Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment

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A rapidly increasing number of chemicals, or their degradation products, are being recognized as possessing estrogenic activity, albeit usually weak. We have found that effluent from sewage treatment works contains a chemical, or mixture of chemicals, that induces vitellogenin synthesis in male fish maintained in the effluent, thus indicating that the effluent is estrogenic. The effect was extremely pronounced and occurred at all sewage treatment works tested. The nature of the chemical or chemicals causing the effect is presently not known. However, we have tested a number of chemicals known to be estrogenic to mammals and have shown that they are also estrogenic to fish; that is, no species specificity was apparent. Many of these weakly estrogenic chemicals are known to be present in effluents. Further, a mixture of different estrogenic chemicals was considerably more potent than each of the chemicals when tested individually, suggesting that enhanced effects could occur when fish are exposed simultaneously to various estrogenic chemicals (as is likely to occur in rivers receiving effluent). Subsequent work should determine whether exposure to these chemicals at the concentrations present in the environment leads to any deleterious physiological effects. — Environ Health Perspect 103(Suppl 7):173–178 (1995)

Key words: vitellogenesis, fish, estrogenic contamination, water quality

Introduction

The issue of contamination of the aquatic environment by chemicals that can mimic the effects of estrogens and the consequences of this contamination is a very recent one. It has, of course, been known for a long time (*1*) that some environmentally persistent man-made chemicals can act as weak estrogens. However, two recent findings have raised some serious concerns about whether exposure of aquatic organisms to estrogenic chemicals contaminating the water might be leading to subtle, but potentially very serious, effects. One of these findings is the growing realization that a wide range of widely used chemicals, and sometimes their major degradation products, can act as weak estrogens (*2–4*). Further, it is likely that this list will lengthen in the foreseeable future, particularly if systematic screening of particular groups of chemicals is undertaken. The other finding is the observation that effluent from sewage-treatment works (STWs) entering rivers and lakes is estrogenic to fish (*5*); that is, the effluent contains a chemical, or more likely a combination of chemicals, which are absorbed by fish and "feminize" the fish, in the sense that they show physiological responses usually associated with high circulating concentrations of estrogens. Other observations of adverse physiological effects on wildlife, which are summarized by Guillette and colleagues (*6*), although not concerned with vitellogenesis, are also indicative of contamination of an aquatic environment by estrogenic chemicals.

In this article we discuss the current situation as we view it, attempt to assess the degree of the problem, and speculate on the consequences to fish of this estrogenic contamination of the aquatic environment. Before doing so, we describe in general terms the control of vitellogenesis in fish.

Vitellogenesis

Vitellogenesis is the process whereby yolky eggs are produced; it entails both the synthesis of vitellogenin by the liver and its uptake by growing oocytes, where it is stored as yolk to serve subsequently as the food reserve of the developing embryos. [For detailed reviews of vitellogenesis in fish, see Tyler (*7*) and Specker and Sullivan (*8*).] Here we are concerned only with the synthesis of vitellogenin.

It appears that expression of the vitellogenin gene, and hence the synthesis of vitellogenin, is (like many genes) under multihormonal control (*9–13*). However, estrogens, particularly 17β-estradiol, play the dominant role (Figure 1). Thus, plasma vitellogenin concentrations rise steadily during sexual maturation of female fish, concomitant with increasing 17β-estradiol concentrations (*14*), to reach tens of milligrams per milliliter in some species, at which time vitellogenin is the major blood protein. Such high concentrations of vitellogenin are required if the female fish is to grow an ovary that can contain thousands of (often very large) yolky oocytes and can comprise 25% of the body weight. Thus, plasma vitellogenin concentrations increase by around one millionfold during the seasonal reproductive cycle of female salmonid fishes. It is this huge range of potential vitellogenin concentrations that provides the ideal basis for a very sensitive bioassay of estrogen exposure of fish.

In contrast, very little if any vitellogenin can be detected in male fish (*15*), presumably because circulating estrogen concentrations in male fish are too low to trigger expression of the vitellogenin gene. Although the vitellogenin gene is normally silent, it can be induced if male fish are treated with estrogens. Exposure of male fish to various concentrations of both natural and synthetic estrogens has shown very pronounced dose–response effects (*16*) and has also shown that male fish are very sensitive to estrogens present in the water. For example,

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Abbreviations used: STWs: sewage treatment works; PCBs, polychlorinated biphenyls; PAHs, polycyclic aromatic hydrocarbons; PCDDs, polychlorinated dibenzodioxins; APEs, alkylphenol polyethoxylates; BCFs; bioconcentration factors.

