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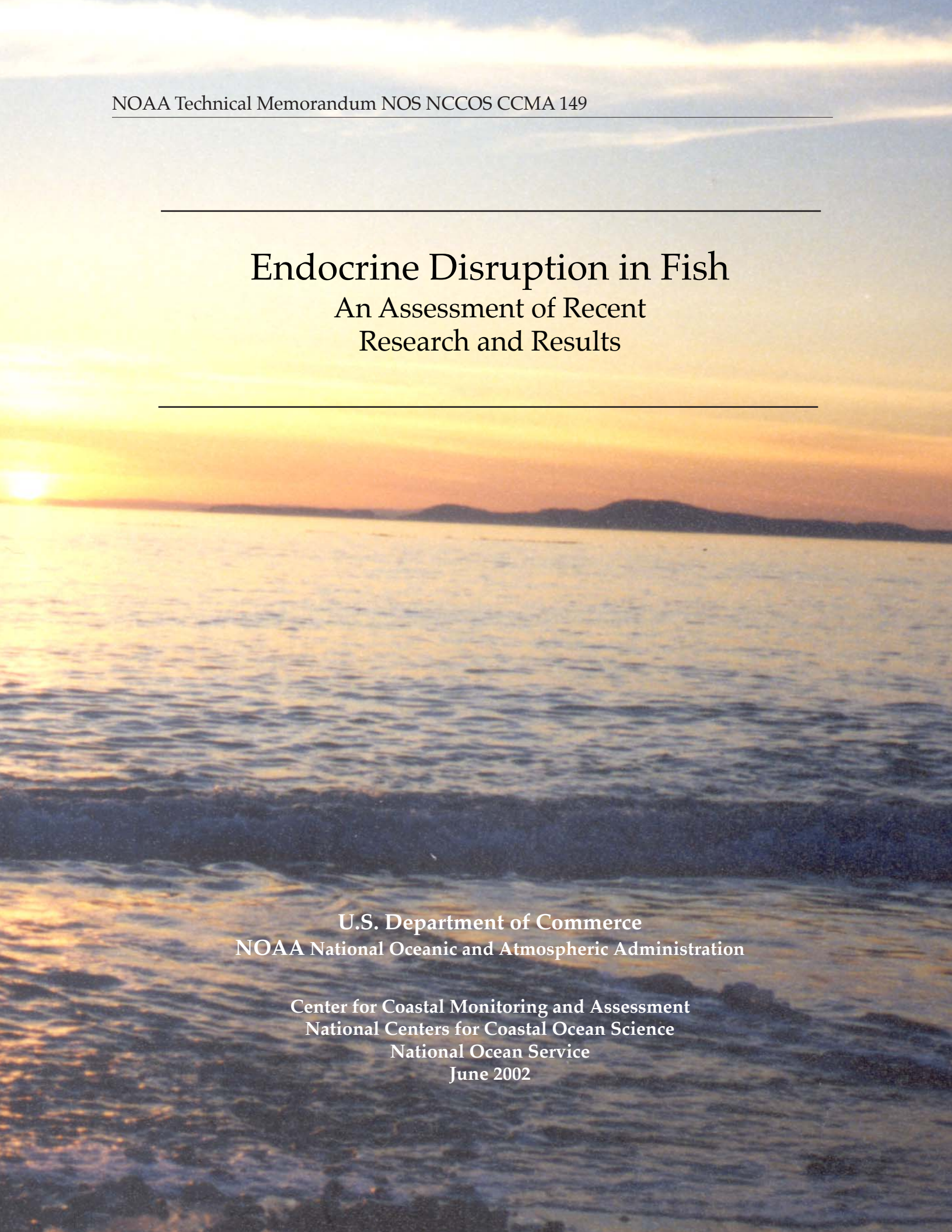

# Endocrine Disruption in Fish

## An Assessment of Recent Research and Results

**U.S. Department of Commerce**
**NOAA National Oceanic and Atmospheric Administration**

**Center for Coastal Monitoring and Assessment**
**National Centers for Coastal Ocean Science**
**National Ocean Service**
**June 2002**

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National Oceanic and Atmospheric Administration
U.S. Department of Commerce
N/SCI-1, SSMC4
1305 East/West Highway
Silver Spring, MD 20910

Notice

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# Endocrine Disruption in Fish
## An Assessment of Recent Research and Results

Anthony S. Pait
*National Oceanic and Atmospheric Administration*

Judd O. Nelson
*University of Maryland*


Silver Spring, Maryland
June 2002



This report should be cited as:
Pait, A.S. and J. O. Nelson. 2002. Endocrine Disruption in Fish: An Assessment of Recent Research and Results. NOAA Tech. Memo. NOS NCCOS CCMA 149. Silver Spring, MD: NOAA, NOS, Center for Coastal Monitoring and Assessment 55pp.

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# Endocrine Disruption in Fish: An Assessment of Recent Research and Results

***Abstract.*** *This report provides an assessment of recent investigations into endocrine disruption in fresh and saltwater species of fish. Most work to date has concentrated on reproductive endocrine disruption. Laboratory studies have shown a variety of synthetic and natural chemicals including certain industrial intermediates, PAHs, PCBs, pesticides, dioxins, trace elements and plant sterols can interfere with the endocrine system in fish. The potency of most of these chemicals, however, is typically hundreds to thousands of times less than that of endogenous hormones. Evidence of environmental endocrine disruption ranges from the presence of female egg proteins in males and reduced levels of endogenous hormones in both males and females, to gonadal histopathologies and intersex (presence of ovotestes) fish.*

*Overt endocrine disruption in fish does not appear to be a ubiquitous environmental phenomenon, but rather more likely to occur near sewage treatment plants, pulp and paper mills, and in areas of high organic chemical contamination. However, more widespread endocrine disruption can occur in rivers with smaller flows and correspondingly large or numerous wastewater inputs.*

*Some of the most severe examples of endocrine disruption in fish have been found adjacent to sewage treatment plants. Effects are thought to be caused primarily by natural and synthetic estrogens and to a lesser extent by the degradation products of alkylphenol polyethoxylate surfactants. Effects found in fish near pulp and paper mills include reduced levels of estrogens and androgens as well as masculinization of females, and has been linked to the presence of β-sitosterol, a plant sterol. Effects seen in areas of heavy industrial activity typically include depressed levels of estrogens and androgens as well as reduced gonadal growth, and may be linked to the presence of PAHs, PCBs, and possibly dioxins. At this time, however, there is no clear indication that large populations of fish are being seriously impacted as a result of endocrine disruption, although additional work is needed to address this possibility.*

## INTRODUCTION

Recently, concern has arisen that certain environmental contaminants as well as some naturally occurring compounds have the potential to impact the endocrine system which regulates vital life processes, including development, growth, metabolism and reproduction. Research on the identification and effects of endocrine-active compounds has become an important area of human and environmental health research. In this report, compounds that either mimic or antagonize the action of naturally occurring hormones are termed endocrine disrupters or endocrine disrupting compounds (EDCs).

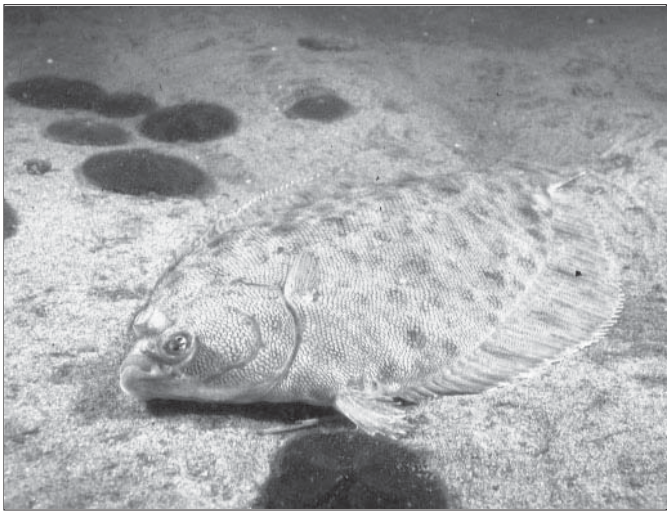
A number of definitions currently exist for EDCs. Kavlock *et al.* (1996) (see inset) proposed a definition that includes not only the primary ways EDCs are thought to interfere with normal functioning of the endocrine system, but also some of the effects EDCs can have on an organism. In 1996, the European Commission defined an endocrine disrupter as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" (European Commission, 1996). As with Kavlock *et al.* (1996), this definition stresses that adverse health effects occur as a result of one or more changes in endocrine function. The National Research Council adopted hormonally active agents as a more neutral mechanistic descriptor, and defined them broadly as substances that possess hormonelike activity, regardless of structure (NRC, 1999).

> *Endocrine disrupters have been defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock et al., 1996).*

Chemicals that can mimic or antagonize the effects of endogenous hormones could potentially have serious effects not only on the development and well being of an individual organism, but perhaps more importantly on the ability of that organism to reproduce,

and its offspring to survive and eventually reproduce. In the aquatic environment, the ability of an EDC to affect an individual or population would depend on a number of factors, including the potency or efficacy of the EDC, its concentration, duration of exposure, bioconcentration potential, presence of other EDCs, life stage exposed, season, other environmental stressors present (e.g., temperature, salinity, and other contaminants), and mobility of the individual.

Many of the investigations into EDCs in the aquatic environment have involved fish because of similarities in the endocrine system to higher vertebrates (Bond, 1979), a demonstrated sensitivity to EDCs in both laboratory and field investigations, ease of working with fish, and the fact that wild populations in a number of locations have exhibited effects thought to be associated with exposure to EDCs. Kime (1999) has also pointed out that both sexes produce large numbers of gametes which can be readily counted or examined for malformations, hatch rates are readily determined, and the resultant offspring can be easily monitored for developmental abnormalities or subsequent reproductive problems.



*Winter flounder camouflaged to match surroundings. Image courtesy of NOAA/NOS.*

*Historical Perspective.* The presence and effects of EDCs is not a new phenomenon. As early as 1949, it was known that crop dusters handling dichlorodiphenyltrichloroethane (DDT) frequently had reduced sperm counts (Patlak, 1996). DDT was shown to produce characteristically estrogenic responses in the reproductive tracts of rats and birds (Bitman and Cecil, 1970). The tragic use of the synthetic estrogen diethylstilbestrol, or DES, by pregnant women from the late 1940s until 1971 to help prevent miscarriages resulted in infertility and increased rates of vaginal clear cell adenocarcinomas in daughters (Colborn *et al*., 1993). In herring gulls, studies beginning in the mid-1960s in Lake Michigan suggested that environmental contaminants were adversely and, in some cases, severely affecting hatching success (Keith, 1966).

More recently, however, the number of compounds identified as suspected or confirmed endocrine disrupters has increased substantially and includes industrial intermediates, such as 4-nonylphenol, bisphenol-A, and the phthalate ester plasticizers, as well as classic contaminants such as the polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, certain pesticides, and even a number of trace elements (Knudsen and Pottinger, 1999). In plants, naturally occurring compounds termed phytoestrogens are also known to have hormonelike properties (Mitksicek, 1995).

