have been conducted, to issues around the sources and fates in the environment of endocrine-disrupting chemicals, and finally to the key role of sewage treatment in controlling the concentrations of these chemicals in the aquatic environment. Having (hopefully) learned our lessons, we have then applied them to the difficult issue of how best to approach future concerns about the potential impacts of other new and emerging contaminants (e.g., pharmaceuticals) on wildlife.

## Introduction

Endocrine disruption has become a major issue in environmental science research and policy. There has been an explosion in the number of papers published on this issue (*1*), which shows no sign of abating. Very significant amounts of money have been spent in the past 10 years on the issue of endocrine disruption. As the results of this (and future) research begin to influence regulation and policy, more will undoubtedly be spent (for one view on the wisdom of this, see ref *2*). Depending on one's perspective, there are a number of ways of summarizing the plethora of information on endocrine disruption. From a strictly scientific perspective, one approach is to review all the available information, with the objective of distilling the essence, whether it be endocrine disruption in humans (see, e.g., ref *3*) or wildlife (see, e.g., ref *4*). Another approach, albeit one rarely taken, is to critically assess the available information, with the aim of ascertaining what proportion of that information advanced knowledge significantly, and to what degree. Both are essentially backward-looking approaches (based on hindsight), and contrast with what we are attempting to do in this paper, which is to look forward, and see if future environmental issues concerned with chemicals (of which there will probably be many) can benefit from the research conducted in the field of endocrine disruption.

To try and achieve this goal, we have chosen to present the key messages to come out of the research on endocrine disruption as a series of "lessons". Our focus is wildlife, not humans, and hence our lessons are derived from what has been learned during the past 10 years from the research conducted on endocrine disruption in wildlife. Nevertheless, some of the lessons are equally relevant to new and emerging issues concerned with the impact of chemicals on human health. Further, as the majority of research on endocrine disruption in wildlife has concerned aquatic organisms (e.g., mollusks, fish, and alligators), our lessons are derived primarily from research focused on the aquatic environment per se, or on the organisms that live within it. However, almost all our lessons apply equally to terrestrial wildlife.

We realize that trying to draw lessons from research, especially other people's research, is fraught with difficulties. No authors are unbiased, and hence, our choices will not be considered by some (perhaps many) as representative and objective. Some of the things we feel were learned (our lessons) may have been obvious to others many years ago. We have different backgrounds (one author is a fish biologist turned ecotoxicologist, the other a hydrologist), which has helped provide breadth, but even collectively our knowledge and understanding of some aspects of endocrine disruption (such as, for example, analytical chemistry issues) is not as good as it could be. Some readers of this paper will consider us, perhaps justifiably, as rather arrogant in considering that we are knowledgeable and farsighted enough to presume we know how the environmental research of the future should be conducted. We are not trying to be that ambitious; instead, all we have tried to do is crystallize our thinking on the lessons we have learned during our involvement in the field of endocrine disruption in wildlife, in the hope that these lessons might stimulate discussion on how best the environmental

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community might address future issues concerned with the impact of chemicals on wildlife. We conclude by examining how, in light of these lessons, the up-and-coming issue of the effects of pharmaceuticals on wildlife could be tackled.

## Lesson 1: Pay Attention to Unusual Biological Observations

Endocrine disruption became an issue when observations of "disrupted" wildlife were linked to environmentally induced alterations in their endocrine systems; that is, the observations drove the issue. In all cases to date, these observations of abnormalities in wildlife were made accidentally; as far as we are aware, nobody has predicted a case of endocrine disruption and then shown that it does indeed occur in wildlife (though this situation may change, as knowledge increases).

Probably the best example of this accidental discovery of endocrine disruption in wildlife is also the best example of endocrine disruption: tributyltin (TBT) and imposex mollusks. The first report of the condition in mollusks now known as imposex was made in 1970: Blaber (*5*) certainly was not looking for this sexual abnormality, as it had not been described at the time. Shortly thereafter, Smith (*6*) reported similar reproductive abnormalities in another species of mollusk on the other side of the Atlantic. It took another 10 years before this condition was linked to exposure to antifouling paints (*7*, *8*), and endocrine disruption suggested as the mechanism (though this term was not used until much later). Even now, over 30 years, and much research, later, the exact mechanism of endocrine disruption is not established (*9*). Nevertheless, whatever the exact mechanism(s) of action, the TBT story provides a very clear example of how an accidental discovery provided one of the cornerstones of endocrine disruption.

Interestingly, the story concerning "feminization" of fish is not dissimilar. The genesis of this example was the unexpected finding, by fish biologists, of an unusually high incidence of hermaphrodite individuals in populations of fish living downstream of two sewage treatment works. The first observation was made in 1978, and supported by further investigations over the next three years (unpublished observations). These investigations showed not only that intersex fish were present, but that the incidence of intersexuality increased with age, and that the older the fish, the greater the degree of reproductive abnormality (results that still await explanation!). It took 15 years or more for the cause of these reproductive abnormalities to be explained (they were due to exposure to estrogenic chemicals present in the sewage treatment works (STW) effluents (*10*, *11*)). We still do not know the consequences of this phenomenon, nor do we know for how long it had been occurring prior to its accidental discovery 25 years ago.

The next example, that of reproductive abnormalities and failure in alligators from contaminated lakes in Florida, was also unexpected, although unlike the previous two examples it arose from purposeful scientific investigations, rather than chance. In this case, wildlife biologists were assessing the status of alligator populations in Florida. They reported that whereas many populations were healthy, in one location, Lake Apopka, a dramatic decline occurred during the 1980s (*12*). It was proposed that the major cause of the population crash was reproductive failure (*13*), possibly due to elevated levels of contaminants in the eggs of these top predators. Subsequent studies examined the reproductive anatomy and endocrinology of young alligators, and suggested that the gonads of juvenile alligators from Lake Apopka had been permanently modified in ovo, so that normal steroidogenesis was not possible, and thus normal sexual maturation was unlikely (though this has yet to be demonstrated) (reviewed in ref *14*). Laboratory studies have, in general, supported the contention that persistent and nonpersistent pesticides in contaminated lakes in Florida, acting as endocrine disruptors, are the cause of the reproductive abnormalities (*15*, *16*).

The only conclusion one can draw is that chance is likely to continue to play a major role in protecting our wildlife from the effects of trace organics. However, becoming more aware of the role that could be played by amateur naturalists may pay dividends, as this group of (often extremely knowledgeable) people may be the first to notice something untoward. Remember that the issue of limb deformities in many amphibian populations was highlighted by the findings of schoolchildren, who posted their results on the Internet (*17*), although wildlife biologists and naturalists had observed and reported the observations (which may or may not be another example of endocrine disruption in wildlife) many years earlier (see, e.g., ref *18*). The recent rapid growth of some specialist "amateur" natural history societies, and their very professional approach to data collection and analysis, means that some of these societies now hold large databases on the particular plants or animals they monitor and study. Such databases may prove useful to ecotoxicologists, as they have already for population ecologists (see, for example, ref *19*).

## Lesson 2: What Is Normal?

It is axiomatic that it is not possible to conclude something is abnormal unless one knows what is normal. However, given our dearth of knowledge about our wildlife, we very often do not know what is normal. Intersexuality in fish, often considered one of the key indicators of endocrine disruption, provides a good example of this dilemma. Many species of fish are gonochoristic, meaning that there are separate male and female sexes. In such species, intersexuality is considered a very uncommon phenomenon; nevertheless, it is regularly reported. For example, Brown and Scott (*20*) reported finding a single intersex powan (*Coregonus lavaretus*) out of 7500 fish they examined, and Borg (*21*) reported finding five intersex stickleback (*Gasterosteus aculeatus*) among "several hundred males". On such occasions, the authors appear to have considered the very low degree of intersexuality as normal. In contrast, a very high incidence of intersexuality in a gonochoristic species is, probably correctly, usually considered as abnormal. For example, Jobling et al. (*11*) reported that 100% of the "male" roach (*Rutilus rutilus*) in two rivers in England were intersex to varying degrees, and suggested that the condition was abnormal, and environmentally induced.

The problem occurs when a relatively low proportion of the animals investigated are found to be intersex: is this normal or not? The correct interpretation is exacerbated if the sample size is low, and hence the absolute number of intersex fish is small. For example, Gereken and Sorydl (*22*) conducted a histopathological assessment of the testes of male fish, as part of a biological survey, and found a few intersex three-spined stickleback (*G. aculeatus*), perch (*Perca fluviatilis*), and eelpout (*Zoarces viviparous*) within relatively small samples of each species. These authors wisely concluded "it is not known whether intersexuality is natural to a certain extent in the species under investigation", rather than jumping to the conclusion that the presence of intersex fish was evidence of endocrine disruption.

Correct interpretation can be aided if different populations of a species, from contaminated and uncontaminated sites, are studied, and incidences of intersexuality compared. Jobling and colleagues (*11*) took such an approach, which allowed them to show that the highest incidences of intersexuality occurred at the sites most contaminated with STW effluent. Similarly, albeit in a smaller study, Vignanò et al. (*23*) found that intersex cyprinid fish were only found in

reaches heavily impacted by a large urban/industrial center in the Po River in Italy, and Allen et al. (*24*) found intersex flounder (*Platichthys flesus*) only in the estuaries associated with large urban/industrial centers. In such instances, even if the "normal" incidence of intersexuality is unknown, it seems reasonable to conclude, on the basis of the different incidences of intersexuality at different locations, that an environmental factor is responsible.