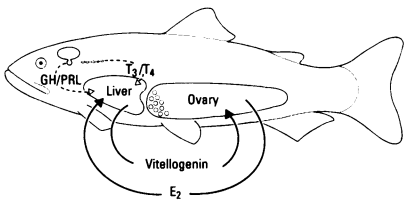

Figure 1. Hormonal control of vitellogenin synthesis. 17β-estradiol (E_2) from granulosa cells of ovarian follicles is considered to be the principal hormone that stimulates vitellogenin synthesis in hepatocytes. In amphibians, there is evidence that growth hormone (GH) and prolactin (PRL) from the pituitary gland and triiodothyronine (T_3) and thyroxine (T_4) from the thyroid glands enhance the effect of estradiol. The same may be true in fishes.

a concentration of the potent estrogen 17β-ethinylestradiol as low as 0.1 ng/l was enough to cause a significant increase in the plasma vitellogenin concentration after only a relatively brief exposure (*5*).

Estrogens act via specific receptors; they diffuse through the cell and nuclear membranes to bind to nuclear estrogen receptors. The detailed mechanisms underpinning their mode of action are under intensive study (*17*). Estrogen receptors are very similar in fish and mammals (*18,19*), which explains why chemicals that act as estrogens do so throughout the vertebrates. The liver of fish, particularly female fish, contains high concentrations of estrogen receptors (*20,21*), which accounts for its ability to synthesize large amounts of vitellogenin when stimulated by estrogen.

Field Studies at Sewage-treatment Works

The stimulus for this work was the discovery that approximately 5% of roach (*Rutilus rutilus*, a common cyprinid), living just downstream from where effluent from an STW entered a river, were hermaphrodites. The usual incidence of hermaphroditism is thought to be extremely small, so small that the finding of just one hermaphrodite fish is often reported in the literature (*22,23*); hence, concern was expressed that something in the effluent might have been responsible for the increased incidence of hermaphroditism. Because sex differentiation in fish is initially labile and can be affected by exposure to steroids (*24,25*), it was suggested that a steroid or steroidlike substance was present in the effluent. To assess this hypothesis, we placed caged male trout directly in the effluent channel at just one STW; that is, the fish were placed in 100% effluent, not in the river downstream from where the effluent entered. Other fish were maintained in springwater as controls. Blood samples were taken after 1, 2, and 3 weeks of exposure, and their contents of vitellogenin were estimated by specific radioimmunoassay (26). The results (Figure 2) demonstrated highly elevated concentrations of vitellogenin in the fish exposed to effluent; even a 1-week exposure was large enough to cause the vitellogenin concentration to increase over 300-fold. Although there may be other explanations, we consider the only likely explanation to be that the effluent contained a chemical, or a mixture of chemicals, that was estrogenic to trout.

Due to the possible consequences of this estrogenic contamination to wildlife living in the river and consumers of water abstracted from the river, we decided to conduct a nationwide survey to assess whether this was a local or general phenomenon. In the nationwide survey, 28 sites covering all 10 Water Authority areas were investigated; tests were conducted throughout England and Wales. At each of the STWs, a cage containing 20 to 30 trout was placed directly in the flow of effluent from the site. Five separate sites (usually commercial trout farms) were chosen as controls on the basis that their water supplies were thought to be uncontaminated by sewage effluent. The fish were left on site for 2 to 3 weeks; after this, they were anesthetized and a blood sample was collected for vitellogenin determination.

At 13 of the 28 sites used for the survey, the trout were unable to survive for the duration of the experiment. This was due mainly to deterioration in effluent quality at some time during the period of the survey. At all 15 sites where fish survived, there was a pronounced increase in the plasma vitellogenin concentration (Figure 3); $p < 0.001$ when compared to appropriate controls, in all cases. The plasma vitellogenin concentrations in the control groups varied because some were all male (and hence had very low vitellogenin concentrations), whereas other groups were immature but of mixed sex (and hence had somewhat higher mean vitellogenin concentrations). However, irrespective of the source of the fish, plasma vitellogenin concentrations in trout held in effluent from STWs were always much higher than in their respective control trout.