The identification of EDCs has come through many avenues of investigation, including chance discoveries while working with various chemicals and cell lines to detailed structure/activity investigations that test specific compounds for their ability to produce a response in cell lines or whole animals. Responses used to detect endocrine active compounds typically include cell proliferation (i.e., in breast cancer cell cultures), binding to the estrogen receptor, and production of specific proteins in cell cultures or whole animals.

*Current Effects*. Examples of apparent reproductive endocrine disruption in aquatic organisms are, in some areas, disturbingly numerous and include intersex fish (male and female gonadal characteristics in gonochoristic or normally separate sex fish) (Jobling *et al.* 1998), elevated levels of a female egg protein in male fish (Lye *et al.*, 1998; Janssen *et al.*, 1997; Folmar *et al.* 1996), and degeneration of gonadal tissue (Lye *et al.*, 1998; Janseen *et al.*, 1997). In Lake Apopka in Florida, a dramatic decline in the alligator population has been attributed to a spill of DDT, dicofol, and possibly 1, 2-dibromo-3-chloropropane (Guillette *et al.*, 1996; Semenza, 1997; Risebrough, 1999).

Feminization and masculinization as well as embryonic deformities in gulls have also been linked to PCBs and halogenated aromatic hydrocarbons (Fox, 1993). Fortunately, it appears that some of the effects seen in fish-eating birds in the Great Lakes are on the decrease (Fox, 1993).

*EDC Debate.* There has been a vigorous debate within the scientific and regulatory communities regarding the extent of endocrine disruption in the environment, the importance of synthetic chemicals relative to naturally occurring EDCs, and the relevance of endocrine disrupters compared to an organism's internal suite of hormones. Some argue that wildlife populations have already been significantly impacted by EDCs, resulting in decreased fertility in fish and shellfish, demasculinization and feminization of male fish, birds, and mammals, and masculinization of female fish and birds (Colborn *et al.*, 1997). Others, however, are doubtful that EDCs in most situations are likely to have a major impact. They point out that EDCs typically bind to receptors (e.g., the estrogen receptor) hundreds or thousands of times more weakly than endogenous hormones. An argument has also been made, at least in the case of environmental estrogens, that the occurrence of natural and synthetic antiestrogens in the environment would act to negate the effect of environmental estrogens (Stone, 1994). While there is considerable and ongoing debate on the occurrence and significance of endocrine disruption, there is little disagreement over the need to better understand the impacts EDCs may be having in the environment.

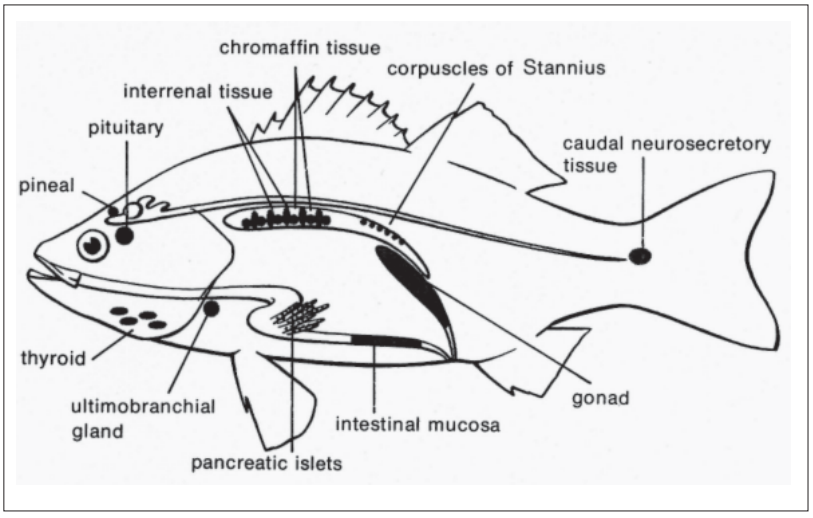



Figure 1. Schematic of typical endocrine system in teleost fish. From Bond (1979). Reprinted by permission.

## ENDOCRINE SYSTEM: OVERVIEW

The endocrine system in fish consists of various glands located throughout the body which synthesize and secrete hormones to regulate an array of biological processes (Figure 1 and Table 1). For example, the thyroid gland secretes the hormones thyroxine ($T_4$) and triiodothyronine ($T_3$), which are believed to aid fish in adapting to changes in temperature and to osmotic stress. The pituitary gland in fish secretes a number of hormones which affect growth, osmoregulation, lipid metabolism and reproductive development and behavior, as well as controlling other endocrine glands (Bone *et al.*, 1995). The corpuscles of Stannius secrete hypocalcin which is thought to be involved in calcium homeostasis and may also be involved in controlling the ratio of calcium to sodium and potassium in the plasma (Bone *et al.*, 1995). As in humans, the fish pancreas secretes insulin which aids in glucose permeability. Secretion of glucagon, also by the pancreas, enables increased glycogen and lipid metabolism.

Although most of what is currently known about the effects of EDCs involves reproduction and reproductive behavior, other areas of the endocrine system, such as the thyroid, may also be targets for EDCs. Investigations into possible effects on other targets are just beginning. At an even more basic level, there is still some uncertainty as to the structure and function of some of the naturally occurring teleost hormones and the glands themselves (Bone *et al.*, 1995), making an assessment of the impact of endocrine disrupting compounds, other than on reproduction, even more difficult.

*Pituitary-Hypothalamus-Gonadal Axis*

One of the most studied pathways that can be affected by EDCs is the pituitary-hypothalamus-gonadal axis, which will be used to illustrate how EDCs can affect the endocrine system in fish. The release of gonadotropin releasing hormone (GnRH) from the hypothalamus in response to a series of environmental cues in fish results in the production and release of gonadotropin hormones (GTH) from the pituitary gland (Figure 2). The gonadotropins released into the systemic circulation elicit increased androgen and estrogen production by the gonads

(Bone *et al.*, 1995). The major estrogen in female fish, 17β-estradiol ($E_2$), is produced primarily in the ovary by the follicular cells. In addition to their importance in eliciting reproductive behavior and the development and maintenance of secondary sex characteristics, the estrogens and androgens are involved in the production of gametes (Bone *et al.*, 1995).

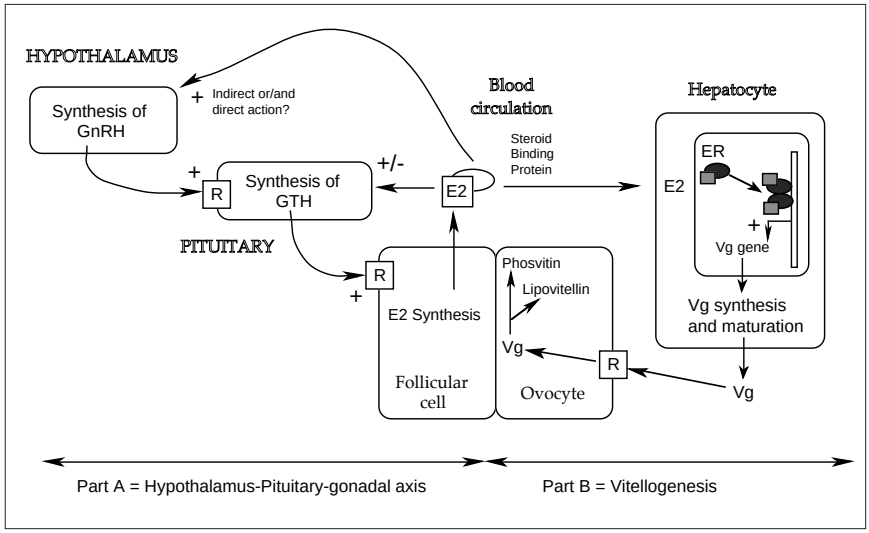



Figure 2. Pituitary-hypothalamus-gonadal axis and the action of estradiol. From Drean (1994). Reprinted by permission.

In oviparous or egg-laying fish, as in other egg-laying animals, the release of $E_2$ from the ovary leads to the synthesis of large amounts of vitellogenin by the hepatocytes (liver cells) (Figure 2). This high density lipoprotein, the precursor of egg yolk, is then transported from the liver via the circulatory system and incorporated into developing oocytes (Anderson *et al.*, 1996a).

Although estrogens are typically associated with females and androgens with males, that demarcation may not be a rigid one (Sharpe, 1997). Research indicates that both male and female vertebrates produce and use estrogens and androgens. It has become fairly well accepted that a minor role for estrogens exists in males, for example, in the regulation of GTH secretion by the pituitary gland. It is also possible that proper levels of estrogens in males may have more widespread effects, and may even be essential for fertility (Sharpe, 1997). Exposure of an organism, however, to levels of natural hormones or EDCs which overwhelm or interfere with the proper functioning of the endocrine system has the potential to seriously affect the health of an organism and its progeny.

Table 1. Selected endocrine glands and hormonal action in fish.

| Gland/Hormone | Target Organ | Effect(s) |
|---|---|---|
| ***Pituitary*** | | |
| Prolactin | various | osmoregulation, reproduction, growth, lipid metabolism, metabolism |
| Growth hormone | various | stimulation of growth |
| Thyroid stimulating hormone | thyroid | stimulation of thryroxine |
| Gonadotrophic hormone | gonads | stimulation of gonads |
| Isotocin, mesotocin | blood vessels | constricts gill blood vessels, systemic vasodilation |
| ***Thyroid*** | | |
| Thyroxin | many | adaptation to environmental changes such as temperature or osmotic stresses |
| Calcitonin | gills and kidney | regulation of calcium metabolism |
| ***Corpuscles of Stannius*** | | |
| Hypocalcin | gills | calcium homeostasis |
| ***Pancreas*** | | |
| Insulin | all cells | increases glucose permeability |
| Glucagon | all cells | glycogen and lipic metabolism |
| ***Chromaffin tissue*** | | |
| Adrenaline | circulation | gill vasodilation, system vasoconstriction |
| Noradrenaline | circulation | increase heart and glucose metabolism |
| ***Intrarrenal*** | | |
| Corticosteroids | gills, kidney | stress response, osmoregulation |
| ***Gonads*** | | |
| Androgens and estrogens | many, including brain | reproductive status and behavior; also of other fish (as pheromones) |

Adapted from Bone et al., 1995. Reprinted by permission.

## MODE OF ACTION

Endocrine disrupting compounds are believed to exert their influence by: 1) mimicking the effects of endogenous hormones, such as the estrogens and androgens; 2) antagonizing the effects of endogenous hormones; 3) altering the pattern of synthesis and metabolism of normal hormones; and 4) modifying hormone receptor levels (Soto *et al.*, 1995). EDCs may also interfere with

the binding proteins that act to transport endogenous hormones to their destination.