A very brief mention of some recent findings illustrates how difficult it is to distinguish between what is normal and what is not. It was recently reported that out of 162 swordfish examined, 40 were intersex (*25*), leading the authors to suggest that these findings "could be due to exposure to estrogen-mimicking substances". Of course they could, but before that conclusion is reached, the natural level of intersexuality in this species needs to be established. To further complicate things, even normal-looking fish might not be what they seem! Currently, fish whose gonads are completely testicular are considered male, and fish with only ovarian tissue in their gonads are considered female. However, this might not be so: using genetic sex-specific probes, Afonso et al. (*26*) have shown that exposure to STW effluent can lead to complete feminization (genetic males with perfectly normal-looking ovaries), and Nakamura et al. (*27*) showed that an estrogenic chemical, nonylphenol, can induce complete feminization of the gonads of genetic male salmon. And to add yet further difficulty, we (Vine et al., unpublished results) have recently found a number of pike (*Esox lucius*) with gonads which appear superficially to be completely ovarian, but which on closer inspection contain small patches of male germ tissue. We do not know whether these fish are almost completely feminized males, slightly masculinized females, or perfectly normal (nondisrupted)! These intersex pike may be an example of the harder one looks, the more one finds.

The central message here is that we lack baseline data about much of our wildlife, even when biodiversity monitoring programs are conducted to assess the "health" of the environment (such as those conducted by many national regulatory bodies). The problem, of course, is that collecting the necessary data to be able to detect evidence of endocrine disruption *before* it led to major effects (such as a population crash) would almost certainly be an unobtainable objective. There are just far too many species (even in the aquatic environment), most of which we know little or nothing about, to ever be able to monitor the health of them all. Even if very large monitoring programs were adopted, it is rather unlikely that they would provide the level of detail required to detect many types of endocrine disruption; for example, fish health monitoring programs are in place in the U.K. (and have been for a long time), but they do not involve histological investigation of organs, and hence failed to detect intersexuality, even though it is quite common (*11*). Further, if "obvious" and major signs of evidence of endocrine disruption are so easily missed, what about more subtle changes? These could be just as important as far as viability of individuals and populations is concerned.

Although we have used intersexuality to illustrate the current uncertainties surrounding what is normal, exactly the same arguments apply equally to just about any parameter considered a biomarker of endocrine disruption (e.g., plasma vitellogenin and sex steroid concentrations): baseline data are usually lacking.

## Lesson 3: One Animal's Poison May Not Be Another's

Very few, if any, well-designed and well-conducted studies aimed at assessing whether different groups of organisms show similar or dissimilar responses to selected endocrine-disrupting chemicals have been reported. Hence, it is not easy to decide if different groups of organisms respond in different ways, or even whether some respond, whereas others do not, to a particular chemical. However, by comparing the results of different studies, done by different groups of researchers for different reasons, it is possible to reach some tentative conclusions on this issue.

What is clear is that most, if not all, vertebrates (fish, amphibians, reptiles, birds, and mammals) do respond in a similar way, with a similar degree of sensitivity, to both steroidal hormones and xenestrogens (and also probably antiandrogens). Thus, for example, estradiol (E2), whether of endogenous or exogenous origin, is a very powerful estrogen, and induces vitellogenin production in all oviparous (egg-laying) vertebrates. This is not surprising when it is realized that there are essentially no differences in the specificities of the estrogen receptors across a wide range of vertebrates (*28*). Likewise, nonylphenol is a weak estrogen, acting through estrogen receptors, in all vertebrates in which it has been studied (*29*). Thus, in the case of vertebrates, it appears that one animal's endocrine disruptor *is* another animal's endocrine disruptor (though we are sure there will be subtle differences between species, many probably caused by differing pharmacokinetics), due largely to the fact that their endocrine systems show many similarities.

It is very unlikely, however, that all invertebrates respond in the same way as do vertebrates to endocrine-disrupting chemicals, and equally unlikely that all invertebrates respond in the same way. For example, steroidal estrogens such as E2 and ethinyl estradiol (EE2) are extremely potent estrogens in fish (see, e.g., ref *30*), but have little, if any, endocrine effect on at least some groups of invertebrates (see, e.g., refs *31* and *32*), though even with this example some authors have reported reproductive effects on one group of invertebrates, mollusks, at concentrations similar to those that cause feminization in fish (*33*). As Oehlmann and Schulte-Oehlmann (*34*) have recently pointed out, there are more than 30 different invertebrate phyla, whereas in contrast all vertebrates comprise only part of a single phylum. The invertebrates are thought to have diverged from the vertebrate lineage over 450 million years ago, and one group (phylum) of invertebrates from another before that time. Hence, there has been plenty of time for endocrine systems in different phyla, and even within a phylum, to evolve and diverge. For example, although not enough good data are available yet on which to base firm conclusions, it seems that the endocrine systems of the various classes of mollusks, and even major groups of gastropods (one class of mollusks), differ greatly, probably as a consequence of pronounced differences in both morphology and life histories (*34*). It would therefore be surprising if an endocrine-disrupting chemical caused the same effect in all invertebrates. Such species specificity makes it extremely difficult, it not impossible, to arrive at a suite of regulatory toxicity tests that will protect most wildlife species from most endocrine disruptors. Currently, and understandably, chemicals are tested on just a few species (perhaps one fish, one or two different invertebrates, and possibly a plant): what chance is there that such a narrow suite of tests will detect all endocrine activities of the chemicals on all organisms likely to be exposed when the chemical reaches the environment?

Probably the best examples to illustrate species specificity are the cases of TBT and insect growth regulators. The effect of TBT on prosobranch snails is one of the best examples of endocrine disruption (reviewed in ref *35*). TBT induces shell deformities in many bivalve mollusks, and also a masculinization, termed imposex. Imposex induced by TBT has been reported in over 150 species of prosobranchs, and serves as a very specific response to organotin compounds. To date, there are no reports of TBT inducing an intersex condition in other groups of invertebrates. A related chemical, triphenyltin (TPT), even induces imposex in some, but not all, species of prosobranch mollusks (*36*). Very recently (*37*), a

**TABLE 1. Representative Median Effective Concentrations of Some Estrogenic Chemicals for Induction of Vitellogenin in Fish[a]**

| chemical | $EC_{50}$ | rel potency[b] | ref |
|---|---|---|---|
| estradiol (E2) | 25 ng/L | 1 | *30* |
| estrone (E1) | 60 ng/L | 0.3 | *30* |
| ethinyl estradiol (EE2) | 1.2 ng/L | 20 | *30* |
| 4-*tert*-nonylphenol (NP) | 8 $\mu$g/L | 0.0025 | *48* |
| 4-octylphenol (OP) | 10 $\mu$g/L | 0.002 | *49* |
| bisphenol A (BPA) | 50 $\mu$g/L | 0.0004 | *50* |
| methoxychlor | 8 $\mu$g/L | 0.0025 | *51* |

[a] All data were obtained using flow-through exposures, supported by chemical analysis of the water to determine actual exposure concentrations. Hence, it is probably acceptable to compare the data from these different studies. Most data come from rainbow trout, although some come from the fathead minnow (because no comparable data are available for trout). [b] E2 was assigned a potency of 1.

strong case has been made that the apparently ubiquitous retinoid X receptor (RXR) and its natural ligand, 9-*cis*-retinoic acid, play major roles in the development of the imposex condition caused by organotin compounds in gastropod mollusks. However, this advance still does not explain why, apparently, only one group of mollusks, and not the many other groups of invertebrates and vertebrates, develop an imposex condition upon exposure to TBT. What is apparently unique about the endocrine control of reproduction in prosobranch mollusks? In a very recent paper (*38*) the authors even question whether this effect is so specific, because TBT appears able to cause masculinization in fish.

Insect growth regulators were developed by the agrochemical industry for use as pesticides. These chemicals have been intentionally developed to disrupt the endocrine systems of different groups of insects, and hence aid their control. Insect growth regulators are a diverse suite of chemicals that, by acting as agonists or antagonists of ecdysone and juvenile hormone, disrupt development, moulting, and/or vitellogenesis and other aspects of reproduction. These growth regulators appear to act quite specifically on insects; some are even specific to one group of insects, such as the Lepidoptera. However, as is often the case, data from tests on other groups of invertebrates (e.g., mollusks) are sparse, if not nonexistent. Until such data are available, one should be slightly cautious about assuming that insect growth regulators are a group of highly specific endocrine-disrupting chemicals, and hence an example of where one animal's poison is not another animal's poison.

It will probably never be possible to conclusively demonstrate that one animal's poison is that animal's poison alone, the major reason being that it will never be possible to test a "poison" on all animals, to be certain of the degree of specificity of the effect. Nevertheless, it does seem likely that certain groups of organisms, with particular physiological control processes, will be selectively targeted by certain endocrine-disrupting chemicals. The very difficult challenge is to be able to accurately predict which group, instead of waiting to find out, as is the situation currently.

## Lesson 4: Potency Is a Key Factor

One thing that has become very clear is the enormous difference in potency of chemicals possessing estrogenic activity (and probably other types of endocrine activity). The most potent are the "real" estrogens, such as the natural estrogen E2 and the synthetic estrogen EE2. Most, and perhaps all, xenestrogens are much less potent, usually by a few orders of magnitude (3 or 4), but sometimes even more (see Table 1 for some representative data). Thus, to obtain the same degree of estrogenic response (such as vitellogenin synthesis), it is usually necessary for the organism to be exposed to a concentration of a xenestrogen very much higher than that of E2 and (especially) EE2.

Obviously potency needs to be allied to environmental concentration. However potent a chemical is, it will not cause effects if it is not present in the environment. In contrast, a weakly estrogenic chemical could cause effects if it was present in the environment at high concentrations, particularly if it also bioconcentrated in aquatic organisms, leading to still higher internal concentrations. However, essentially all of the evidence to date suggests that it is the potent steroidal estrogens that are the primary causative agents leading to feminization of fish (see, e.g., ref *39*). Both laboratory (see, e.g., refs *30* and *40*) and field (*41*) studies have demonstrated that extremely low concentrations (nanograms per liter, or even lower in the case of EE2) of steroidal estrogens affect fish. In fact, the concentration of EE2 that causes profound effects on fish (they cannot reproduce successfully) is so low that it is difficult to measure reliably and accurately in "dirty" samples such as effluents and river water.

Despite the general agreement that steroidal estrogens cause much of the feminization of fish that has been reported, there appear to be at least a few specific locations where concentrations of alkylphenolic chemicals, in particular nonylphenol, are high enough that they contribute to the feminization, or may even be the major causative chemicals (see, e.g., refs *42*−*44*). In contrast, we are unaware of any evidence suggesting that bisphenol A (a very weak estrogen to fish when exposure is through the water) contributes to the reported estrogenic effects in fish.