There was variability in the degree of response—from 500-fold (Site 11) to over 50,000-fold (Site 8). The variability was probably caused by a number of factors, which included the composition of the effluent (i.e., which estrogenic chemicals were present and at what concentrations), the time on site, the water temperature during the trials, and the age and sex of the test fish. The STWs chosen all handled a large amount of domestic

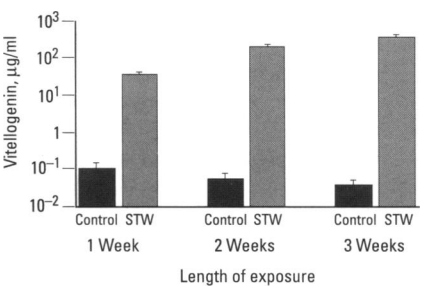

Figure 2. The effect of effluent from a sewage-treatment works on the plasma vitellogenin concentration of male rainbow trout. One cage containing 20 male trout was placed directly in the effluent channel of a sewage-treatment works (STW), and another (control) was maintained in a laboratory supplied with high-quality spring water. Plasma samples were collected after 1, 2, and 3 weeks and assayed for vitellogenin. Exposure to effluent caused a pronounced increase in the plasma vitellogenin concentration ($p < 0.001$ at all times). Results are mean ± SEM ($n = 20$).

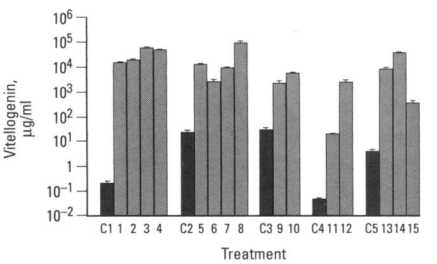

Figure 3. Results of a nationwide survey to assess whether effluents from all sewage-treatment works are estrogenic. Rainbow trout in cages were placed in the effluent channels of 15 different sewage-treatment works located throughout England and Wales (coded 1 to 15). The trout were obtained from five different fish farms and in some cases were all male (C1 and C4), but in other cases only mixed-sex immature trout were available (C2, C3, and C5). Caged trout maintained at the fish farms, all of which were supplied with high-quality water, served as controls (C1 to C5). The fish were maintained in the cages for between 2 and 3 weeks before a plasma sample was collected and subsequently assayed for vitellogenin. In all 15 cases, trout maintained in effluent had much higher plasma vitellogenin concentrations than their respective controls ($p < 0.001$ when compared to appropriate controls, in all cases). Note that the vitellogenin concentrations are expressed on a log scale. Results are mean ± SEM ($n = 20$ in all cases).

waste but varied in the amount and composition of the industrial waste water they also received. The only viable explanation for these results appears to be that effluents from all STWs in the United Kingdom are estrogenic to fish. We are aware of similar results from one site in France where eels were used as the test fish (R Billard, personal communication); this suggests that the phenomenon is not confined to the United Kingdom but is likely to occur internationally. The nature of the estrogenic compound or compounds in effluent is presently unknown; there are many possibilities.

Our most recent field studies have focused on the situation in rivers and reservoirs, rather than in effluent. Thus, we have placed trout in cages along entire river systems and in reservoirs of various sizes. We found that there was an effect (elevated vitellogenin concentrations) throughout one entire river system; the magnitude of the effect was small except at the five places where effluent from STWs entered the river, when the effect was very pronounced. We have not observed any estrogenic activity in any reservoirs (Harries et al., unpublished observations).

Estrogenic Chemicals in the Aquatic Environment

A surprisingly wide range of chemicals are estrogenic, including natural chemicals, such as phytoestrogens and mycoestrogens, and man-made chemicals, such as some organochlorine pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs), surfactants, and plasticizers (*27*). In many cases the estrogenic activity of these chemicals has been discovered accidentally (*2,3*). There has not been a systematic investigation of any groups of chemicals to assess which ones (if any) are estrogenic. Hence, on the basis that over 60,000 man-made chemicals are in regular use (and an unidentified number of their degradation products are also present in the aquatic environment), it is likely that other chemicals or groups of chemicals will prove to be estrogenic.