The most frequently studied and best understood type of EDCs are those that mimic estrogens (Gillesby and Zacharewski, 1998; Kime, 1999). A current model for the binding and action of $E_2$ is shown in Figure 3. Estradiol produced by the ovaries and transported via the circulatory system is passively taken up by the cell (e.g., hepatocyte) and then crosses the nuclear membrane. The unliganded estrogen receptor (ER) is maintained in an inactive conformation through interactions with a number of proteins, primarily heat shock proteins Hsp 59, 70, and 90 (Gillesby and Zacharewski, 1998). Following the binding of estrogen to the receptor, the heat shock proteins dissociate allowing the ER to change its conformation to the active form.

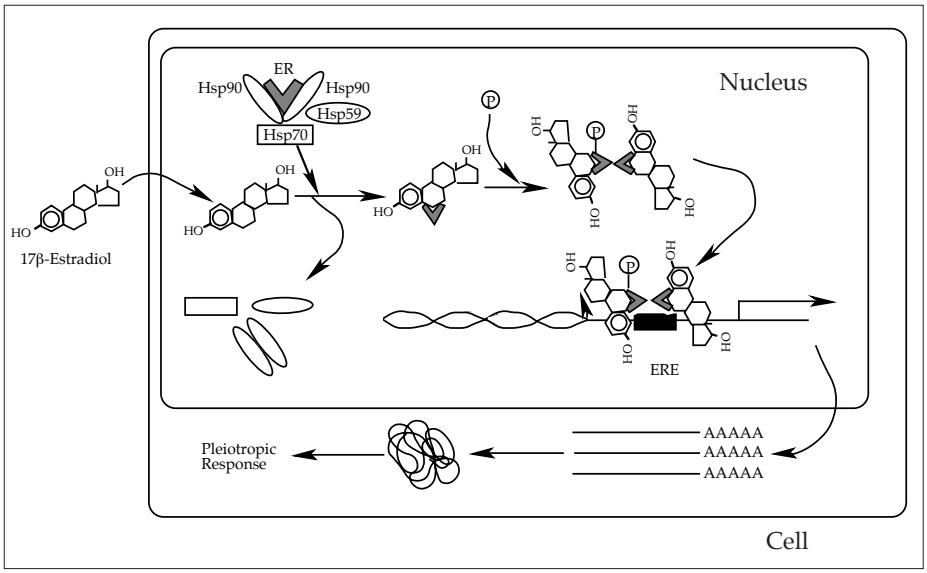



Figure 3. Mechanism of action of the estrogen receptor. From Gillesby and Zacharewski (1998). Reprinted by permission.

Once activated, the receptor forms a homodimer complex which seeks out specific DNA segments, in this case the estrogen response elements (EREs). Binding of the complex to the ERE results in a rearrangement of the chromatin and transcription of the gene, followed by production of the target protein (Figure 3). A compound able to bind to the estrogen receptor in the cell might very well result in transcription and pleiotropic responses potentially affecting numerous functions within the organism.

In fish hepatocytes, the protein vitellogenin is produced via the pathway illustrated in Figure 3. While vitellogenin is normally associated with female fish, male fish also possess the hepatocyte ER and can synthesize vitellogenin when exposed to $E_2$ or to estrogen mimics. The production of this egg protein in oviparous fish, particularly in males, has become an important biomarker in the investigation of EDCs, both in the laboratory and in the field. Normally, vitellogenin in males is either absent or at very low concentrations (Sumpter and Jobling, 1995; Panter *et al.*, 1998; Harries *et al.*, 1997). In females vitellogenin is taken up by the ovaries, in male fish vitellogenin produced as a result of exposure to estrogens or to estrogen mimics is only slowly metabolized, making it a valuable biomarker. Currently, however, the ecological significance of elevated levels of vitellogenin in fish, particularly in males, is unclear (Jobling *et al.*, 1998).

Although the mode of action of estrogenic compounds on the production of vitellogenin in male fish is fairly well understood, there still remains much uncertainty as to how EDCs impact the overall development of aquatic organisms. In most cases, timing of exposure during development seems to be critical. It appears there is a labile period when fish are most susceptible to endocrine pertubation occurring just after hatching or at a juvenile stage, the time prior to morphological sex differentiation (Jobling *et al.*, 1998). Sex reversal in fish by treating eggs or larvae with $E_2$ or testosterone has found widespread use in aquaculture (Yamaziki, 1983). There is also some evidence that adult gonadal organization can be affected, but these changes appear to be reversible once the EDC is removed.

## TYPES AND SOURCES OF EDCS

EDCs include a diverse group of synthetic industrial and agricultural chemicals and even some naturally occurring compounds (Santodonato, 1997). Listed in

Table 2 and diagramed in Figure 4, they include industrial intermediates such as bisphenol-A (Celius *et al.*, 1999; Sumpter and Jobling, 1995) and 4-nonylphenol (Jobling and Sumpter, 1993; White *et al.*, 1994), PAHs (Santodonato, 1997; Thomas, 1988), pesticides such as the insecticides endosulfan (Chakravorty *et al.*, 1992), carbofuran (Sukumar and Karpagaganapathy, 1992), lindane (α-HCH) (Celius *et al.*, 1999), DDT (Khan and Thomas, 1998; Celius *et al.*, 1999), and the herbicide atrazine (Wetzel *et al.*, 1994), PCBs (Monosson *et al.*, 1994; Thomas, 1988; Thomas 1989), and a number of metals including lead (Thomas, 1988), cadmium (Thomas, 1989; Ruby *et al.*, 2000; Kime *et al.*, 1996), and mercury (Rurangwa *et al.*, 1998).

Table 2. Confirmed or suspected endocrine disrupting compounds.

| **Chemical/Class** | **Use/Source** |
|---|---|
| *Industrial Chemicals/Byproducts* | |
| 4-Nonylphenol | Surfactant intermediate/breakdown product |
| Octylphenol | Surfactant intermediate/breakdown product |
| Bisphenol-A | Monomer of polycarbonate |
| 4-tert-pentylphenol | Industrial intermediate |
| Benzo*[a]* pyrene | Fossil fuel combustion product |
| Phenanthrene | Fossil fuel combustion product |
| Polychlorinated biphenyls | Transformer oil |
| Dioxins | Industrial and waste incineration byproducts |
| Polybrominated diphenyl ethers | Flame retardants |
| Butyl benzyl phthalate | Plasticizer |
| Butylbenzyl phthalate | Plasticizer |
| Di-n-butyl phthalate | Plasticizer |
| *Pesticides* | |
| Atrazine | Herbicide |
| Carbofuran | Insecticide |
| Toxaphene | Insecticide |
| Endosulfan | Insecticide |
| Lindane | Insecticide |
| DDT | Insecticide |
| DDE | Degradation product of DDT |
| *Metals* | |
| Mercury | Industry (e.g., chloralkali plants) |
| Cadmium | Industry (e.g., metal plating, battery production) |
| Lead | Industry (e.g., battery production) |
| *Natural Products* | |
| b-Sitosterol | Pulp and paper industry by-product/plant sterol |
| Genistein | Plant sterol |
| Daidzein | Plant sterol |
| Enterodiol | Plant sterol |

Many of the chemicals currently known to interact with the estrogen receptor can be found in the effluent from sewage treatment plants (STPs) (Jobling *et al.*, 1998). Although the efficacy or potency of all these compounds is less than that of the endogenous hormones, there is concern that some have the ability to bioaccumulate to active or harmful levels over time. Additional compounds will likely be identified as more work is completed and better structure/activity relationships are developed. As noted by Santodonato (1997), a number of estrogenic EDCs or their primary oxidative metabolites, share a common structural relationship with the phenolic A ring in $E_2$ (Figure 4).

Alkylphenols and alkylphenol polyethoxylates, or APEs, have received much of the recent attention because of their estrogenic effects in laboratory studies, and their presence in the aquatic environment. APEs are used as surfactants in many applications from soaps and detergents to pesticide formulations. Once in the environment, microbial degradation results in loss of the ethoxylates, eventually leaving the more persistent alkylphenol (e.g., 4-nonylphenol). Jobling *et al.* (1998) have shown that exposure to alkylphenolic compounds result in the synthesis of vitellogenin in male fish.

APEs enter the aquatic environment via discharges from STPs, textile, and pulp and paper mills (White *et al.* 1994; Field and Reed 1996). 4-Nonylphenol, one of the degradation products of the nonylphenol polyethoxylates, has been the subject of numerous studies because of its estrogenic effects and presence in the environment. Approximately 575 million pounds are manufactured annually in the U.S. (C&EN, 1997).

Bisphenol-A is the monomer of the plastic polycarbonate. Approximately 1.6 billion pounds are produced in the U.S. each year (C&EN, 1997). Much of the polycarbonate eventually winds up in landfills. Bisphenol-A appears to be an estrogen mimic, with a demonstrated affinity for rat ER (Krishnan *et al.*, 1993).

Polycyclic aromatic hydrocarbons, or PAHs, are found in fossil fuels such as oil and coal and are

released into the environment through combustion, surface runoff, oil spills, recreational boating and shipping, municipal waste effluents and atmospheric deposition (Kime, 1998). In urban areas it is thought that the majority of PAHs are the result of atmospheric deposition from the combustion of fossil fuels (McElroy *et al.*, 1989). Santodonato (1997) noted that while PAHs may function as weak ER agonists, they are expected to bind preferentially to the Ah receptor, triggering the induction of Ah-responsive genes which can lead to an antiestrogenic effect.

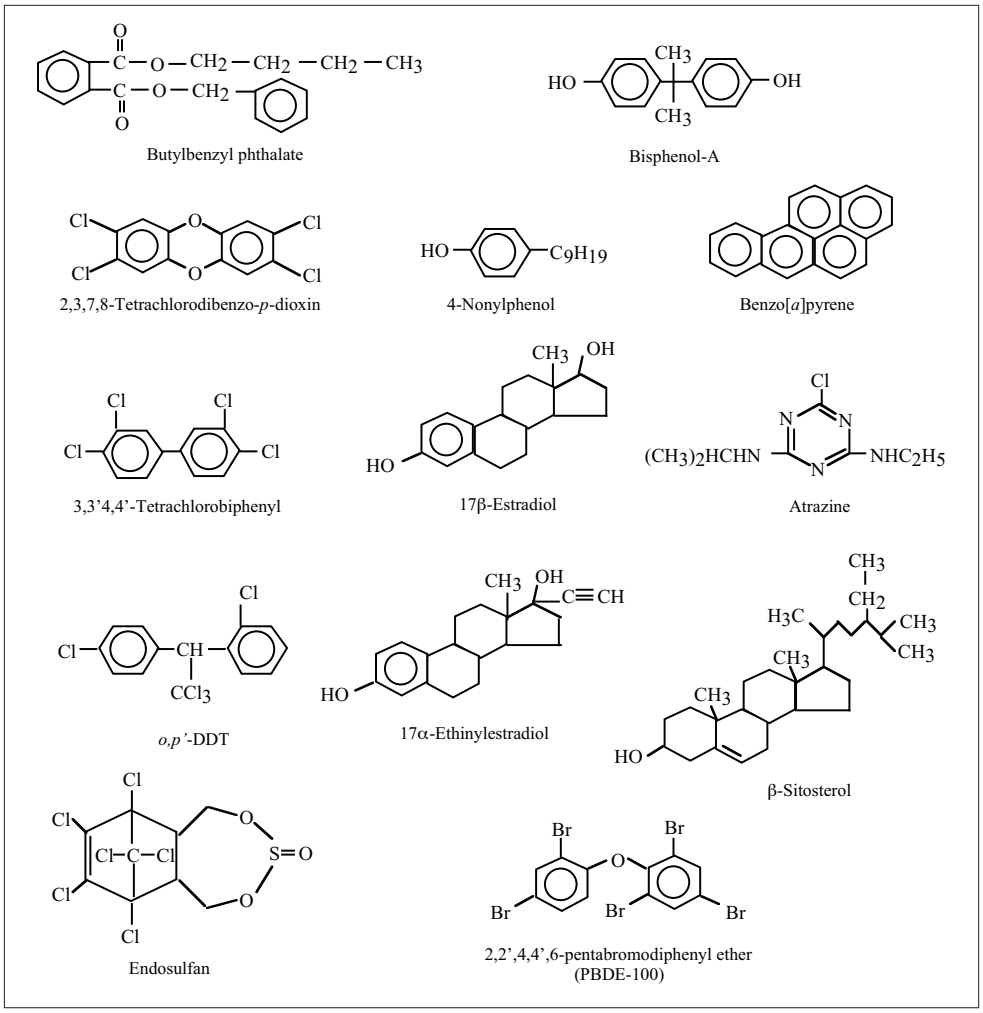



Figure 4. Molecular structure of 17β-estradiol, along with a number of confirmed or suspected endocrine disrupting compounds.