TBT serves as a second example of the extraordinary high potency of an endocrine-disrupting chemical. It is extremely well documented that water concentrations only have to be in the low nanogram per liter range for imposex to be induced (*45*, *46*). Well out to sea, in shipping lanes, where TBT concentrations may be less than 1 ng/L, effects on mollusks are still observed (*47*). However, for E2 and EE2, high potencies were well established, and hence their effects were predictable, while those of TBT were not (because the mechanism of action remains unknown).

## Lesson 5: Degradation Products May Bite!

For decades, alkylphenol polyethoxylates (APEs) have been economically important as nonionic surfactants used in a variety of industrial and household applications. Early research concentrated on the biodegradation of the parent polyethoxylates, which were clearly demonstrated to be successfully eliminated in an activated sludge environment (*52*). A wide variety of breakdown products were subsequently identified as being formed during sewage treatment (*53*). Apart from nonylphenol (NP) itself, these included shorter chain ethoxylates, particularly nonylphenol mono- and diethoxylates and also nonylphenoxy carboxylated varieties, principally nonylphenol mono- and diethoxy carboxylates. Some breakdown products such as the 4-*tert* isomers of nonyl and octylphenol were shown to be estrogenic to fish (*54*). In effect, biodegradation of the parent compound, which began by reducing the length of the ethoxylate chain, had the effect of converting a nonestrogenic molecule into a series of (weakly) estrogenic degradation products (*55*). The significance of these breakdown products, particularly 4-*tert*-NP, as the most important endocrine disrupters in some contaminated waters should not be overlooked (*42*−*44*).

Permethrin is a commonly used insecticide in agriculture and is degraded in soil with a half-life of 7−112 days (*56*, *57*). A number of breakdown products are formed in soil, including 3-phenoxybenzyl alcohol, phenoxybenzoic acid, and cyclopropane permethrin acid (*58*, *59*). Although not perhaps in the same category as NP, these breakdown products have been found to be weak endocrine disrupters, albeit only in

vitro to date. Thus, 3-phenoxybenzyl alcohol was found to be 100-fold more potent as an estrogen than the parent, while 3-phenoxybenzoic acid and cyclopropane permethrin acid were antiestrogens, with potencies only 100−1000-fold less than 4-hydroxytamoxifen, an activity not shared by the parent permethrin (*60*). Hot spots of permethrin water and sediment contamination have been found in the Aire and Calder Rivers in the U.K., and are believed to be related to the local textile industry (*61*). Thus, the exposure of aquatic organisms to these endocrine-disrupting breakdown products would be a possibility.

Another, more recent, example of a possibly significant breakdown product has come from triclosan. Although there are some acute toxicity concerns with this commonly used antibacterial agent, some recent studies (*62*) have indicated that a proportion of the parent compound could be converted into a much more harmful dioxin form in sunlight-irradiated river waters (some dioxins are extremely potent endocrine disrupters).

Finally, it is often forgotten that steroidal estrogens, when excreted by people and animals in their urine, enter the sewer as largely biologically inactive conjugates (*63*). These glucuronide or sulfate conjugates possess little estrogenic potential (*64*), but biodegradation within both the sewer (*65*) and the treatment works (*66*) can cleave the conjugates, releasing the free active hormone as the initial breakdown product. What these stories tell us is that a risk assessment strategy based solely on testing the active ingredients may overlook the potentially harmful effects of breakdown products!

A very clearly written summary of our current knowledge of the presence and toxicity of degradation products (degradates) has recently been published (*67*).

## Lesson 6: Beware of Continual Exposure to Low Concentrations and Mixtures

The (often very high) impact of some endocrine-disrupting chemicals on some faunas is only observed when the organisms are continuously exposed. In fact, continuous exposure will be the common exposure scenario for aquatic organisms where these chemicals are discharged in sewage effluent. Although both E2 and E1 are readily biodegraded in river water (*68*), because they are discharged in sewage effluent, which continually enters the aquatic environment, they are present in receiving waters every day of the year. Although their concentrations in the receiving waters will vary with the season, due primarily to changes in dilution, their continual presence at these locations justifies the term "pseudopersistent".

It is probably this continuous exposure to, for example, nanogram per liter concentrations of EE2 that accounts for the extreme effects it can cause (see, e.g., refs *40* and *41*). In contrast, microgram and even milligram amounts need to be injected to produce comparable effects (*69*−*71*), and these are then only transitory, and attenuate as the chemical is metabolized and excreted. Unfortunately, there have not been any thorough studies that have compared, for example, the effects induced by low-level, chronic (continuous) exposure to those induced by higher level, acute (short-term) exposure. However, there is now no doubt that continuous exposure via the water can lead to effects when the actual concentration of the chemical is surprisingly low (see the discussion in ref *72* for some thoughts on why this may be). Whatever the explanation, a large body of evidence, much of it obtained from well-conducted laboratory experiments (see, for example, the references in Table 1), but also some from field studies (see, e.g., refs *73*−*75*), demonstrates very conclusively that low concentrations of endocrine-disrupting chemicals can cause effects if exposure is continuous. Thus, it would be a mistake to focus all attention on the (high-volume) chemicals present in the environment at high concentrations at the expense of the less prevalent, but more potent, chemicals.

Finally, it is important to constantly keep in mind that aquatic organisms are rarely, if ever, exposed to a single estrogenic chemical, but instead are usually exposed simultaneously to many estrogenic chemicals (i.e., to a mixture of estrogenic chemicals), of varying potencies, each present at a unique concentration. Thus, to understand the risk posed by exposure to estrogenic chemicals, it is necessary to understand how chemicals interact in mixtures (*76*). Our current knowledge of this important issue is relatively poor, especially when mixtures containing a reasonable number of estrogenic chemicals (the situation wild organisms face), and what effects these mixtures have in vivo, are considered (see refs *30* and *51* for results from binary mixtures). However, it seems likely that even if each estrogenic chemical is present at a concentration below that which causes an effect, the mixture might nevertheless cause an effect (*77*). Understanding, and hence being able to predict, the effects of mixtures of endocrine-disrupting chemicals, which vary enormously in both concentration in the environment and potency, is a real challenge, but one that must be addressed if wildlife are to be adequately protected from the effects of the complex mixtures of endocrine-active chemicals that they can be exposed to. Particular difficulties may be faced in determining the net effect of a mixture of endocrine-active chemicals that have different hormonal activities. For example, could the presence of antiestrogens cancel out the effect of estrogenic chemicals, or an androgenic chemical maintain the "masculinity" of males that might otherwise be "feminized" by the estrogenic chemicals they are simultaneously exposed to?

## Lesson 7: Beware of Nontraditional Pollutants from Unexpected Sources

There has been a tendency to assume that any adverse impacts on aquatic wildlife living in water close to urban areas must be due in some way to the presence of xenobiotic (industrial) chemicals. Following the discovery that sewage effluent was estrogenic (*10*), the research over the next four years concentrated on xenobiotic estrogen mimics such as the estrogenic alkylphenol isomers (*49*, *52*, *55*). However, when a toxicity identification and evaluation procedure was carried out on municipal sewage effluent, using an in vitro yeast estrogen assay, the steroidal estrogen component was identified as contributing the greatest proportion of the overall estrogenic activity (*39*). Similarly, use of an in vitro test using human breast cancer MCF-7 cells, which proliferate when exposed to estrogens, suggested the xenobiotic estrogens, such as NP, in the sewage effluent constituted only 1−4% of the overall estrogenic potency (*78*). Two related messages emerge very clearly from the study of endocrine disruption of fish. One is that natural organic molecules, such as E2 and E1, can, if present in the wrong place at the wrong time, cause adverse effects. One would hardly call E2 a pollutant, yet perhaps it can be. Natural chemicals, synthesized to regulate normal physiological processes, have generally been thought of as "good", not "bad". The second is that E2 is not exactly a "foreign" chemical to a fish; they naturally synthesize it. Yet, if exogenous estradiol is present at the "wrong" time, and/or at the wrong concentration, it has severe effects (*79*).

E2 and E1 have been found in a large number of streams impacted by agricultural wastes in the U.S. (*80*, *81*). It is clear that intensive animal husbandry could generate large quantities of both the steroidal estrogens and androgens from urinary and fecal deposition (*82*, *83*), and indeed high

concentrations (up to 2 $\mu$g/L) of estradiol and testosterone have been found in runoff from poultry manure (*84*). A study has indicated elevated vitellogenin in female turtles living in ponds impacted by runoff from beef cattle (*85*), and more recently, endocrine disruption has been reported in wild fathead minnows living in streams receiving effluent from cattle feedlots (*86*). Might the natural steroidal hormones excreted by farm animals have a greater disruptive effect on the local stream fauna than the episodic releases of the xenobiotic pesticides? Even natural high concentrations of animals, such as occurs during the mass spawning of salmon, can be a source of sex steroidal hormones (*87*).

Considerable quantities of androgens are synthesized and excreted naturally by mammals (*88*), and consequently, a proportion of these hormones will arrive at sewage treatment works. The low hydrophobicity of androgens such as testosterone suggests that accumulation in sludge will be low. Testosterone and androstenedione have been found in the effluent of some American sewage treatment works (*89*), but in the U.K., surveys found androgens present in the effluent of works with only primary, or biological, filter sewage treatment (*90*, *91*), suggesting they are more rapidly degraded than the estrogens. Thus, androgens are only likely to be present, if at all, in the effluent of biological filter treatment works, but not in the effluent of the more efficient activated sludge plants (*90*). This might lead to the intriguing possibility that endocrine disruption in a catchment might include androgenic effects in the upper, less populated reaches (where more basic sewage treatment might predominate) but be entirely estrogenic in the more densely populated lower river reaches (where a higher standard of biological sewage treatment would be expected).