Many of these man-made chemicals are widely used in major industries such as agriculture, the petrochemical industry, the plastics industry, and the soap and detergent industry. Very high volumes of some of these estrogenic chemicals are used, which leads to the appearance of significant amounts in the aquatic environment. For example, around 300,000 tons of alkylphenol–polyethoxylates are used annually; about 60% of these end up in the aquatic environment where they are degraded to environmentally persistent estrogenic chemicals (*28*). It is also possible that some synthetic estrogens, particularly ethinylestradiol, may contaminate the aquatic environment. This very potent estrogen is widely used as a contraceptive throughout the world. However, it is excreted by women almost exclusively in conjugated forms, which are considered biologically inactive; both ethinylestradiol glucuronide and sulfate are inactive as estrogens in trout (our unpublished results). Notwithstanding, it has been claimed that ethinylestradiol can be detected in river water in the United Kingdom (*29*).

When trying to assess the impact of this contamination of the aquatic environment by estrogenic chemicals, two factors of major importance are the estrogenic potencies of these chemicals and their concentrations in the environment. It is impossible to provide a realistic estimate of the concentration of any estrogenic chemical in the aquatic environment, because reported values (when they are available) vary so much. This variability is understandable, given that different techniques have been used and different samples (such as influent, effluent, river water, groundwater, etc.) have been analyzed in different areas of the world. A long list of all published concentrations of all chemicals known to be estrogenic would be of very little use. As an example of the difficulties, consider the situation with alkylphenol–polyethoxylates (APEs) and their degradation products (*30*). Concentrations of APEs in the influent to STWs are in the milligram per liter range, as they are in the effluent from specific industries such as pulp mills and textile works. Effluent from STWs contains hundreds of micrograms per liter, whereas river-water concentrations of the major degradation products are in the tens of micrograms per liter range or less. Even drinking water contains detectable amounts of these chemicals (*31*). Thus, specifying exactly what concentration a fish is exposed to is impossible and may not even be particularly meaningful, because the concentration in the fish is what is important.

All of the environmental estrogens discovered to date are relatively weak estrogens. Their potencies vary, but most are many orders of magnitude (often 3 or 4) less potent than 17β-estradiol. Although relative potencies probably depend on the assay system used to assess them, it is fair to say that these chemicals are weak estrogens. Nevertheless, they appear to possess full activity and interact with the estrogen receptor in exactly the same manner as the natural ligand, 17β-estradiol (*4*).

Laboratory Investigations of Estrogenic Chemicals

To assess the efficacy of estrogenic chemicals to stimulate synthesis of vitellogenesis, we have used an *in vitro* system based on primary cultures of hepatocytes from trout (*32*).

The Potency of Individual Chemicals

We have tested a representative range of chemicals that have been reported to be estrogenic, usually in mammalian systems, to determine whether they are also estrogenic to trout, and if so, assess approximately how potent. Some of the results obtained are shown in Figure 4. All of the chemicals tested stimulated synthesis of vitellogenin in a dose-dependent manner (at very high concentrations, one of the chemicals was toxic to the cells); all were fairly weakly estrogenic. Nevertheless, some of these chemicals stimulated vitellogenin synthesis at concentrations reported to be present in the aquatic environment. Further, this assay system is a relatively insensitive one because the naive cells from liver tissue of male trout are exposed for only 2 days before the response is assessed; in natural situations fish are subjected continuously to any estrogenic chemicals present in the water.

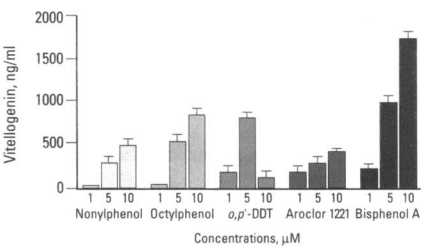

Figure 4. Estrogenic activity of some environmentally persistent chemicals. The estrogenic activity of these chemicals was investigated by assessing their ability to stimulate vitellogenin synthesis in cultured hepatocytes obtained from male rainbow trout. Nonylphenol and octylphenol are degradation products of widely used surfactants; *o,p'*–DDT is a pesticide; Aroclor is used primarily in electrical capacities and transformers; and bisphenol A is a plasiticizer. All five chemicals are aquatic pollutants. Note that in each case the stimulatory effect was dose related, with the exception of *o,p'*–DDT in which the highest concentration tested (10 μM) was toxic to the hepatocytes. Results (mean ± SEM; $n = 6$) are expressed as the vitellogenin concentration in the culture medium after a 2-day exposure to the chemicals.