Dioxins are not intentionally manufactured but are typically formed and released through industrial activities such as chlorine bleaching at pulp and paper mills, chlorination at waste and drinking water treatment plants, and from municipal solid waste and industrial incinerator emissions. Anderson *et al.* (1996a) have shown *in vitro* that both the dioxin TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxin) and the furan 2,3,4,7,8-pentachlorodibenzofuran are estrogen antagonists. As with some of the PAHs, there appears to be a relationship between $E_2$ antagonism and induction of the CYP1A1 protein.

Polychlorinated biphenyls (PCBs) were manufactured and used widely as coolants and lubricants in transformers, capacitors, and other electrical equipment. There are no known natural sources of PCBs. The manufacture of PCBs in the United States ceased in 1977 due to their persistence, ability to bioaccumulate and related health concerns. PCBs generally appear to produce antiestrogenic and possibly antiandrogenic responses.

Phthalate esters are used in the manufacture of polyvinyl chloride as a softening agent and have been shown to be somewhat estrogenic. Compounds identified to date in descending order of potency include butylbenzyl phthalate (BBP), dibutyl phthalate, diisobutyl phthalate, and diethyl phthalate (Harris *et al.*, 1997).

Pesticides are used to control a wide variety of insect and plant pests. Pesticides are usually applied as a formulation containing the active ingredient, along with other materials, such as solvents, wetting agents or carriers (Pait *et al.*, 1992). Some active ingredients have the potential to impact the endocrine system, as do some of the surfactants used in the formulations. The carbamate insecticide carbofuran, has been shown to inhibit oocyte development in at least one

species of fish (Sukumar and Karpagaganapathy, 1992). Tennant *et al.* (1994), working with rats, concluded that while the chloro-s-triazine herbicides atrazine and simazine did not possess any intrinsic estrogenic activity, these two compounds were capable of weak inhibition of estrogen-stimulated responses in the rat uterus (i.e., effect on progesterone receptor binding, and thymidine incorporation into uterine DNA). More recently, Crain *et al.* (1997) showed that atrazine has the ability to stimulate production of the enzyme aromatase which converts androgens to estrogens, and presumably could interfere with sexual differentiation and development.

DDT, the pesticide banned in early 1970s, has been shown to induce production of the egg protein vitellogenin in primary fish hepatocytes (Celius *et al.*, 1999). The metabolites of DDT also appear capable of impacting the endocrine system. Donohoe and Curtis (1996) have shown that *o'p'*-DDE is estrogenic, however, *p,p'*-DDE, the dominant persistent metabolite of DDT, is not estrogenic using a variety of assays (Safe, 1995; Donohoe and Curtis, 1996). Kelce *et al.* (1995) while confirming that it had little ability to bind to the estrogen receptor in rats, found that *p,p'*-DDE was a potent androgen antagonist in male rats.

*Industrial activities can be the source of a variety of endocrine disrupting compounds including surfactants and polycyclic aromatic hydrocarbons. Image courtesy of NOAA/NOS.*

Trace elements occur naturally in the earth's crust but are concentrated and introduced into the environment through mining and manufacturing processes. Kime (1999) has pointed out that trace elements which induce the production of metallothioneins in the liver or gonads might disrupt gamete production by disturbing normal zinc homeostasis, essential for the development of both eggs and sperm. Cadmium, at aqueous concentrations of 50 ppm, has been shown to significantly decrease sperm motility (Kime *et al.*, 1996). Mercury has been shown to have a major impact on sperm motility at a concentration of only 1 ppb (Rurangwa *et al.*, 1998), and to have a direct effect on the egg micropyle, preventing entry of sperm (Khan and Weis, 1993). In rainbow trout, exposure to lead resulted in smaller oocytes (eggs) (Ruby *et al.*, 2000).

A number of phytoestrogens, including genistein, daidzein and enterodiol are known to affect the endocrine system. An infertility syndrome in sheep, known as clover disease, can be found in animals grazing on subterranean clover (Cheek *et al.*, 1998). Hughes (1988) has gone as far to suggest that phytoestrogens might actually be a defense strategy by plants to limit the fertility of grazing herbivores. Fungi are also known to produce several toxins which can affect the endocrine system (Celius *et al.* 1999). One of these is the mycotoxin zearalenone produced by *Fusarium*, a common contaminant in cereals and other plant products.

Effluents from pulp and paper mills can affect reproductive function in fish (Tremblay and Van Der Kraak, 1998). One of the compounds that could be responsible is β-sitosterol, a major by-product of wood pulp delignification, which appears to be activated or produced in the presence of *Mycobacterium smegmatis* (Bortone and Cody, 1999; MacLatchy *et al.*, 1997).

In addition to the compound types discussed above, there is growing acknowledgment that natural and synthetic estrogens, when present, are likely responsible for at least some of the endocrine-related effects seen in fish, particularly near STPs (Harries *et al.*, 1999; Purdom *et al.*, 1994; Larsson *et al.*, 1999). The synthetic estrogen 17α-ethinylestradiol (Figure 4), used in oral contraceptives, has been found in STP effluents along with $E_2$ and the $E_2$ metabolites estrone and estriol. The concentration of the natural and synthetic hormones needed to elicit a response, such as vitellogenin production in male fish, is typically hundreds or thousands of times less than that of an EDC such as 4-nonylphenol.

## LABORATORY STUDIES

This section contains a review of some of the laboratory studies that have addressed effects of EDCs in fish. A review of all laboratory investigations was beyond the scope of the report. Kime (1998) has completed an excellent and exhaustive review of the effects of EDCs in fish, primarily from laboratory studies.

*Alkylphenol Polyethoxylates*. The APEs are estrogenic and are perhaps the most studied of the EDCs, particularly in male fish, in part because of their potency and presence in the environment. Effects associated with APEs typically include elevated levels of the egg protein vitellogenin in males, reduction in gonadal development, and even intersex fish.

Jobling and Sumpter (1993) conducted a series of *in vitro* laboratory experiments using nonylphenol ethoxylate metabolites in rainbow trout (*Oncorhynchus mykiss*). In that paper, they introduced the production of vitellogenin in male fish as an indication of an estrogenic effect. Vitellogenin is now a widely used biomarker of exposure to estrogenic compounds. Jobling and Sumpter (1993) found that the metabolites of nonylphenol polyethoxylate, namely 4-nonylphenol, nonylphenol diethoxylate (NP2EO) and nonylphenoxycarboxylic acid (NP1EC) were all estrogenic in male rainbow trout, with potencies, however, between 1 x $10^{-4}$ to 1 x $10^{-6}$ the activity of the natural estrogen $E_2$ (Table 3).

Table 3. Relative potencies of estrogenic compounds.

| Compound | Relative Potency[a] | Mean ED 50 |
|---|---|---|
| 17β-estradiol | 1.0 | 1.81 nM |
| 4-nonylphenol (4NP) | 9.00E-06 | 16.15 μM |
| 4-tert-butylphenol (4-tBP) | 1.60E-04 | 2.06 μM |
| 4-tert-octylphenol (4-tOP) | 3.70E-05 | 2.11 μM |
| nonylphenol diethoxylate (NP2EO) | 6.00E-06 | 17.27 μM |
| nonylphenoxycarboxylic acid (NP1EC) | 6.30E-06 | 15.25 μM |

[a]Refers to the mean potency of each compound relative to 17β-estradiol
Source: Jobling and Sumpter, 1993. Reprinted by permission.

White *et al.* (1994) also investigated the ability of selected APEs to stimulate vitellogenin synthesis in rainbow trout. They found that octylphenol, 4-nonylphenol, NP1EC and NP2EO were each capable of inducing vitellogenin synthesis. A summary of vitellogenin synthesis from cultured hepatocytes in comparison to $E_2$ is shown in Figure 5 for each of the compounds tested on a molar basis. The concentration of the alkylphenols or APEs needed to elicit a response similar to $E_2$, however, was much higher. For example, at $10^{-5}$ M octylphenol, the amount of vitellogenin produced was roughly equal to that in cells exposed to only $10^{-8}$ M $E_2$, roughly a 1,000-fold concentration difference.

Jobling *et al.* (1996) assessed the effects of APEs and their metabolites in whole rainbow trout. They looked at vitellogenin synthesis and inhibition of testicular growth using 4-nonylphenol, NP1EC and NP2EO. After a 3-week exposure, all of the alkylphenolic compounds tested caused significant elevations in plasma vitellogenin. The order of potency was octylphenol > 4-nonylphenol > NP2EO > NP1EC, similar to what White *et al.* (1994) found working with rainbow trout hepatocytes.

At a concentration of 30 μg/L, octylphenol produced a million-fold increase in the concentration of vitellogenin, while the concentration of vitellogenin in fish exposed to 4-nonylphenol, NP2EO, and NP1EC were elevated less, yet still by 100- to 1,000-fold over background levels. The potent synthetic estrogen 17α-ethinylestradiol ($EE_2$) (Figure 4) at a concentration of only 2 ng/L produced a similar effect. They also conducted a dose-response experiment using 4-nonylphenol and found that the lowest concentration needed to induce a significant elevation of plasma vitellogenin was approximately 20 μg/L.