Masculinization of fish associated with the receiving water of paper mills has been reported (*92*−*94*). But what chemicals are causing this masculinization? Research appears to show that plant sterols such as $\beta$-sitosterol, stigmasterol, and stigmastanol may be transformed by microorganisms to produce androgenic steroids (*95*). Androstenedione is certainly one natural hormone that has been reported in the sediment of a river receiving paper mill effluent, where masculinized fish have been found (*96*). Although the major androgenic component(s) have yet to be identified, it seems probable that they originate from the plant material. Perhaps the natural phytosterols and resin acids which emanate from cut timber may be having a greater impact on the aquatic wildlife downstream of paper mills than some of the xenobiotic chemicals now used in the paper process (*94*). However, it is important to keep in mind that these effluents are ill-defined and complex, and hence, it is quite possible that chemicals other than phytosterols play a role in the effects observed in fish that are thought to be caused by exposure to these effluents.

Apart from the estrogens and androgens, humans and farm animals also excrete quantities of the natural hormone progesterone (*97*, *98*). Like the steroidal estrogens and androgens, it is highly probable that progestins will arrive at STWs, and a proportion be discharged with the effluent. This appears to be the case for the synthetic progesterone medroxyprogesterone, which is used in hormone replacement therapy (*89*). Because some progestins are potent preovulatory pheromones for fish (*99*), these compounds may cause "sensory disruption" in fish (*89*). Although fish are very specific in the pheromone they respond to (for example, male goldfish are sensitive to 17,20$\beta$-dihydroxyprogesterone at 0.03 ng/L (*100*)), it may be that inadvertently some species of fish will react to the progestins excreted by the human population. Perhaps we should consider whether the discharge of the natural progestin hormones may be causing another form of nontoxic disruption to some fish.

In summary, we should not immediately assume any disruptive effects we observe in aquatic wildlife downstream of sewage works, pulp mills, or livestock farms as being due to xenobiotic chemicals, since the potential of natural hormones cannot be excluded, and these chemicals may, in fact, be the major "culprits".

## Lesson 8:Acute Toxicity Tests May Not Be Very Helpful

One lesson that has come through time and time again during the 10 years of research on endocrine disruption is that the current testing regime used to determine the toxicity of a chemical to aquatic organisms has "failed", in the sense that it has not detected the endocrine activity of many chemicals (e.g., nonylphenol and bisphenol A). Further, endocrine disruption research on these and other chemicals (such as the steroidal estrogens and androgens) has shown that there can be an extremely large difference between the concentration of a chemical that causes mortality in the well-established, routinely conducted acute toxicity tests (this concentration is usually reported as the $LC_{50}$) and that which causes endocrine disruption. This latter point is very well demonstrated by the example of EE2.

Acute toxicity tests using the fathead minnow produced an $LC_{50}$ (the concentration of the chemical that killed half the fish during the days of the test) of 1.6 mg/L (*101*). This could be taken to suggest that EE2 is not a very toxic chemical (at least to fish). However, the results of recent chronic (long-term) tests (see, e.g., ref *40*) give an entirely different picture. Endocrine-mediated effects, such as vitellogenin synthesis, occur at very low (nanogram per liter) concentrations: the $EC_{50}$ for vitellogenin is about 1 ng/L: this is 1600000-fold lower than the $LC_{50}$! Further, concentrations of EE2 above a few nanograms per liter completely inhibit reproduction (*40*, *41*). Clearly, chronic toxicity tests can give a message entirely different from that of acute tests; in the former, EE2 appears to be an extremely toxic chemical. Few chemicals present in the aquatic environment cause effects at such low concentrations.

Perhaps even more surprisingly, it transpired that EE2 *is* acutely toxic to fish, but this effect is delayed long enough that it is not detected in the acute toxicity test protocols used currently. These last for a maximum of four days, but the acute toxicity of EE2 is not evidenced until somewhat later. The reason for this is that the mortality is due to excessive production of vitellogenin, which causes kidney failure (other organs probably also suffer severe toxicity), followed by death (*102*), but it takes a few days for the blood vitellogenin concentration to reach a level high enough to severely disrupt kidney function.

As a consequence of the realization that current toxicity tests (chronic as well as acute) may not detect endocrine-mediated effects, regulatory agencies (in particular the OECD) are in the process of establishing and validating new testing procedures aimed at detecting the endocrine activities of chemicals.

## Lesson 9: Central Role Played by Sewage Treatment

The centralization of sewerage and the widespread introduction of secondary biological sewage treatment have brought enormous benefits to society and the environment. Given the short hydraulic residence time (a few hours), the large reduction in the amount of natural and xenobiotic organic molecules that occurs in a STW is remarkable (*103*). However, in a key paper for endocrine disruption research, Jobling et al. (*11*) demonstrated that the biggest impacts in fish were associated with their proximity to sewage effluent. In the case of the natural and synthetic steroidal estrogens, environmental chemists have shown that, for normally operating activated sludge plants, up to 40% of these

compounds can survive sewage treatment (*104*−*107*). Thus, the STW will be the major conduit by which many of the thousands of chemicals used, or excreted, by the human population each day in the developed world will reach the aquatic environment. There are four key factors that are critical in predicting the impact of an individual STW on the immediate receiving water: (1) the size of the human population connected to the STW, (2) the flow through the works, (3) the type of treatment employed, and (4) the available dilution in the receiving water.

While there are many reports on the presence, or absence, of endocrine disruption in aquatic organisms living in waters receiving sewage effluent, usually very little consideration is given to how the precise makeup of the associated treatment works might have influenced the results. This disconnection between the biological effects and the upstream sewage treatment/hydrology makes it impossible for the reader to judge the significance of the reported relationship.

The importance of knowing the size of the human population connected to the works, and the available dilution in the receiving water, is self-evident. However, it is probably worth exploring briefly the significance of flow through the works, and treatment type. Differences in influent strength (L of water/head) and dilution could result in a 100-fold difference in concentration in the receiving waters (*104*). The flow into the works, and consequently dilution, will change not only with respect to the seasons (sometimes due to water-table rise and infiltration into the sewers), but also with rainfall entry into the sewers (*93*). Additionally, flow into the works will change over the course of the day, due to peaks in "human activity", such as can occur around 8:00 a.m. (*108*, *109*). Thus, taking into account sewer transit, and the typical activated sludge treatment, the 8:00−9:00 a.m. peak "flush" would probably not emerge in the effluent until 8:00 p.m. Given these variations in "human discharge" and flow, the most valuable way to make measurements of hormones, or chemicals intimately associated with human activity, in effluents is from 24 h composite samples collected during summer dry weather conditions.

Studies on endocrine disrupters have also shown that the level of biological treatment can have a dramatic impact on the amount of these compounds passing into the effluent. For example, works which have sedimentation alone, or sedimentation with chemical precipitation (to remove phosphate), achieved little or no removal of steroidal estrogens/endocrine-disrupting chemicals (*39*, *110*). More advanced works contain biological, or trickling, filters comprising tanks with biofilms supported on coarse media upon which the sewage liquor is sprayed following primary sedimentation (*108*). The biostep water contact time (hydraulic residence time, or HRT) is quite short, perhaps 30 min, and this often appears to be not quite as effective as the activated sludge system (*110*−*112*). These works are still commonly used to treat the sewage of smaller communities. Activated sludge is a more intensive biological treatment in which bacteria are suspended in a tank and vigorously aerated, with a HRT of 5−20 h plus (*104*, *113*). The tanks often have a first anoxic, or anaerobic, stage to encourage denitrification, which may also play a role in removing trace organics such as steroidal estrogens (*114*). There are many treatment types in between, but biological filters and activated sludge are still the most common. More sophisticated works with tertiary treatment such as UV, ozonation, nanofiltration, and reverse osmosis will be likely to reduce yet further the microorganic contaminant content of the effluent (*115*−*117*). Clearly, the greater the extent of biological activity deployed by the works, the greater the removal of biodegradable organic compounds that can be expected, and consequently the lower the effluent concentration. With biological treatment it is logical to assume that both the duration of biological treatment and the amount of active biomass present will influence the amount of biodegradation of an organic molecule in sewage (*110*, *116*, *118*, *119*), although we must be aware that, in the real world of biological treatment, these correlations are not always as clear as might be expected (*120*). Another significant factor may be whether the plant is nitrifying or not. Activated sludge plants with long sludge retention times (to encourage the slower growing nitrifying bacteria) may also possess microbial communities which fortuitously have a good ability to degrade some of the more recalcitrant organic molecules, such as has been reported for EE2 (*114*, *121*, *122*).

## Lesson 10: Hydrology Will Tell You Where To Look!

Traditional risk assessment involves a comparison of the predicted environmental concentration (PEC) of a chemical with the predicted no effect concentration (PNEC) on target species. The PEC is derived from the amount of substance used per year, the size of the human population, the volume of wastewater produced per capita per day, and the amount of removal in treatment, with the environmental dilution factor set at 10 (*123*, *124*). However, the available dilution in river catchments can be considerably less and is enormously variable in both the temporal and spatial senses (see, e.g., ref *125*). For example, in a study on steroidal estrogens in the Nene and Lea Rivers in southern England it was determined that effluent made up 34% and 75% of the flow, respectively, during an August 2000 sampling period (*126*). In northern England, of the 33 STWs discharging into the Aire−Calder catchment, during dry weather periods (95th percentile), more than half will be contributing greater than 10% of the flow at the point of discharge and some, such as the large Knostrop Low STW serving Leeds, will be contributing more than 60% of the flow (*127*)! While some might consider the U.K. as an extreme case, even a superficial look at the map in Europe shows that there are some big population centers discharging sewage into modest rivers where a dilution factor of 10 is likely to be an overestimate; Brussels and the Zenne River, Madrid and the Manzanares River, and Milan and the Lambro River would be examples. Similar situations probably occur in other densely populated areas of the world.