Effects of Mixtures of Chemicals

In the real world, fish are unlikely to be exposed to just one estrogenic chemical, but instead are likely to live in water that contains many different estrogenic chemicals. This is particularly so if the estrogenic activity in the water originated from STW effluent because this is a very heterogeneous mixture of chemicals. Thus, ideally we need to know how a fish responds to a mixture of estrogenic chemicals rather than to an individual chemical. However, it is very difficult to mimic the real world, primarily because we do not know at present which chemicals, in what concentrations, contribute to the estrogenic activity of effluent (this is likely to vary depending on the site). Nevertheless, it is possible to assess the effect of prepared mixtures of chemicals of known composition. Essentially, the question being addressed is "Can the response to a mixture of estrogenic chemicals be different from the response to a single chemical?" We have conducted some preliminary experiments to try to answer this question. Some representative results are shown in Figure 5; relatively small responses were obtained when hepatocytes were exposed to submaximal concentrations of five different chemicals, but a considerably greater response was obtained when hepatocytes were treated with a mixture of the five chemicals. Thus, it is possible that fish living in an estrogenic environment might show a more pronounced "feminizing" response when exposed to the mixture of chemicals than they would if they were exposed to a single estrogenic chemical at the same concentration.

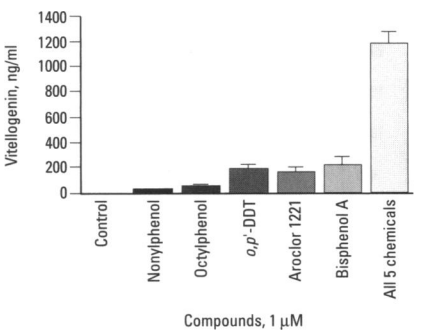

Figure 5. Enhanced effect of a mixture of weakly estrogenic chemicals. The estrogenic activity of five chemicals was assessed by their ability to stimulate vitellogenin synthesis in cultured hepatocytes of rainbow trout. After 2 days of exposure to the chemicals, either individually or together (all five chemicals, each at a concentration of 1 μM, were present in the culture medium), the vitellogenin concentration in the medium was determined (results are expressed as mean ± SEM). Note that when all five chemicals were present in the culture medium, a situation analogous to that in the aquatic environment when many different estrogenic chemicals are likely to be present simultaneously, there was a much greater effect ($p<0.001$) than in response to any individual chemicals.

Phylogenetic Considerations

It is important to know whether a chemical that mimics the effects of estrogen in one species will do so in others; that is, is there any species specificity in the response to estrogenic chemicals? Generally, the answer appears to be no. For example, White et al. (*4*) showed that a number of different alkylphenolic compounds (derived from the degradation of one class of nonionic surfactants) were estrogenic to fish, avian, and mammalian cells. Further, the relative potencies (to each other and to 17β-estradiol) were approximately the same, irrespective of the origin of the cells. However, one recent report (*33*) has claimed that several DDT derivatives and PCB mixtures do not bind to the estradiol receptor in one species of fish, the spotted seatrout (*Cynoxion nebulosus*) and, hence, would presumably not be estrogenic in this species. To determine if this situation is common, we have done preliminary receptor-binding studies using estradiol receptor preparations prepared from the livers of rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*). These fish are classified into two distinctly different orders; the more primitive salmoniformes containing the trout, and the cypriniformes containing the roach. Our findings showed that three quite distinct estrogenic chemicals, namely nonylphenol, genistein, and *o,p'*-DDT, are equally estrogenic in both species (Figure 6). Thus, there was no evidence of species specificity when these two species were compared; however, there are over 20,000 species of fish, so care should be taken in generalizing a conclusion reached from comparing just two! Nevertheless, most evidence supports the idea that if a chemical is estrogenic in one species it will be in all others. The high degree of conservation of the structure of the estradiol receptor, particularly in the parts involved in ligand binding and transactivation (*18,19*), support this notion.