Jobling *et al.* (1996) noted that the concentrations required to induce a response *in vivo* were two orders of magnitude lower than the concentrations needed in earlier *in vitro* experiments (Jobling and Sumpter, 1993). They suggested the disparity could be due to the bioaccumulative properties of the compounds and/or the metabolism of chemicals by the fish to more active metabolites. This suggests that over time accumulation of lower concentrations of APEs and/

or their metabolites could elicit a response from fish in the environment. Table 4 summarizes some of the effects of EDCs on whole fish in the laboratory, while Table 5 summarizes some of the *in vitro* effects with primary hepatocyte cultures.

Hemmer *et al.* (2001) investigated the effects of 4-nonylphenol on vitellogenin production in male sheepshead minnows (*Cyprinodon variegatus*). Fish were exposed to 0.6, 5.4, 11.8, 23.3, and 42.7 µg/L 4-nonylphenol in a flow through system. Sampling occurred on days 0, 2, 5, 13, 21, 35, and 42. Vitellogenin was measured using a direct enzyme-linked immunosorbent assay (ELISA). The sheepshead minnows demonstrated a rapid, dose-dependent induction of plasma vitellogenin (Hemmer *et al.*, 2001). By the fifth day, fish exposed to 5.38 µg/L 4-nonylphenol or higher had significantly higher vitellogenin concentrations than fish in the control tanks. Fish produced as much as 100 mg vitellogenin/ml of plasma by day 13 in the highest exposure (42.7 µg/L), and by day 42, males had produced an even higher vitellogenin concentration, approximately 130 mg/ml.

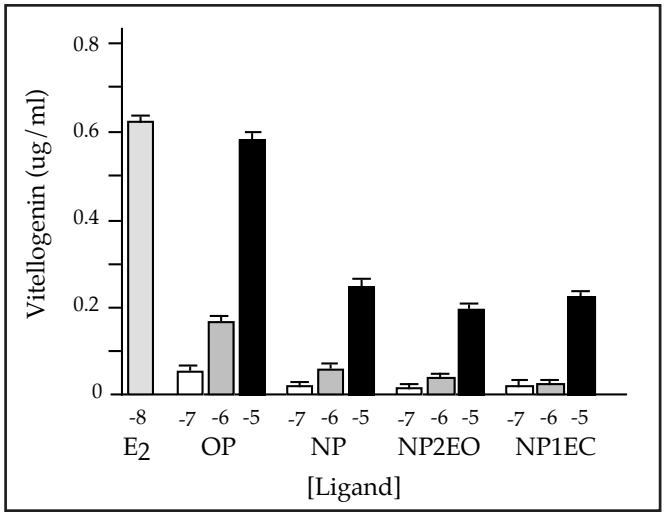



Figure 5. Vitellogenin production from cultured hepatocytes of rainbow trout in response to alkylphenolic compounds and 17b-estradiol (E2). Compounds were tested at $10^{-8}$ M (-8), $10^{-7}$M (-7), $10^{-6}$M (-6), and $10^{-5}$M (-5). From White *et al.*, 1994. Reprinted by permission.

Pait and Nelson (in prep.) assessed the ability of male killifish *(Fundulus heteroclitus)* to produce vitellogenin in response to intraperitoneal injections of 4-nonylphenol over a period of 8 days (Figure 6). *F. heteroclitus* responded significantly to injections of 50 mg/kg and greater 4-nonylphenol, with the 150 mg/kg exposure resulting in a plasma vitellogenin concentration of approximately 23 mg/ml.

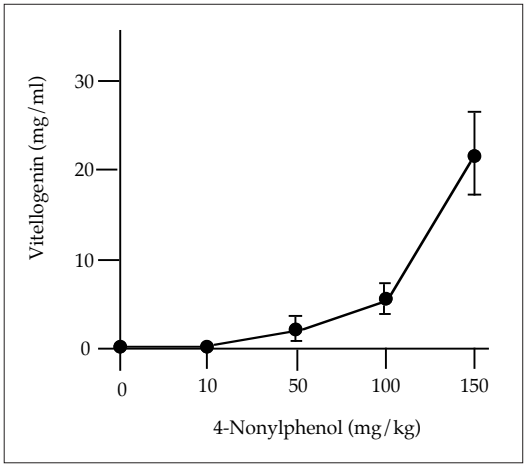



Figure 6. Production of vitellogenin (mean ±SEM) in male killifish *(Fundulus heteroclitus)* exposed to 4-nonylphenol. (Pait and Nelson, in prep.)

Lewis and Lech (1996) investigated the uptake, disposition, and persistence of $^{14}$C-nonylphenol in rainbow trout. The compound was rapidly taken up into most tissues and had an apparent half-life of 19-20 hours in muscle and fat. Using thin layer chromatography, three glucuronide metabolites were separated, however, the structure of these metabolites was not positively identified. They concluded that 4-nonylphenol appears to be metabolized through oxidation and glucuronic acid conjugation with subsequent excretion in the bile.

Arukwe *et al.* (2000) assessed the metabolism and organ distribution of 4-nonylphenol in Atlantic salmon (*Salmo salar*). Fish were given a single dose of $^{3}$H-nonylphenol (25 µg) and then sampled at 24, 48, and 72 hours. Similar to Lewis and Lech (1996), Arukwe *et al.* (2000) found that 4-nonylphenol was conjugated to glucuronic acid. Nonylphenol-glucuronide was the main metabolite found in both biliary and urinary samples. In biliary samples, between 80 and 95 percent of the total radioactivity was present as nonylphenol-glucuronide. Hydroxylated and oxidated compounds made up the remainder of the metabolites identified.

Gray and Metcalfe (1997) investigated the effects of 4-nonylphenol on Japanese medaka *(Oryzias latipes)*. Medaka were exposed from hatch to 3 months of age to

Table 4. Effects of endocrine disrupting compounds on whole fish in the laboratory.

| Fish/Species | Sex | Compound | Method of Exposure | Concentration | Duration | Vtg | T | E2 | GSI | GD | I | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rainbow trout *(Oncorhynchus mykiss)* | male | 4-Nonylphenol | whole fish (water) | 30 µg/L | 3 weeks | + | | | + | | | Jobling *et al.*, 1996 |
| Killifish *(Fundulus heteroclitus)* | male | 4-Nonylphenol | whole fish (intraperitoneal inj.) | 50-150 mg/kg | 8 days | + | | | + | | | Pait and Nelson, in prep. |
| Sheepshead minnow *(Cyprinodon variegatus)* | male | 4-Nonylphenol | whole fish (water) | 0.6-42.7 µg/L | 42 days | + | | | | | | Hemmer *et al.*, 2001 |
| Japanese medaka *(Oryzias latipes)* | male | 4-Nonylphenol | whole fish (water) | 50 µg/L | 3 months | | | | + | – | + | Gray and Metcalfe, 1997 |
| Japanese medaka *(Oryzias latipes)* | juvenile | 4-Nonylphenol | whole fish (water) | 17.7 - 51.5 µg/L | 104 days | | | | | | + | Yokota *et al.*, 2001 |
| Summer flounder *(Paralichthys dentatus)* | male | Octylphenol | whole fish (intraperitoneal inj.) | 2 - 200 mg/kg | 8 weeks | | | | – | | | Mills *et al.*, 2001 |
| Fathead minnows *(Pimphales promelas)* | male | Bisphenol-A | whole fish (water) | 1 - 1,280 µg/L | 164 days | + | | | – | – | | Sohoni *et al.*, 2001 |
| Rainbow trout *(Oncorhynchus mykiss)* | juvenile | Bisphenol-A | whole fish (water) | 10 - 500 µg/L | 12 days | + | | | | | | Lindholst *et al.*, 2000 |
| Rainbow trout *(Oncorhynchus mykiss)* | juvenile | Bisphenol-A | whole fish (intraperitoneal inj.) | 50 mg/kg | 18 days | + | | | | | | Christiansen *et al.*, 2000 |
| Common carp *(Cyprinus carpio)* | male | 4-*tert* -Pentylphenol | whole fish (water) | 3-1,000 µg/L | 3 months | + | | | | – | | Gimeno *et al.*, 1998a |
| Common carp *(Cyprinus carpio)* | male | 4-*tert* -Pentylphenol | whole fish (water) | 1,000 µg/L | 140 days | | | | | – | + | Gimeno *et al.*, 1998b |
| Japanese medaka *(Oryzias latipes)* | male | 17α–Ethinylestradiol | whole fish (water) | 0.1 µg/L | 2 months | | | | | | + | Scholz and Gutzeit, 2000 |
| Fathead minnows *(Pimphales promelas)* | male | 17α–Ethinylestradiol | whole fish (water) | 0.064 µg/L | 305 days | | | | | – | + | Lange *et al*., 2001 |
| Japanese medaka *(Oryzias latipes)* | juvenile | 17α–Ethinylestradiol | whole fish (water) | 0.1 µg/L | 110 days | | | | | | + | Metcalfe *et al.*, 2001 |
| Rainbow trout *(Oncorhynchus mykiss)* | female | β-Napthoflavone and 17β-estradiol | whole fish (intraperitoneal inj.) | 50 mg/kg β-NF and 0.5 mg/kg $E_2$ | 24 hours | – | | | | | | Anderson *et al.*, 1996b |
| Atlantic croaker *(Micropogonias undulatus)* | female | Benzo[*a* ]pyrene | whole fish (diet) | 0.4 mg/70g | 30 days | | | – | – | – | | Thomas 1988 |

NE, no effect; Vtg, vitellogenin; T, testosterone; E2, 17β-estradiol; GSI, gonadosomatic index; GD, gonadal development; I, intersex condition

Table 4. Effects of endocrine disrupting compounds on whole fish in the laboratory (continued).