With the increasing sophistication of geographic information system (GIS) based hydrological models, such as Low Flows 2000 (*128*, *129*), which predict flows throughout a catchment, it is possible to map "hot spots" for determinands associated with the human population. Such hydrological models can be married to other models which predict the input load for specific chemicals from an STW, such as that recently developed for steroidal estrogens (*63*) which requires only the head of population and flow from the STW. Examples of GIS hydrology combined with chemical process models are GREAT-ER, which is used for some model catchments in Europe (*127*, *130*), and PhATE, used for some catchments in North America (*131*). These generate color-coded maps showing the predicted concentrations throughout the catchment. The models can encompass the in-river degradation rates, which we know to be temporarily and spatially variable (*67*).

Discovering the significance of the key environmental processes which determine the fate of chemicals is one of the most important benefits of a modeling exercise. For example, if a microbiologist were asked when the concentration of endocrine-disrupting chemicals might be highest, he or she might answer "the winter, because then biodegradation will be slow and the chemicals become more persistent". But if the same question were asked of a hydrologist, he or she might answer "the summer, because then the available dilution is lowest, and consequently the chemical concentration will be at its highest". A process model such as EXAMS, which incorporates hydrology and all the potential chemical fate processes to predict concen-

trations for river reaches, will rapidly cut short such arguments (*126*, *132*).

These models will represent an increasingly important resource for environmental chemists, biologists, and regulators alike, their principal advantage being in telling scientists where, and potentially when, to look for human-derived chemicals and their effects. Future developments will probably refine such models, to take into account differences in efficiencies of different sewage treatment types across the catchment. Other subtle refinements may be incorporated on the basis of expert knowledge on differing human behaviors and consumption patterns. For example, by taking into account the age profile of a town (some may have a preponderance of retired people, or the presence of a large student population), more accurate predictions of the concentrations of certain chemicals, such as pharmaceuticals, should be possible.

## Keeping Focused

It is an instructive exercise to consider whether all the effort (and money) invested in research on endocrine disruption was wisely spent. One approach to doing this exercise is to identify the key issues and then examine to what degree they were addressed and, preferably, answered. Leaving aside the issue of endocrine disruption in humans, which is outside the scope of this paper, the key issues concerning endocrine disruption, or indeed any compounds with disruptive effects in the environment, might be the following: (a) To what extent is this type of disruption occurring in wildlife? (b) What species are being affected? (c) What chemicals are responsible for these effects, and how do they behave in the environment? (d) What are the consequences of these effects, at both the individual and, probably more importantly, population levels? (e) What could, and should, be done?

Although we have not tried to "map" all the published papers (a few thousand by now!) to these key issues, it would seem that a significant proportion of the research funded did not focus on these key issues. We realize that this is a very bold assertion that not everyone would agree with. However, we think that many would agree that sometimes the focus of what we should be trying to do (addressing the really important questions about the sustainability of our environment) seems to have been lost, or at least to not be evident to us.

To provide just one example, consider the issue of what chemicals are estrogenic to fish. As discussed already (see lesson 4), fairly strong evidence exists to suggest that the steroidal estrogens are, in most cases, the major estrogenic chemicals causing the effects on fish, with alkylphenolic chemicals also contributing occasionally. Hence, the focus should probably be on these chemicals. There seems little, if any, reason to demonstrate that yet another chemical is weakly estrogenic to fish, if that chemical is not affecting wild fish.

## The Way Forward?

There are now an increasing number of questions being asked about whether the presence of some pharmaceuticals and personal care products (PPCPs) in effluent are harming aquatic flora and fauna. Answering these questions will not be easy: the number and range of PPCPs is large, and the number of organisms they could affect equally large. Of particular concern are pharmaceuticals, because all of these are biologically active chemicals (they would not do their jobs if they were not). Further, some are biologically active at low (sometimes extremely low) concentrations, as the example of EE2 illustrates only too well. In response to this understandable concern, and in good faith, industry and regulatory authorities are now expending considerable efforts to determine whether these chemicals pose a risk, and if so, to what, and to what degree. The approach being used is the traditional one of conducting a small range of acute and chronic toxicity tests for each chemical in turn (that is, each chemical is assessed individually). On the basis of what we consider we have learned from endocrine disruption, we are concerned that this approach will be unlikely to protect wildlife to the degree we, and others, would like. Therefore, perhaps in parallel, we think that other approaches should be considered.

One possible strategy would involve first assembling a multidisciplinary team consisting of, at a minimum, one or more toxicologists, ecotoxicologists, wildlife biologists, chemists, hydrologists, and regulators. Because environmental risk assessment is a multidisciplinary task, the objective is to start with a group of people who collectively have all the range of expertise that will undoubtedly be required.

From here on, two approaches are possible. One is what we have called "compound-specific", by which we mean that the risks posed by a single chemical are considered; for example, a pharmaceutical company may want to determine the environmental risks (if any, of course) posed by one of its new or currently used drugs. The other is what we have called "issue-specific", by which we mean defining the risks posed by a group of related chemicals (related in the sense of a common use). Examples of the latter would be "defining the risk to the aquatic environment posed by the presence of pharmaceuticals", or "defining the risk posed by the presence of cosmetics". We will consider each in turn.

**1. Compound-Specific Environmental Risk Assessment.** First, collect and assess all available information on the chemical of interest. Knowledge of the chemical and physical properties of the PPCP should enable the fate and behavior of that chemical in the aquatic environment to be predicted with a reasonable degree of accuracy. This knowledge should further enable one to predict which organisms are likely to receive the highest exposure, and to what degree the chemical will bioaccumulate.

For pharmaceuticals (but possibly not for other groups of PPCPs, such as those present in toiletries and cosmetics), the amounts used (and excreted) are, sometimes with effort, obtainable, which is an important first step in grappling with the scale of the issue (although always keep in mind that a surprisingly small amount of EE2—the amount used annually in the U.K. is around 40 kg—can cause a lot of problems!).

Next, use the information in hydrological modeling to predict where the worst impacted reaches of a catchment are likely to be. Focus exclusively there. Then, using any information that is available on possible effects of the compound of interest (for example, if the drug of interest is a $\beta$ blocker, look for effects on the cardiovascular system), target the physiological systems and responses most likely to be affected by the compound (we accept that sometimes there will probably be completely unexpected effects in organisms not targeted by the investigations).

Analytic investigation aimed at determining the concentrations of the compound in both plasma and bile would be very informative, as these would provide information on uptake and body burdens, which (if the chemical were a pharmaceutical) could be compared to data obtained from therapeutic use (in humans) to predict if effects were likely. This approach to intelligently trying to predict whether human pharmaceuticals will affect wildlife (fish in particular) has been very cogently argued by Huggett and co-workers (*133*).

**2. Issue-Specific Environment Risk Assessment.** Rather than agonize over which chemical/metabolite/mode of action we should focus on, perhaps it would be wise to *begin* by examining wildlife, to determine if any effects are noticeable. This is essentially a "bottom up, listening to the environment"

approach. Recall that the natural and synthetic steroidal estrogen story did not begin because someone suggested that there would be a problem with these chemicals, but rather because detective work pointed at these chemicals *after* problems in fish were observed. So, for example, if the issue of concern is pharmaceuticals in the aquatic environment, the aim would not be to assess the impact of each pharmaceutical in turn (and there are hundreds in everyday use), but instead to determine if the collective mixture of pharmaceuticals (and their often ill-defined degradation products) is, or is not, adversely affecting biodiversity and its sustainability.

This approach would start by using geographical/hydrological modeling to identify which reaches in a country have little available dilution and are associated with significant STWs of a modest standard with negligible industrial input. This would provide a range of "human impact factors" across the country (or area of study). The biology of sites with the highest human impact factor (i.e., those at which concentrations of chemicals of interest are predicted to be highest) would be compared in the widest sense with that of nearby sites with very low impact factors. The aim would be to have these two sites (or groups of sites) as similar as possible, to maximize the chances that any biological differences are caused by the chemicals of concern, over and above that traditionally associated with elevated organic pollution (*134*). If unusual observations were made that were unique to the high impact sites, more traditional approaches could then be used to (hopefully) verify the causative relationship.

Either of these approaches may offer a more direct route to understanding what harm, if any, is occurring in native flora and fauna as a result of pharmaceutical or personal care product discharges. We are not claiming a monopoly of wisdom here (variants on our suggestions have already been used to tackle some environmental problems), but are attempting to stimulate innovative thinking about how to conduct environmental risk assessments. We consider new approaches will be necessary if we wish to conserve our natural heritage in the face of a rapidly changing world.

## Literature Cited


(1) Matthiessen, P. Historical perspective on endocrine disruption in wildlife. *Pure Appl. Chem.* **2003**, *75*, 2249−2262.

(2) Durodie, B. The true cost of precautionary chemicals regulation. *Risk Anal.* **2003**, *23*, 389−398.

(3) Sharpe, R. M.; Skakkebaek, N. E. Male reproductive disorders and the role of endocrine disruption: advances in understanding and identification of areas for future research. *Pure Appl. Chem.* **2003**, *75*, 2923−2038.

(4) Tyler, C. R.; Jobling, S.; Sumpter, J. P. Endocrine disruption in wildlife: a critical review of the evidence. *Crit. Rev. Toxicol.* **1998,** *23*, 319−361.

(5) Blaber, S. J. M. The occurrence of a penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus* (L.). *Proc. Malac. Soc. London* **1970**, *39*, 231−233.

(6) Smith, B. S. Sexuality in the American Mud Snail, *Nassarius obsoletus* Say. *Proc. Malac. Soc. London* **1971**, *39*, 377−378.

(7) Smith, B. S. Reproductive abnormalities in stenoglossan snails related to pollution from marinas. *J. Appl. Toxicol.* **1981a**, *1*, 15−21.

(8) Smith, B. S. Male characteristics on female mud snails caused by antifouling bottom paints. *J. Appl. Toxciol.* **1981b**, *1*, 22−25.

(9) Oberdörster, E.; McClellan-Green, P. Mechanisms of imposex induction in the mud snail. *Ilyanassa obsolete*: TBT as a neurotoxin and aromatose inhibitor. *Mar. Environ. Res.* **2002**, *54*, 715−718.

(10) Purdom, C. E.; Hardiman, P. A.; Bye, V. J.; Eno, N. C.; Tyler, C. R.; Sumpter, J. P. Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **1994**, *8*, 275−285.