Bioaccumulation

Most of the estrogenic chemicals discussed above are lipophilic and hydrophobic and, hence, have a strong tendency to bioconcentrate and bioaccumulate in aquatic organisms, both plants and animals. For example, bioconcentration factors (BCFs) for many PCBs and other organochlorine pesticides in fish are between 1000 and 100,000 (*34*), leading to concentrations in fish of the order of micrograms per gram of fat. Similarly, estrogenic PAHs and PCDDs bioconcentrate to a similar degree, as does nonylphenol, for which BCFs in fish between 13 and 1300 have been reported (*35,36*). One consequence of this bioaccumulation is that chemicals that are weakly estrogenic *in vitro*, as is the case with most if not all of the environmental estrogens, may be active *in vivo* at considerably lower concentrations.

Different organisms will bioconcentrate different estrogenic chemicals to different degrees. Even within a single organism, the bioconcentrated compound is unlikely to be equally spread through all tissues; it is much more likely to be preferentially concentrated in a few tissues, such as fat. What happens to these compounds once bioconcentrated within an organism is essentially unknown; they may be physiologically inactive while stored in adipose tissue, but when this fat is mobilized (which often occurs during reproduction), the compounds may be freed to act elsewhere or they may be metabolized into other compounds that may or may not be active as estrogens.

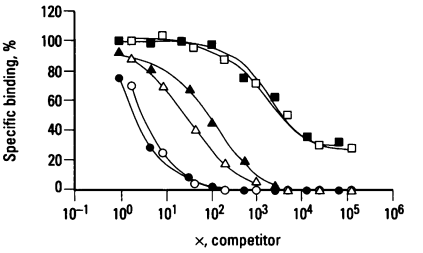

Figure 6. A comparison of the estrogenic activity of a phytoestrogen (genistein) and nonylphenol (a degradation product of some surfactants) in two different species of fish, the rainbow trout (open symbols) and roach (closed symbols). Estrogenic activity was assessed by the ability of the chemical to compete with tritiated 17β-estradiol for binding to estrogen receptors in receptor-binding assays. The receptor preparations were prepared from liver tissue. Results are expressed as percent specific binding of tritiated estradiol in the presence of increasing concentrations of chemical; for example, 10^3 means that the concentration of the test chemical was 1000 times higher than the concentration of tritiated estradiol. The test chemicals were 17β-estradiol (trout, ○; roach, ●), genistein (trout, △; roach, ▲), and nonylphenol (trout, □; roach, ■).

Consequences to Fish of Estrogenic Contamination of Water

This is the most important issue: what, if any, are the consequences to aquatic

organisms of living in a "sea of estrogen"? The simple answer is that we do not know. To date, there is no evidence to suggest that fish, or any other aquatic organisms, are affected adversely by living in and bioaccumulating estrogenic chemicals. The possible effects are almost endless because of the multitude of roles played by endogenous estrogens in normal physiology; these range from sex differentiation at the egg/embryo stage to sexual maturation of adults (*24,25,37*). The most likely process to be affected is reproduction, because estrogens are pivotal to successful reproduction, particularly in females where they play major roles in controlling gonadotrophin secretion (*38*), vitellogenesis, synthesis of the eggshell proteins, and many other processes (*39*).

It is established that fish placed in undiluted effluent (before it enters a river or lake) show rapid and pronounced physiological responses; males respond as though they have been feminized (*5*). Because effluent from STWs can contribute a very significant amount of the flow of U.K. rivers—in periods of low rainfall, this is often over 50%—it is likely that effects will be noticed in some river systems. Indeed, our recent data (J Harries, unpublished data) show that entire river systems can be estrogenic to fish. We cannot presently ascribe any deleterious consequences to the unnatural synthesis of vitellogenin reported in fish, but it seems likely that many processes regulated by estradiol are affected (vitellogenin synthesis being only one of them); it is probable that these changes from the normal pattern will adversely affect reproduction. Only a thorough study of wild populations of fish can directly answer the question of whether there are serious consequences to aquatic wildlife from the widespread contamination of the aquatic environment by estrogenic chemicals.

Conclusions

A rapidly increasing number of chemicals, or their degradation products, are being recognized as estrogenic, albeit usually weakly so. These chemicals enter waterways via the effluent from STWs (and possibly from other sources of effluent) and are absorbed and bioaccumulated in sufficient concentrations to induce physiological responses in fish, which are indicative of exposure to estrogens. The consequences of this exposure and the responses to it are unknown presently, but they could include adverse effects on physiological processes, particularly reproduction. Further work is required to elucidate which chemicals are estrogenic and to what degree and, particularly, to explain whether exposure to these chemicals at the concentrations present in the environment leads to deleterious physiological effects.

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