| Fish/Species | Sex | Compound | Method of Exposure | Concentration | Duration | Vtg | T | E2 | GSI | GD | I | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| European flounder *(Platichthys flesus)* | female | Phenanthrene and chrysene | whole fish (diet) | 0.4 - 12.5 nM/g food | 12 weeks | | | – | | | | Monteiro *et al.*, 2000b |
| Rock sole *(Lepidopsetta bilineata)* | female | Prudhoe Bay crude oil | whole fish (intraperitoneal inj.) | 0.1 - 1.0 mg/kg | 7 days | | | – | | | | Johnson *et al.*, 1993 |
| Atlantic croaker *(Micropogonias undulatus)* | female | Aroclor 1254 | whole fish (diet) | 0.24 mg/70g | 30 days | | | | – | | | Thomas 1988 |
| Atlantic croaker *(Micropogonias undulatus)* | female | Aroclor 1254 | whole fish (diet) | 0.24 mg/70g | 30 days | | | – | | | | Thomas 1989 |
| White perch *(Morone americana)* | female | 3,3',4,4'-Tetrachlorobiphenyl | whole fish (intraperitoneal inj.) | 0.2-5.0 mg PCB/kg | 9 weeks | NE | NE | | – | | | Monosson *et al.*, 1994 |
| Starry flounder *(Platichthys stellatus)* | female | Natural PCB exposure | whole fish (water/diet) | unknown | life | | | | | – | | Spies and Rice, 1988 |
| Japanese medaka *(Oryzias latipes)* | juvenile | *o'p*-DDT | whole fish (water) | 7.5 µg/L | 2-8 weeks | + | | | | | + | Cheek *et al.*, 2001 |
| Atlantic croaker *(Micropogonias undulatus)* | female | *o'p*-DDT | whole fish (diet) | 0.02 and 0.01 µg DDT/g | 3-7 weeks | | | | + | | | Khan and Thomas, 1998 |
| Sheepshead minnow *(Cyprinodon variegatus)* | male | Endosulfan | whole fish (water) | 0.28 - 0.79 µg /L | 40 days | NE | | | | | | Hemmer *et al.*, 2001 |
| Walking catfish *(Clarias batrachus)* | female | Endosulfan | whole fish (water) | 1.5 µg/L | 16 days | – | | | | | | Chakravorty *et al.*, 1992 |
| Dwarf gourami *(Colisa lalia)* | female | Carbofuran | whole fish (water) | 0.7 µg/L | 20 days | | | | | – | | Sukumar and Karpagaganapathy, 1992 |
| Sheepshead minnow *(Cyprinodon variegatus)* | male | Methoxychlor | whole fish (water) | 12.1 µg /L | 35 days | + | | | | | | Hemmer *et al.*, 2001 |
| Rainbow trout *(Oncorhynchus mykiss)* | juvenile | Butylbenzyl phthalate | whole fish (water) | 500 mg/kg | 9 days | + | | | | | | Christiansen*et al.*, 1998 |
| Rainbow trout *(Oncorhynchus mykiss)* | female | Lead | whole fish (water) | 10 µg/L | 12 days | | | | – | | | Ruby *et al.*, 2000 |
| Atlantic croaker *(Micropogonias undulatus)* | female | Lead | whole fish (diet) | 1.34 mg/70g | 30 days | | | | – | | | Thomas, 1988 |
| Atlantic croaker *(Micropogonias undulatus)* | female | Cadmium | whole fish (water) | 1 mg/L | 30 days | | | + | + | | | Thomas, 1989 |
| Rainbow trout *(Oncorhynchus mykiss)* | juvenile | β-Sitosterol | whole fish (water) | 75 - 100 µg/L | 3 weeks | + | – | | | | | Tremblay and van Der Kraak, 1998 |
| Goldfish | males/females | β-Sitosterol | whole fish (water) | 75- 1,200 µg/L | 12 days | | – | – | | | | MacLatchy *et al.*, 1997 |

NE, no effect; Vtg, vitellogenin; T, testosterone; E2, β-estradiol; GSI, gonadosomatic index; GD, gonadal development; I, intersex condition

Table 5. *In vitro* (fish hepatocyte) effects of endocrine disrupting compounds.

| Fish/Species | Compound | Concentration | Duration | Vtg | Vtg Production | Reference |
|---|---|---|---|---|---|---|
| Rainbow trout *(Oncorhynchus mykiss)* | 4-Nonylphenol | 10 μM | 48 hours | + | 0.25 μg/ml | Jobling and Sumpter, 1993 |
| Rainbow trout *(Oncorhynchus mykiss)* | 4-Nonylphenol | 1-10 μM | 48 hours | + | 0-0.45 μg/ml (approx.) | Sumpter and Jobling, 1995 |
| Rainbow trout *(Oncorhynchus mykiss)* | 4-Octylphenol | 10 μM | 48 hours | + | 0.95 μg/ml (approx.) | Jobling and Sumpter, 1993 |
| Rainbow trout *(Oncorhynchus mykiss)* | 4-Octylphenol | 10 μM | 48 hours | + | 0.2-0.4 μg/ml (approx.) | White *et al.*, 1994 |
| Rainbow trout *(Oncorhynchus mykiss)* | Bisphenol-A | 1-10 μM | 48 hours | + | 0.2 - 1.7 μg/ml (approx.) | Sumpter and Jobling, 1995 |
| Atlantic salmon *(Salmo salar)* | Bisphenol-A | 1-10 μM | 48 hours | + | Absorbence units; >controls | Celius *et al.*, 1999 |
| Rainbow trout *(Oncorhynchus mykiss)* | 4-nonylphenoxycarboxylic acid (4-NPEO) | 10 μM | 48 hours | + | 0-0.17 μg/ml (approx.) | White *et al.*, 1994 |
| Rainbow trout *(Oncorhynchus mykiss)* | β-Napthoflavone and 3-methylcholanthrene | 0.021 - 6.2 μM | 48 hours | – | NA | Navas and Segner, 2000 |
| Rainbow trout *(Oncorhynchus mykiss)* | Soot and 17β-estradiol | 0.05-25μg/L | 48 hours | – | NA | Villalobos *et al.*, 1996 |
| Rainbow trout *(Oncorhynchus mykiss)* | Aroclor 1221 | 1-10μM | 48 hours | + | 0.2 - 0.4 μg/ml (approx.) | Sumpter and Jobling, 1995 |
| Rainbow trout *(Oncorhynchus mykiss)* | 2,3,4,7,8-Pentachlorodibenzofuran and 17β-estradiol | $10^{-12}$-$10^{-8}$M | 48 hours | – | Production < with $E_2$ alone | Anderson *et al.*, 1996a |
| Rainbow trout *(Oncorhynchus mykiss)* | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 17β-estradiol | $10^{-11}$-$10^{-8}$M | 48 hours | – | Production < with $E_2$ alone | Anderson *et al.*, 1996a |
| Atlantic salmon *(Salmo salar)* | *o'p'*-DDT and lindane | 1-10 μM | 96 hours | + | Absorbence units; >controls | Celius *et al.*, 1999 |

Vtg, vitellogenin; NA, not available from article

nominal concentrations of 10, 50, and 100 μg/L. At 50 and 100 μg/L, 50 and 86 percent of the males, respectively, developed ovotestes, an intersex condition characterized by both testicular and ovarian tissue within the gonad. In addition, while the ratio of males to females in the control was 2:1, the ratio declined to 1:2 in the higher 4-nonylphenol treatment.

In a more recent study, Yokota *et al.* (2001) addressed life cycle toxicity of 4-nonylphenol to Japanese medaka. Exposure began at 24 hours postfertilization and continued for up to 104 days posthatch for the $F_0$, and 60 days for the $F_1$ generation. Both generations of medaka were exposed under flow through conditions to 4-nonylphenol concentrations of 4.2, 8.2, 17.7, 51.5 and 183 μg/L. The highest concentration significantly reduced survival (hatching and swim-up) of the $F_0$ fish. At 60 days posthatch, external secondary sex characteristics appeared totally skewed towards females in the 51.5 μg/L treatment. Gonadal histology revealed that 20 percent of the fish in the 17.7 μg/L treatment, and 40 percent in the 51.5 μg/L treatment had ovotestes. Because of the skewed sex ratio, no fish from the 51.5 μg/L treatment could be used to examine fertility and fecundity in the $F_0$ generation, and only three pairs could be selected from the 17.7 μg/L treatment. Fish were exposed from day 71 to 103 posthatch with concentrations ranging from 4.2 to 17.7 μg/L. While fecundity was not affected by the treatments, mean fertility was reduced by 76 percent in the 17.6 μg/L treatment. In the $F_1$ generation, significant ovotestes were observed in the 8.2 (4 percent) and the 17.7 μg/L (18 percent) concentrations.

Mills *et al.* (2001) investigated the effects of octylphenol on juvenile male summer flounder (*Paralichthys dentatus*). Flounder were injected twice with octylphenol in a coconut oil carrier during the experiment for total concentrations of 2, 20, and 200 mg/kg plus a control. Experiments were run for 8 weeks in flow-through aquariums, and fish were sampled at 4, 6 and 8 weeks. None of the doses of octylphenol, however, resulted in the production of detectable amounts of vitellogenin in the juvenile male flounder as determined by direct ELISA, although the gonadosomatic index, or GSI (gonad weight/body weight X 100) showed some evidence of a decline in fish exposed to 200 mg/kg 4-nonylphenol.

*Bisphenol-A.* This compound is also estrogenic, capable of inducing vitellogenin in male fish. The myriad of uses and associated high production volume for bisphenol-A has heightened concern for its possible effects in the aquatic environment.

Lindholst *et al.* (2000) studied the estrogenic response of rainbow trout exposed to bisphenol-A in a flow-through system. Juvenile fish were exposed to nominal concentrations of 10, 40, 70, 100, and 500 μg/L of bisphenol-A for 6 and 12 days. Plasma samples were analyzed for vitellogenin using a direct sandwich ELISA. While exposure to increasing concentrations of bisphenol-A resulted in increasing levels of vitellogenin after 6 days, only the 500 μg/L concentration produced significantly higher vitellogenin levels (approximately 1 mg/ml) than controls. Interestingly, after 12 days the concentration of plasma vitellogenin in those fish exposed to 70 or 100 μg/L bisphenol-A remained the same or decreased slightly. Lindholst *et al.* (2000) attributed this to a possible increase in the degradation rate of bisphenol-A as a result of cytochrome P450 induction. After 12 days, vitellogenin levels in fish exposed to 500 μg/L continued to increase, which the authors suggested was the result of detoxification processes not being able to keep pace with the higher concentration of bisphenol-A.

Sohoni *et al.* (2001) investigated the long-term reproductive effects of bisphenol-A in fathead minnows (*Pimphales promelas*). Mature male and female fish were exposed to nominal bisphenol-A concentrations of 1, 16, 160, 640 or 1,280 μg/L in flow-through chambers. At days 43, 71, and 164, selected fish were sacrificed for measurements of somatic growth, GSI, and plasma vitellogenin (ELISA). The testes were processed for histologic analysis. At concentrations of 640 and 1,280 μg/L, bisphenol-A had a significant inhibitory effect on weight and length in males by Day 71.

In males, exposure to bisphenol at concentrations of 160 μg/L and higher for at least 71 days resulted in vitellogenin concentrations between 120 and 144 μg/L. The GSIs were also significantly decreased in both males and females exposed to bisphenol-A at concentrations of 640 μg/L and higher for 164 days. Histological examination revealed a decrease in spermatozoa in fish exposed to nominal bisphenol-A concentrations of 16 μg/L and greater for 164 days. Results from breeding pair experiments begun on Day 42 indicated that egg production was inhibited at 1,280 μg/L, and that hatchability was reduced at a nominal bisphenol-A concentration of 640 μg/L.

Christiansen *et al.* (2000) investigated the potential for a number of xenoestrogens, including bisphenol-A, to induce vitellogenesis in juvenile rainbow trout. Fish were injected with 50 mg/kg in a peanut oil carrier at days 0, 6, and 12 of an 18 day exposure. The average plasma vitellogenin concentration after 18 days was 0.066 mg/ml. Christiansen *et al.* (2000) noted there was substantial variability between fish, with vitellogenin concentrations ranging from 0.07 to 482 μg/ml.

Celius *et al.* (1999) looked at the production of vitellogenin and the induction of zona radiata proteins (eggshell proteins) in primary hepatocytes of Atlantic salmon exposed to bisphenol-A. Cells treated with 1, 5, or 10 uM of bisphenol-A produced both vitellogenin and zona radiata proteins in an approximate dose-dependant manner after 96 and 48 hours, respectively.