(11) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32*, 2498−2506.

(12) Woodward, A. R.; Jennings, M. L.; Percival, J. F.; Moore, T. Low clutch viability of American alligators on Lake Apopka. *Florida Sci.* **1993**, *56*, 52−63.

(13) Jennings, M. L.; Percival, H. F.; Woodward, A. R. Evaluation of alligator hatchling and egg removal from three Florida lakes. *Proceedings of the Annual Conference of the Southeastern Association of Fish and Wildlife Agencies*; Southeastern Association of Fish and Wildlife Agencies: Tallahassee, FL, 1988; Vol. 42, pp 283−294.

(14) Guillette, L. J. Jr.; Crain, D. A.; Gunderson, M. P.; Kools, S. A. E.; Milnes, M. R.; Orlando, E. F.; Rooney, A. A.; Woodward, A. R. Alligators and endocrine disrupting contaminants: a current perspective. *Am. Zool.* **2000**, *40*, 438−452.

(15) Bergeron, J. M.; Crews, D.; McLachlan, J. A. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. *Environ. Health Perspect.* **1994**, *102*, 780−786.

(16) Willingham, E.; Crews, D. Sex reversal effects of environmentally relevant xenobiotic concentrations on the red-eared slider turtle, a species with temperature-dependent sex determination. *Gen. Comp. Endocrinol.* **1999**, *113*, 429−435.

(17) Schmidt, C. W. Amphibian deformities continue to puzzle researchers. *Environ. Sci. Technol.* **1997**, *31*, 324A−326A.

(18) Van Valen, L. A natural model for the origin of some higher taxa. *J. Herpetol.* **1974**, *8*, 109−121.

(19) Houlahan, J. E.; Findlay, C. S.; Schnidt, B. R.; Meyer, A. H.; Kuzmin, S. L. Quantitative evidence for global amphibian population declines. *Nature* **2000**, *404*, 752−755.

(20) Brown. E. A. R.; Scott, D. B. C. A second hermaphrodite specimen of *Coregonus lavaretus* (L.) (Salmonidae, Coregoninae) from Loch Lomond, Scotland. *J. Fish Biol.* **1988**, *33*, 957−958.

(21) Borg, B. Oocytes in the testes of the three-spined stickleback, *Gasterosteus aculeatus*. *Copeia* **1983**, No. 1, 259−261.

(22) Geroken, J.; Sorydl, H. Intersex in feral marine and freshwater fish from northeastern Germany. *Mar. Environ. Res.* **2002**, *54*, 651−655.

(23) Vignanò, L.; Arillo, A.; Mansari, A.; Mandich, A. First observation of intersex cyprinids in the Po River (Italy). *Sci. Total Environ.* **2001**, *269*, 189−194.

(24) Allen, Y.; Matthiessen, P.; Scott, A. P.; Haworth, S.; Feist, S.; Thain, J. E. The extent of estrogenic contamination in the UK estuarine and marine environments—further surveys of flounder. *Sci. Total Environ.* **1999**, *233*, 5−20.

(25) De Metrio, G.; Corriero, A.; Desantis, S.; Zuani, D.; Cirillo, F.; Deflorio, M.; Bridges, C. R.; Eicker, J.; de la Serna, J. M.; Megalofonou, P.; Kime, D. E. Evidence of a high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius* L.). *Mar. Pollut. Bull.* **2003**, *46*, 358−361.

(26) Afonso, L. O. B.; Smith, J. L.; Ikonomou, M. G.; Devlin, R. H. Y-chromosomal DMA markers for discrimination of chemical substance and effluent effects on sexual differentiation in salmon. *Environ. Health Perspect.* **2002**, *110*, 881−887.

(27) Nakamura, M.; Nagoya, H.; Hirai, T. Nonylphenol induces complete feminization of the gonad in genetically controlled all-male amago salmon. *Fish. Sci.* **2002**, *68*, 1387−1389.

(28) Sumida, K.; Ooe, N.; Saito, K.; Kaneko, H. Limited species differences in estrogen receptor alpha-mediated reporter gene transactivation by xenestrogens. *J. Steroid Biochem. Mol. Biol.* **2003**, *84*, 33−40.

(29) White, R. Jobling, S.; Hoare, S. A.; Sumpter, J. P.; Parker, M. G. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **1994**, *135*, 175−182.

(30) Thorpe, K. L.; Cummings, R. I.; Hutchinson, T. H.; Scholze, M.; Brighty, G.; Sumpter, J. P.; Tyler, C. R. Relative potencies and combination effects of steroidal estrogens in fish. *Environ. Sci. Technol.* **2003**, *37*, 1142−1149.

(31) Breitholz, M.; Bengtsson, B.-E. Estrogens have no hormonal effect on the development and reproduction of the harpecticoid copepod, *Nitocra spinipes*. *Mar. Pollut. Bull.* **2001**, *42*, 879−886.

(32) Segner, H.; Caroll, K.; Fenske, M.; Janssen, C. R.; Maack, G.; Pascoe, D.; Schäfers, C.; Vandenbergh, G. F.; Watts, M.; Wenzel, A. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicol. Environ. Saf.* **2003**, *54*, 302−314.

(33) Jobling, S.; Casey, D.; Rodgers-Gray, T.; Oehlmann, J.; Schulte-Oehlmann, U.; Pawlowski, S.; Baunbeck, T.; Turner, A. P.; Tyler, C. R. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol.* **2003**, *65*, 205−220.

(34) Oehlmann, J.; Schulte-Oehlmann, U. Endocrine disruption in invertebrates. *Pure Appl. Chem.* **2003**, *75*, 2207−2218.

(35) Matthiessen, P.; Gibbs, P. E. Critical appraisal of the evidence of tributyltin-mediated endocrine disruption in molluscs. *Environ. Toxicol. Chem.* **1998**, *17*, 37−43.
(36) Schulte-Oehlmann, U.; Tillmaan, M.; Markert, B.; Oehlmann, J. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyltin as a xeno-androgea. *Ecotoxicology* **2000**, *9*, 399−412.
(37) Nishikawa, J.; Mamiya, S.; Kanayama, T.; Nishikawa, T.; Shiraishi, F.; Horiguchi, T. Involvement of the Retinoid X Receptor in the development of imposex caused by organotins in gastropods. *Environ. Sci. Technol.* **2004**, *38*, 6271−6276.
(38) Shimasaki, Y.; Kitano, T.; Oshima, Y.; Inoue, S.; Imada, N.; Honjo, T. Tributyltin causes masculinization in fish. *Environ. Toxicol. Chem.* **2003**, *22*, 141−144.
(39) Desbrow, C.; Routledge, E. J.; Brighty, G. C.; Sumpter, J. P.; Waldock, M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **1998**, *32*, 1549−1558.
(40) Lange, R.; Hutchinson, T. H.; Croudace, C. P.; Siegmund, F.; Schweinfurth, H.; Hampe, P.; Panter, G. H.; Sumpter, J. P. Effects of the synthetic estrogen 17α-ethinylestradiol over the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2001**, *20*, 1216−1227.
(41) Palace, V. P.; Evans, R. E.; Wautier, K..; Baron, C.; Vandenbyllardt, L.; Vandersteen, W.; Kidd, K. Induction and vitellogenin and hitological effects in wild fthead minnows from a lake experimentally treated with the synthetic estrogen, ethinylestradiol. *Water Qual. Res. J. Can.* **2002**, *37*, 637−650.
(42) Solé, M.; de Alda, M. J. L.; Castillo, M.; Porte, C.; Ladegaard-Pederson, K.; Barcelo, D. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). *Environ. Sci. Technol.* **2000**, *34*, 5076−5083.
(43) Sheahan, D. A.; Brighty, G. C.; Daniel, M. Jobling, S.; Harries, J. E.; Hurst, M. R.; Kennedy, J.; Kirby, S. J.; Morris, S.; Routledge, E. J., et al. Reduction in the estrogenic activity of a treated sewage effluent discharge to an English river as a result of a decrease in the concentration of industrially-derived surfactants. *Environ. Toxicol. Chem.* **2002**, *21*, 515−519.
(44) Todorov, J. R.; Elskus, A. A.; Schlenk, D.; Ferguson, P. L.; Brownawell, B. J. Estrogenic responses of larval sunshine bass (Morone saxatilis x M. chrysops) exposed to New York city sewage effluent. *Mar. Environ. Res.* **2002**, *54*, 691−695.
(45) Gibbs, P. E.; Pascoe, P. L.; Burt, G. R. Sex change in the female dog whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J. Mar. Biol. Assoc.* **1998**, *68*, 715−731.
(46) Mensink, B. P.; Everaarts, J. M.; Kralt, H.; Tenhallerstjabbes, C. C.; Boon, J. P. Tributyltin exposure in early life stages induces the development of male sexual characteristics in the common whelk, Buccinum undatum. *Mar. Environ. Res.* **1996**, *42*, 151−154.
(47) Fent, K. Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* **1996**, *26*, 1−117.
(48) Harris, C. A.; Santos, E. M.; Janbakhsh, A.; Pottinger, T. G.; Tyler, C. R.; Sumpter J. P. Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female rainbow trout. *Environ. Sci. Technol.* **2001**, *35*, 2909−2916.
(49) Jobling, S.; Sheahan, D.; Osborne, J. A.; Matthiessen, P.; Sumpter, J. P. Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals. *Environ. Toxicol. Chem.* **1996**, *15*, 194−202.
(50) Sohoni, P.; Tyler, C. R.; Hurd, K.; Caunter, J.; Hetheridge, M.; Williams, T.; Woods, C.; Evans, M.; Toy, R.; Gargas, M.; Sumpter, J. P. Reproductive effects of long-term exposure to Bisphenol-A in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* **2001**, *35*, 2917−2925.
(51) Thorpe, K. L.; Hutchinson, T. J.; Hetheridge, M. J.; Scholze, M.; Sumpter, J. P.; Tyler, C. R. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Oncorhynchus mykiss)*. *Environ. Sci. Technol.* **2001**, *35*, 2476−2481.
(52) Rudling, L.; Solyom, P. The investigation of biodegradability of branched nonylphenol ethoxylates. *Water Res.* **1974**, *8*, 115−119.
(53) Ahel, M.; Giger, W.; Koch, M. Alkylphenol polyethoxylate surfactants in the aquatic environment -I. Occurrence and transformation in sewage treatment. *Water Res.* **1994**, *28*, 1131−1141.
(54) Jobling, S.; Sumpter, J. P. Detergent components in sewage effluent are weakly estrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.* **1993**, *27*, 361−372.
(55) Routledge, E. J.; Sumpter, J. P. Structural features of alkylphenolic chemicals associated with estrogenic activity. *J. Biol. Chem.* **1997**, *272*, 3280−3288.
(56) Kaufman, D. D.; Haynes, S. C. Permethrin degradation in soil and microbial culture. *ACS Symp. Ser.* **1977**, *42*, 147−161.
(57) Kaneko, H.; Ohkawa, H.; Miyamoto, J. Degradation and movement of permethrin isomers in soil. *J. Pestic. Sci. (Int. Ed.)* **1978**, *3*, 43−51.
(58) Roberts, T. R.; Standen, M. E. Degradation of the pyrethroid insecticide WL 41706, (+)-α-cyano-3-phenoxybenzyl-2,2,3,3-tetramethylcyclopropanecarboxylat e, in soil. *Pestic. Sci.* **1977**, *8*, 600−610.
(59) Roberts, T. R.; Standen, M. E. Further studies on the degradation of the pyrethroid insecticide cypermethrin in soil. *Pestic. Sci.* **1981**, *12*, 285−296.
(60) Tyler, C. R.; Beresford, N.; van der Woning, M.; Sumpter, J. P.; Thorpe, K. Metabolism and environmental degradation of pyrethroid insecticides produce compounds with endocrine activities. *Environ. Toxicol. Chem.* **2000**, *19*, 801−809.
(61) Long, J. L. A.; House, W. A.; Parker, A.; Rae, J. E. Micro-organic compounds associated with sediments in the Humber rivers. *Sci. Total Environ.* **1998**, *210/211*, 229−253.
(62) Latch, D. E.; Packer, J. L.; Arnold, W. A.; McNeill, K. Photochemical conversion of Triclosan to 2,8-dichlorodibenzo-p-dioxin in aqueous solution. *J. Photochem. Photobiol., A* **2003**, *158*, 63−66.
(63) Johnson, A. C.; Williams, R. J. A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ. Sci. Technol.* **2004**, *38*, 3649−3658.
(64) Beresford, N.; Routledge, E. J.; Harris, C. A.; Sumpter, J. P. Issues arising when interpreting results from an *in vitro* assay for estrogenic activity. *Toxicol. Appl. Pharmacol.* **2000**, *162*, 22−23.
(65) D'Ascenzo, G. D.; Di Corcia, A.; Gentili, A.; Mancini, R.; Mastropasqua, R.; Nazzari, M.; Samperi, R. Fate of natural estrogen conjugates in the municipal aqueous environment. *Sci. Total Environ.* **2003**, *302*, 199−209.
(66) Belfroid, A. C.; Van der Horst, A.; Vethaak, A. D.; Schafer, A. J.; Rijs, G. B. J.; Wegener, J.; Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. *Sci. Total Environ.* **1999**, *225*, 101−108.
(67) Boxall, A. B. A.; Sinclair, C. J.; Fenner, K.; Kolpin, D.; Maund, S. J. When synthetic chemicals degrade in the environment. *Environ. Sci. Technol.* **2004**, *38*, 369A−375A.
(68) Jürgens, M. D.; Holthaus, K. I. E.; Johnson, A. C.; Smith J. J. L.; Hetheridge, M.; Williams, R. J. The potential for estradiol and ethinylestradiol degradation in English rivers. *Environ. Toxicol. Chem.* **2002**, *21*, 480−488.
(69) Denslow, N. D.; Bowman, C. J.; Ferguson, R. J.; Lees, H. S.; Hemmer, M. J.; Folmar, L. C. Induction of gene expression in Sheepshead Minnows (*Cyprinodon variegates*) treated with 17$\beta$-estradiol, diethylstilbestrol, or ethinylestradiol: the use of mRNA fingerprints as an indicator of gene regulation. *Gen. Comp. Endocrinol.* **2001**, *121*, 250−260.
(70) Larkin, P.; Sabo-Attwood, T.; Kelso, J.; Denslow, N. D. Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and p,p$^1$-DDE. *Comp. Biochem. Physiol., B* **2002**, *133*, 543−557.
(71) Schultz, I. R.; Orner, G.; Merdink, J. L.; Skillman, A. Dose−response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17α-ethinylestradiol. *Aquat. Toxciol.* **2001**, *51*, 305−318.
(72) Pickford, K. A.; Thomas-Jones, R. E.; Wheals, B.; Tyler, C. R.; Sumpter, J. P. Route of exposure affects the estrogenic response of fish to 4-ter-octylphenol. *Aquat. Toxicol.* **2003**, *65*, 267−279.
(73) Harries, J. E.; Sheahan, D. A.; Jobling, S.; Matthiessen, P.; Neall, P.; Sumpter, J. P.; Tylor, T.; Zaman, N. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* **1997**, *16*, 534−542.
(74) Hemmings, J. M.; Waller, W. T.; Chow, M. C.; Denslow, N. D.; Venables, B. Assessment of the estrogenicity and toxicity of a domestic wastewater effluent flowing though a constructed wetland system using biomarkers in male fathead minnows (*Pimephales promelas* Rafinesque, 1820). *Environ. Toxicol. Chem.* **2001**, *20*, 2268−2275.
(75) Kirby, M. F.; Allen, Y. T.; Dyer, R. A.; Feist, S. T.; Katsiadaki, I.; Matthiessen, P.; Scott, A. P.; Smith, A.; Stentiford, G. D.; Thain, J. E.; Thomas, K. V.; Tolhurst, L.; Waldock, M. J. Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruptin by estrogenic contamination