*Rainbow trout, Oncorhynchus mykiss, a fish frequently used in endocrine disrupter research. Image courtesy of USDOI/ FWS - Duane Raver.*

Sumpter and Jobling (1995) assessed the ability of bisphenol-A to induce vitellogenin production at a concentration of 1 μM in male rainbow trout hepatocytes. Bisphenol-A induced significant vitellogenin production after 2 days.

*4-tert-pentylphenol.* This compound has also shown some estrogenic potential. Gimeno *et al.* (1998a) investigated the effects of 4-*tert*-pentylphenol (TPP), an industrial intermediate, on the common carp *Cyprinus carpio* during spermatogenesis. Sexually mature male carp were exposed for up to 3 months to nominal concentrations ranging from 32 to 1,000 μg/L using an intermittent flow-through dosing system.

Exposure of the male carp to TPP resulted in elevated vitellogenin levels and the inhibition of spermatogenesis. Exposure of fish to each concentration also resulted in reduced testicular growth, with a correspondingly lower testicular weight. In three out of five fish exposed to the higher TPP concentrations, disorganization of the seminiferous lobules, atrophy of the germinal epithelium, and some tissue necrosis was observed. While exposure of adult male carp to TPP for 3 months did not result in intersex fish, exposure to a high concentration of $E_2$ resulted in the development of ovotestes in one mature fish.

Gimeno *et al.* (1998b) investigated the feminizing effects of TPP on young male carp. Genetic males (XY) were exposed at 50 days post hatch to nominal TPP concentrations of 100, 320, and 1,000 μg/L using an intermittent flow-through dosing system. Histological examination of the gonads was carried out beginning at day 20 and then continued every 10 days until 140 days post hatch. Gimeno *et al.* (1998b) found that spermatogenesis was severely inhibited in the testes of the young carp. At the two highest TPP concentrations, a few oocytes were detected in the testes, along with an oviduct.

*Polycyclic Aromatic Hydrocarbons (PAHs).* The PAHs appear to depress the production or levels of circulating estrogens as well as vitellogenin in female fish. There does not appear to be much information on the effects of PAHs in male fish. PAHs have also been shown to result in lower GSIs.

A number of studies have examined the relationship between compounds that induce the cytochrome P4501A1 isozyme (CYP1A1), namely the polycyclic and some of the halogenated aromatic hydrocarbons, and endocrine disruption. This effect has been linked to compounds that bind to the aryl hydrocarbon (Ah) receptor, which functions in an analogous way to the ER, by forming a complex with the xenobiotic. In a paper by Anderson *et al.* (1996b), a set of experiments were run *in vivo* with juvenile rainbow trout. Fish were injected with various amounts of $E_2$ along with βNF (β-napthoflavone), a PAH. Trout injected with 0.5 mg/kg $E_2$ plus 25 or 50 mg/kg βNF had depressed vitellogenin synthesis by the liver relative to $E_2$ injected alone. They attributed inhibition of vitellogenin synthesis to an estrogen receptor "down regulation", that is, a lowered amount of estrogen receptor present or available as evidenced by a depressed estradiol-estrogen receptor binding capacity of liver in trout injected with βNF.

At higher levels of $E_2$ the effect was reversed, which the authors attributed to a possible phosphorylation

of certain cellular proteins which might synergistically activate ER-mediated gene transcription with $E_2$, independent of any changes in cellular ER content, or changes in the binding affinity of the ER for the ligand (Anderson *et al.*, 1996b). They also noted that the antiestrogenic effect of βNF at lower concentrations could be caused by increased metabolism of $E_2$, as a result of Phase II conjugation. Santodonato (1997) noted that although PAHs may function as weak ER agonists, they are expected to bind preferentially to the Ah receptor, triggering the induction of the Ah-responsive genes (e.g., P450) more likely leading to an antiestrogenic effect.

Navas and Segner (2000) investigated the antiestrogenicity of PAHs to cultured rainbow trout hepatocytes along with the role of the Ah receptor. Cells were co-exposed to PAHs and $E_2$, and the effects on 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Ah receptor-regulated), and vitellogenin production ($E_2$-regulated) were assessed. Cells were first pre-exposed for 24 hours to serial dilutions (0.021-6.2 uM) of the test compounds (3-methylcholanthrene, anthracene, or β-napthoflavone), followed by addition of 1 uM $E_2$ along with the test compounds. Anthracene had no effect on either vitellogenin synthesis or EROD activity. However, vitellogenin synthesis decreased with increasing concentrations of 3-methylcholanthrene or β-napthoflavone, even in the presence of $E_2$. EROD activity, however, was found to increase with increasing concentrations of all these compounds. The compound with the highest EROD induction activity, β-napthaflavone, was also the strongest inhibitor of vitellogenin production. The addition of either of two Ah receptor inhibitors, α-napthoflavone, or 8-methoxypsoralen (6.25 or 12.54 uM) resulted in a reversal of the inhibition. Because there were no significant differences in the $E_2$ concentrations in the control or induced cultures, Navas and Segner (2000) concluded that elevated levels of CYP1A1 were not associated with significant biotransformation of $E_2$ in the treatment cultures, which could have resulted in lower vitellogenin levels. Instead, the authors hypothesized that lower vitellogenin levels observed in those cultures treated with PAHs may have been the result of the activated Ah receptor and xenobiotic response element interacting or possibly inhibiting the regulatory regions of the vitellogenin gene.

Monteiro *et al.* (2000a) assessed the ability of PAHs to inhibit *in vitro* ovarian steroidogenesis in the European flounder *Platichthys flesus*. Fully vitellogenic ovarian tissue was incubated for 24 hours with either phenanthrene, benzo[*a*]pyrene or chrysene (15μM), along with the steroid precursor 17a-hydroxyprogesterone, at a concentration of 0.15 μM. All three PAHs significantly reduced the production of androstenedione and $E_2$.

In a followup study, Monteiro *et al.* (2000b) assessed the effect *in vivo* of phenanthrene or chrysene on plasma steroid levels in female European flounder. Fish were given the PAHs as part of the diet for a period of 12 weeks during the previtellogenic phase of the reproductive cycle. While neither phenanthrene or chrysene appeared to affect ovarian development, there was a significant effect on plasma $E_2$ levels. In fish exposed to 0.4 nmol chrysene/gram of food, or 0.5-12.5 nmol phenanthrene/gram of food, plasma $E_2$ levels decreased significantly. In female flounder exposed to 12.5 nmol phenanthrene, plasma $E_2$ levels were 21 pg/ml, compared to 107 pg/ml in control fish.

Thomas (1988) investigated dietary effects of the PAH benzo[*a*]pyrene on endocrine function in female Atlantic croaker, *Micropogonias undulatus*. The experiment was carried out over 30 days during gonadal recrudescence (seasonal maturation). Benzo[*a*]pyrene at a rate of 0.4 mg/70g fish/day significantly impaired ovarian development in the fish. In females, the GSI was only 66 percent of controls (Figure 7). In addition, reduced ovarian growth was accompanied by a significant decline in circulating $E_2$ levels (Thomas, 1988).

Johnson *et al.* (1993) injected gravid female rock sole (*Lepidopsetta bilineata*) and flathead sole (*Hippoglossoides elassododon*) from a nonurban area in Puget Sound with Prudhoe Bay Crude Oil (PBCO) at doses of 0.1, 0.5, and 1.0 mg oil/kg body weight. Injection of the fish with either fresh or weathered PBCO resulted in decreased plasma $E_2$ levels after 7 days. Experiments with ovarian tissue fragments exposed to weathered PBCO also appeared to inhibit ovarian $E_2$ production in a dose-dependent manner.

In work containing aspects of both a laboratory and field study, Knudsen *et al.* (1997) exposed male rainbow trout in a series of 500 L tanks to treated effluent collected from an oil refinery. Treatment involved skimming, sedimentation and biological degradation. The tanks containing the fish received drinking quality water mixed with either 0, 2.5, 10, or 20 percent treated refinery effluent. At an effluent concentration of 20 percent, fish that were exposed for either 2 or 3 weeks produced a significantly

higher concentration of vitellogenin than control fish. Knudsen *et al.* (1997) noted that while a rather high concentration of the effluent was needed to produce an effect, the treated effluent clearly contained bioactive substances. Although the chemical(s) responsible for the elevated vitellogenin levels were not identified, the authors felt that removal of certain refinery process chemicals as opposed to enhanced treatment might be a cost effective means of addressing the observed effects.

Villalobos *et al.* (1996) exposed a primary liver cell culture from rainbow trout to an extract of soot. Exposure of the cells to the extract at a concentration of 0.05-25 μg/L along with an $E_2$ concentration of 1 uM caused an induction of CYP1A1 protein after binding to the Ah receptor, followed by a reduction in $E_2$-dependent vitellogenin synthesis, an antiestrogenic effect.

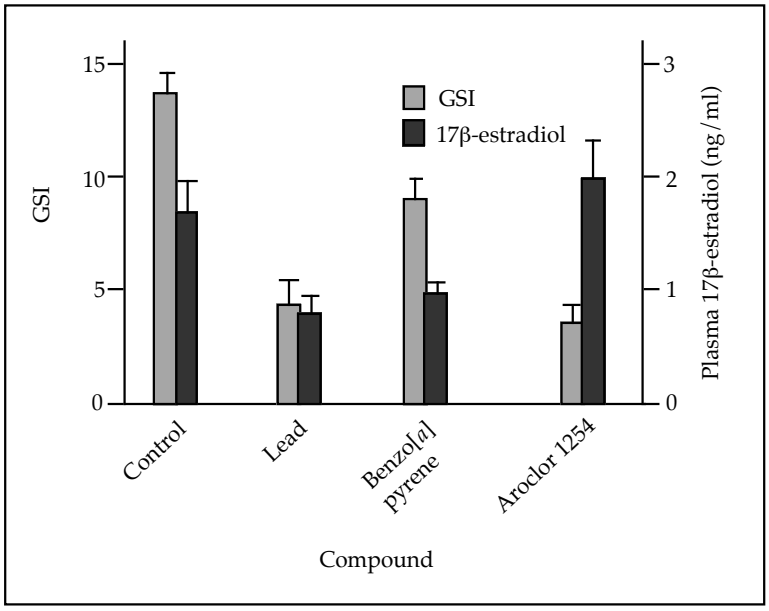



Figure 7. Effect of various compounds on GSI and plasma 17β-estradiol in female Atlantic croaker (*Micropogonias undulatus*). From Thomas, 1988. Reprinted by permission.

*Polychlorinated Biphenyls*. In general, PCBs appear to depress levels of estrogens as well as gonadal development and the GSI in female fish. Little information was found on effects in male fish. Although no longer manufactured, the widespread occurrence of PCBs in the environment coupled with their effects on the endocrine system is of concern.