in United Kingdom estuaries: temporal trends, 1996 to 2001. *Environ. Toxicol. Chem.* **2004**, *23*, 748−758.
(76) Payne, J. P.; Rajapakse, N.; Wilkins, M.; Kortenkamp, A. Prediction and assessment of the effects of mixtures of four xenestrogens. *Environ. Health Perspect.* **2000**, *108*, 983−987.
(77) Silva, E.; Rajapakse, N.; Kortenkamp, A. Something from "nothing"—Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* **2002**, *36*, 1751−1756.
(78) Körner, W.; Bolz, U.; Süssmuth, W.; Hiller, G.; Schuller, W.; Hanf, V.; Hagenmaier, H. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. *Chemosphere* **2000**, *40*, 1131−1142.
(79) Panter, G. H.; Thompson, R. S.; Sumpter J. P. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural estrogens E2 and oestrone. *Aquat. Toxicol.* **1998**, *42*, 243−253.
(80) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. Pharmaceuticals, hormones and other organic wastewater contaminants in US streams, 1999−2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202−1211.
(81) Soto, A. M.; Calabro, J. M.; Prechtl, N. V.; Yau, A. Y.; Orlando, E. F.; Daxenberger, A.; Kolok, A. S.; Guillette, L. J.; le Bizec, B.; Lange, I. G.; Sonnenschein, C. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nabraska, USA. *Environ. Health Perspect.* **2004**, *112*, 346−352.
(82) Hanselman, T. A.; Graetz, D. A.; Wilkie, A. C. Manure-borne estrogens as potential contaminants: A review. *Environ. Sci. Technol.* **2003**, *37*, 5471−5478.
(83) Raman, D. R.; Williams, E. L.; Layton, A. C.; Burns, R. T.; Easter, J. P.; Daugherty, A. S.; Mullen, M. D.; Sayler, G. S. Estrogen content of dairy and swine wastes. *Environ. Sci. Technol.* **2004**, *38*, 3567−3573.
(84) Finlay-Moore, O.; Hartel, P. G.; Cabrera, M. L. 17α-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. *J. Environ. Qual.* **2000**, *29*, 1604−1611.
(85) Irwin, L. K.; Gray, S.; Oberdorster, E. Vitellogenin induction in painted turtle, Chrysemys picta, as a biomarker of exposure to environmental levels of estradiol. *Aquat. Toxicol.* **2001**, *55*, 49−60.
(86) Orlando, E. F.; Kolok. A. S.; Binzcik, G. A.; Gtes, J. L.; Horton, M. K.; Lambright, C. S.; Gray, L. E.; Soto, A. M.; Guillette, L. J. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the Fathead Minnow. *Environ. Health. Perspect.* **2004**, *112*, 353−358.
(87) Kolodziej, E. P.; Harter, T.; Sedlak, D. L. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environ. Sci. Technol.* **2004**, *38*, 6377−6384.
(88) Hoffmann, B.; Evers, P. In *Drug residues in animals*; Rico, A. G., Ed.; Academic Press: New York, 1986; pp 111−146.
(89) Kolodziej, E. P.; Gray, J. L.; Sedlak, D. L. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environ. Toxicol. Chem.* **2003**, *22*, 2622−2629.
(90) Thomas, K. V.; Hurst, M. R.; Matthiessen, P.; McHugh, M.; Smith, A.; Waldock, M. J. An assessment of in vitro androgenic activity and the identification of environmental androgens in United Kingdom estuaries. *Environ. Toxicol. Chem.* **2002**, *21*, 1456−1461.
(91) Kirk, L. A.; Tyler, C. R.; Lye, C. M.; Sumpter, J. P. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. *Environ. Toxicol. Chem.* **2002**, *21*, 972−979.
(92) Howell, W. M.; Black, D. A.; Bortoni, S. A. 1980. Abnormal expression of secondary sex characters in a population of mosquito fish, *Gambusia affinis hollbrooki*: evidence for environmentally induced masculinization. *Copeia* **1980**, No. 4, 676−681
(93) Parks, L. G.; Lambright, C. S.; Orlando, E. F.; Guillette, L. J.; Ankley, G. T.; Gray, L. E. Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol. Sci.* **2001**, *62*, 257−267.
(94) Ellis, R. J.; Van Den Heuvel, M. R.; Bandelj, E.; Smith, M. A.; McCarthy, L. H.; Stuthridge, T. R.; Dietrich, D. R. In vivo and in vitro assessment of the androgenic potential of pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**, *22*, 1448−1456.
(95) Howell, W. M.; Denton, T. E. Gonopodial morphogenesis in female mosquitofish, *Gambusia afinis afinis* masculinised by exposure to degradation products from plant sterols. *Environ Biol. Fish* **1989**, *24*, 43−51.
(96) Jenkins, R. L.; Wilson, E. M.; Angus, R. A.; Howell, W. M.; Kirk, M. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. *Toxicol. Sci.* **2003**, *73*, 53−59.
(97) Santoro, N.; Goldsmith, L. T.; Heller, D.; Illsley, N.; McGovern, P.; Molina, C.; Peters, S.; Skurnick, J. H.; Forst, C.; Weiss, G. Luteal progesterone relates to histological endometrial maturation in fertile women. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 4207−4211.
(98) Rabiee, A. R.; Macmillan: K. L.; Schwarzenberger, F. Plasma, milk and faecal progesterone concentrations during the oestrous cycle of lactating dairy cows with different milk yields. *Anim. Reprod. Sci.* **2002**, *74*, 121−131.
(99) Lower, N.; Scott, A. P.; Moorer, A. Release of sex steroids in the water by roach. *J. Fish Biol.* **2004**, *64*, 16−33.
(100) Sorensen, P. W.; Hara, T. J.; Stacy, N. E.; Dulka, J. G. Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17-α, 20-$\beta$ -dihydroxy-4-pregnen-3-one. *J. Comp. Physiol., A* **1990**, *166*, 373−383.
(101) Schweinforth, H.; Laenge, R.; Miklautz, H.; Schauer, G. Umweltverhalten und aquatische toxizitaet von ethinylestradiol. *Stoffe mit endokriner wirkung im wasser*; Institute for Water Management; R. Oldenbourg Publishers: Munich, 1997; pp 39−54.
(102) Zillioux, E. J.; Johnson, I. C.; Kiparissis, Y.; Metcalfe, C. D.; Wheat, J. V.; Ward, S. G.; Liu, H. The Sheepshead Minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17α-ethinylestradiol. *Environ. Toxicol. Chem.* **2001**, *20*, 1968−1978.
(103) Johnson, A. C and Sumpter J. P. Removal of endocrine disrupting chemicals in activated sludge treatment works. *Environ. Sci. Technol.* **2001**, *35*, 4697−4703.
(104) Johnson, A. C.; Belfroid, A.; Di Corcia, A. Estimating steroid estrogen inputs to activated sludge treatment works and observations on their removal from the effluent. *Sci. Total Environ.* **2000**, *256*, 163−173.
(105) Baronti, C.; Curini, R.; D'Assenzo, G.; Di Corcia, A.; Gentili, A.; Samperi, R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ. Sci. Technol.* **2000**, *34*, 5059−5065.
(106) Lee, H.-B.; Peart, T. E. Determination of 17$\beta$-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *J. AOAC Int.* **1998**, *81*, 1209−1216.
(107) Nasu, M.; Goto, M.; Kato, H.; Oshima, Y.; Tanaka, H. Study on endocrine disrupting chemicals in wastewater treatment plants. *Water Sci. Technol.* **2001**, *43*, 101−108.
(108) Boon, A. G.; Hemfry, J.; Boon, K.; Brown, M. Recent developments in the biological filtration of sewage to produce high-quality nitrified effluents. *Water Environ. Manage.* **1997**, *11*, 393−412.
(109) Heberer, T. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *J. Hydrol.* **2002**, *266*, 175−189.
(110) Svensen, A.; Allard, A.-R.; Ek, M. Removal of estrogenicity in Swedish municipal sewage treatment plants. *Water Res.* **2003**, *37*, 4433−4443.
(111) Stumpf, M.; Ternes, T. A.; Wilken, R.-D.; Rodrigues, S. V.; Baumann, W. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brasil. *Sci. Total Environ.* **1999**, *225*, 135−141.
(112) Ternes, T. A.; Stumpf, M.; Mueller, J.; Haberer, K.; Wilken, R.-D.; Servos, M. Behaviour and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **1999**, *225*, 81−90.
(113) Cooper, P. F.; Downing, A. L. Milestones in the development of the activated sludge process over the past eighty years. *J. Chart. Inst. Water Environ. Manage.* **1998**, *12*, 303−313.
(114) Andersen, H.; Siegrist, H.; Halling-Sørensen.; Ternes, T. A. Fate of estrogens in a municipal sewage treatment plant. *Environ. Sci. Technol.* **2003**, *37*, 4021−4026.
(115) Walker, D. Estrogenicity and wastewater recycling: Experience from Essex and Suffolk Water. *J. Chart. Inst. Water Environ. Manage.* **2000**, *14*, 427−431.
(116) Drewes, J. E.; Heberer, T.; Redderen, K. Fate of pharmaceuticals during indirect potable reuse. *Water Sci. Technol.* **2002**, *46*, 73−80.
(117) Snyder, S. A.; Westerhoff, P.; Yoon, Y.; Sedlak, D. L. Pharmaceuticals, personal care products, and endocrine disrupters in

water: Implications for the water industry. *Environ. Eng. Sci.* **2003**, *20*, 449−469.
(118) Holbrook, R. D.; Novak, J. T.; Grizzard, T. J.; Love, N. G. Estrogen receptor agonist fate during wastewater and biosolids treatment processes: A mass balance analysis. *Environ. Sci. Technol.* **2002**, *36*, 4533−4539.
(119) Metcalfe, C. D.; Koenig, B. G.; Bennie, D. T.; Servos, M.; Ternes, T. A.; Hirsch, R. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environ. Toxicol. Chem.* **2003**, *22*, 2872−2880.
(120) Johnson, A. C.; Aerni, H.-R.; Gerritsen, A.; Gibert, M.; Giger, W.; Hylland, K.; Jürgens, M.; Nakari, T.; Pickering, A.; Suter, M. J-F.; Svenson, A.; Wettstein, F. E. Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. *Water Res.* **2005**, *39*, 47−58.
(121) Vader, J. S.; Van Ginkel, C. G.; Sperling, F. M. G. M.; de Jong, J.; de Boer, W.; de Graaf, J. S.; van der Most, M.; Stokman, P. G. W. Degradation of EE2 by nitrifying activated sludge. *Chemosphere* **2000**, *41*, 1239−1243.
(122) Joss, A.; Andersen, H.; Ternes, T.; Richle, P. R.; Siegrist, H. Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: Consequences for plant optimization. *Environ. Sci. Technol.* **2004**, *38*, 3047−3055.
(123) Hilton, M. J.; Thomas, K. V.; Ashton, D. *Targeted monitoring programme for pharmaceuticals in the aquatic environment*; R&D Technical Report P6-012/06/TR; Environment Agency: Bristol, U.K., 2003.
(124) Jones, O. A. H.; Voulvoulis, N.; Lester, J. N. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Res.* **2002**, *36*, 5013−5022.
(125) Kolpin, D. W.; Skopec, M.; Meyer, M. T.; Furlong, E. T.; Zaugg, S. D.; Urban contribution of pharmaceuticals and other organic waste water contaminants to streams during differing flow conditions: *Sci. Total Environ.* **2004**, *328*, 119−130.
(126) Williams, R. J.; Johnson, A. C.; Smith, J. J. L.; Kanda, R. Detailed investigation of steroid estrogen profiles along river stretches arising from sewage treatment works discharges. *Environ. Sci. Technol.* **2003**, *37*, 1744−1750.
(127) Schowanek, D.; Fox, K.; Holt, M.; Schroeder, F. R.; Koch, V.; Cassani, G.; Matthies, M.; Boeje, G.; Vanrolleghem, P.; Young, A.; Morris, G.; Gandolfi, C.; Feijtel, T. C. J. GREAT-ER: a new tool for management and risk assessment of chemicals in river basins Contribution to GREAT-ER#10. *Water Sci. Technol.* **2001**, *43*, 179−185.
(128) Holmes, M. G. R.; Young, A. R.; Gustard, A. G.; Grew, R. A region of influence approach to predicting flow duration curves in ungauged catchments. *Hydrol. Earth Syst. Sci.* **2002**, *6* (*4*), 721−731.
(129) Young A. R.; Grew R.; Holmes M. G. R. Low Flows 2000: a national water resources assessment and decision support tool. *Water Sci. Technol.* **2003**, *48*, 119−126.
(130) Schowanek, D.; Webb, S. Exposure simulation for pharmaceuticals in European surface waters with GREAT-ER. *Toxicol. Lett.* **2002**, *131*, 39−50.
(131) Anderson, P. D.; D'Aco, V. J.; Shanahan, P.; Chapra, S. C.; Buzby, M. E.; Cunningham, V. L.; Duplessie, B. M.; Hayes, E. P.; Mastrocco, F. J.; Parke, N. J.; Rader, J. C.; Samuelian, J. H.; Schwab, B. W. Screening analysis of human pharmaceutical compounds in US surface waters. *Environ. Sci. Technol.* **2004**, *38*, 838−849.
(132) Williams, R. J.; Jürgens, M. D.; Johnson, A. C. Initial predictions of the concentrations and distribution of 17b-E2, oestrone and ethinylE2 in 3 English rivers. *Water Res.* **1999**, *33*, 1663−1671.
(133) Huggett, D. B.; Cook, J. C.; Ericsson, J. F.; Williams, R. T. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. *Hum. Ecol. Risk Assess.* **2003**, *9*, 1789−1799.
(134) Clarke RT, Wright JF, Furse MT. RIVPACS models for predicting the expected macroinvertebrate fauna and assessing the ecological quality of rivers. *Ecol. Monit.* **2003**, *160*, 219−233.

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