Thomas (1988) investigated the effect of Aroclor 1254 on Atlantic croaker. Females fed Aroclor 1254 in a prepared diet (0.24 mg/70g fish/day) for 30 days had substantially lower GSIs (25 percent of controls) (Figure 7). Thomas (1989) conducted additional experiments with Aroclor 1254 and found that in addition to reduced ovarian growth in croaker, plasma $E_2$ levels were significantly reduced. Thomas (1989) hypothesized that the lower $E_2$ levels decreased vitellogenesis and hence ovarian growth.

Monosson *et al.* (1994) injected groups of female adult white perch (*Morone americana*) with the coplanar PCB 3,3',4,4'-tetrachlorobiphenyl (TCB) at three concentrations ranging from 0.2-5.0 mg PCB/kg body weight prior to the spawning season. In those fish receiving the highest dose of the PCB, fewer matured, and in those that did, the GSI was approximately half that of the control fish. Interestingly, however, levels of $E_2$, testosterone and plasma vitellogenin were not significantly affected by any of the TCB exposures (Monosson *et al.*, 1994). Survival of the larvae was also affected by parental exposure. Larvae produced from females dosed with 1.0 and 5.0 mg/kg had significantly lower survival rates.

Sumpter and Jobling (1995) found that a 1 μM concentration of Aroclor 1221 actually induced vitellogenin production in male rainbow trout hepatocytes. In Figure 8, an apparent increase in vitellogenin production resulted from increasing concentrations of Aroclor 1221. This is a seemingly opposite effect of that found by Thomas (1988) for Aroclor 1254. Interestingly, when the hepatocytes were exposed to a mixture of chemicals, all at a concentration of 1 μM, Sumpter and Jobling (1995) found an enhanced effect (Figure 9).

In the laboratory, Spies and Rice (1988) investigated reproductive success in starry flounder (*Platichthys stellatus*) from two localities in San Francisco Bay with differing degrees of contamination. Mature females from both sites were induced to spawn in the laboratory using carp pituitary extract, and relationships between measures of survival through successive early life stages, and the degree of contamination in both parent and spawned eggs were assessed.

Significant negative correlations existed between PCB content of the eggs and percent fertilized eggs that hatched, termed embryological success. There were also negative correlations between maternal hepatic aryl hydrocarbon hydroxylase (AHH) activity and percent viable eggs, fertilization success and embryological success. In addition, Spies and Rice (1988) found that females captured from the more urbanized location (Berkeley) had significantly fewer viable eggs, lower fertilization rates and higher AHH activity than fish from the less contaminated site in San Pablo Bay.

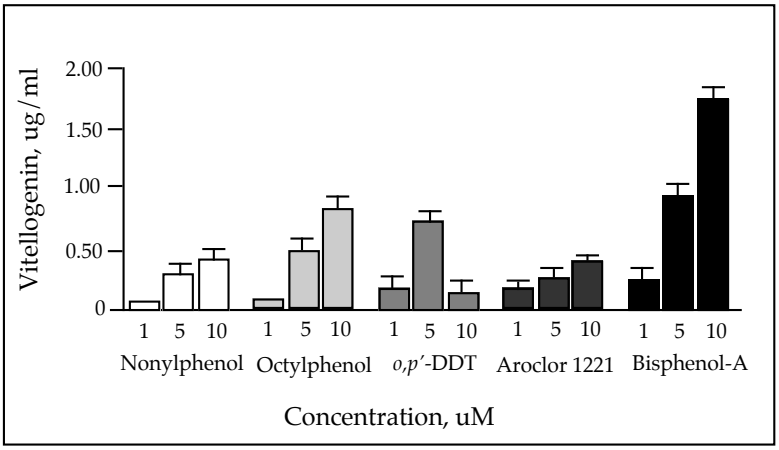



Figure 8. Estrogenic activity of selected compounds in male rainbow trout. Results are presented as the mean ±SEM. From Sumpter and Jobling, 1995. Reprinted by permission.

*Furans and Dioxins.* Although little work has been done on the effects of furans and dioxins on the endocrine system in fish, they appear to be antiestrogenic, at least *in vitro*, perhaps sharing a similar mechanism with the PAHs. Using rainbow trout hepatocytes, Anderson *et al.* (1996a) investigated the *in vitro* modulation of $E_2$-induced vitellogenin synthesis by a group of compounds, including 2,3,4,7,8-pentachlorodibenzofuran, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and 2,3,7,8-tetrachlorodibenzofuran. All were antiestrogenic, that is, reducing the amount of vitellogenin synthesis in trout cells coexposed to $E_2$. Anderson *et al.* (1996a) noted that the potency of inhibition was directly related to the strength of the compound as an inducer of CYP1A1 protein, similar to that found by Navas and Segner (2000) for PAHs.

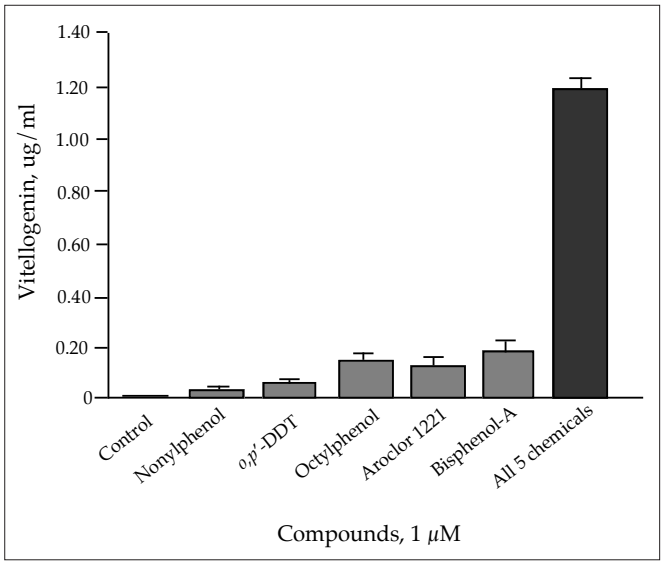



Figure 9. Effect of several estrogenic compounds, including a mixture of all five, on vitellogenin production in male rainbow trout. From Sumpter and Jobling, 1995. Reprinted by permission.

*Polybrominated Diphenyl Ethers (PBDEs).* Since the 1960s, polybrominated diphenyl ethers or PBDEs (Figure 4) have found widespread use as flame retardants in products from television sets to rug and drapery textiles. Commercial PBDE mixtures range from penta- to deca-BDEs, with worldwide annual production estimated at 148 million pounds (Hooper and McDonald, 2000). PBDEs have been found in humans, wildlife, and sediments, and residues appear to be on the increase (McDonald, 2002). Meerts *et al.* (2001) found that a number of PBDEs were estrogenic using an estrogen responsive human cell line. In addition to being estrogenic, there is also evidence that PBDEs can interfere with thyroid hormone balance and function. Mice dosed with penta-BDE showed decreased levels of $T_4$ after 8 days (Fowles *et al.*, 1994). In addition, a number of PBDEs or their metabolites bind with high affinity to the thyroid hormone transport protein transthyretin (Meerts *et al.*, 2000), and to the thyroid hormone receptors (Marsh *et al.*, 1998). Although no

articles were located on endocrine related effects of PBDEs in fish, this emerging class of EDCs would appear to be good candidates for future laboratory and field investigations.

*Pesticides*. A number of pesticides have been identified as EDCs. Some appear to be antiestrogenic, while others, such as *o,p'*-DDT and methoxychlor, appear capable of inducing vitellogenesis in male fish.

Chakravorty *et al.* (1992) investigated the effect of the insecticide endosulfan on vitellogenesis in the catfish *Clarias batrachus*. Female fish were exposed to a nominal concentration of 1.5 μg/L endosulfan in a static renewal system for a period of 16 days. There was a significant decline in plasma vitellogenin compared to controls (approximately 0.8 mg/ml) after 16 hours, with a maximum low (approximately 0.1 μg/ml) reached after 48 hours (Chakravorty *et al.* 1992).

*Certain pesticides or compounds used in pesticide formulations are suspected of being endocrine disrupters. Image courtesy of NOAA/NOS.*

Hemmer *et al.* (2001) assessed the effects of endosulfan on vitellogenin production in male sheepshead minnows. Fish were exposed to measured concentrations ranging from 0.28 - 0.79 μg/L for up to 40 days in a flow through system. None of these exposures, however, resulted in the accumulation of detectable levels of vitellogenin in the plasma.

Hemmer *et al.* (2001) also assessed the production of vitellogenin in sheepshead minnows exposed to methoxychlor. They noted that while the parent compound is not estrogenic, the demethylated metabolites are active and bind to the estrogen receptor. Exposure of male sheepshead minnows to a methoxychlor concentration of 5.6 and 12.1 μg/L resulted in fairly rapid increases in plasma vitellogenin. By day 35, fish exposed to 12.1 μg/L produced a substantial amount of vitellogenin, approximately 120 mg vitellogenin/ml of plasma.

Sukumar and Karpagaganapathy (1992) investigated the effects of sublethal concentrations of the carbamate insecticide carbofuran on the dwarf gourami, *Colisa lalia*. Female fish were exposed to a concentration of 0.7 μg/L technical grade carbofuran in a static renewal exposure for a period of 20 days. The fish exposed to the carbofuran had fewer mature oocytes, and the mature oocytes that were observed appeared atretic (Sukumar and Karpagaganapathy,1992).

Ghosh and Thomas (1995) assessed the antagonistic effects of the insecticide kepone (chlordecone) on oocyte maturation *in vitro* in Atlantic croaker. The breakdown of the germinal vesicle in Atlantic croaker is controlled by the maturation-inducing hormone 17α,20β,21–trihydroxy-4-pregnen-3-one (20β-S). Ghosh and Thomas (1995) exposed fully grown and primed oocytes to either 20β-S, or 20β-S and kepone. Kepone concentrations ranged from 100 nM to 100 μM. Exposure of the oocytes for as little as one minute to 0.1 μM kepone resulted in a significantly lower breakdown of the germinal vesicles when compared to controls. Exposure of the oocytes to kepone followed by washing and then incubation with 20β-S resulted in a normal germinal vesicle breakdown process, leading the authors to conclude that kepone competes with the hormone for the receptor, rather than simply being toxic to the oocytes.

Celius *et al.* (1999) investigated the production of vitellogenin and zona radiata (eggshell) proteins in primary hepatocytes of Atlantic salmon exposed to a variety of suspected EDCs including *o,p'*-DDT and lindane (γ–HCH). Cells treated with 1, 5, and 10 μM DDT and lindane induced vitellogenin and zona radiata proteins in an approximate dose-dependant manner.

Cheek *et al.* (2001) reported on reproductive impairment in medaka by *o,p'*-DDT. Fish were exposed for either 2 or 8 weeks post hatch to nominal concentrations ranging from 0 to 7.5 μg/L in intermittent flow through chambers. Fish were then sampled at 2, 4, or 8 weeks for vitellogenin content and gonad development. The remaining fish were then transferred to clean water and grown to sexual maturity and placed in mating pairs. Measurements of fecundity, fertility and hatching success were made on the eggs collected. Cheek *et al.* (2001) found that *o,p'*-DDT had no effect on vitellogenin expression after a 2 week exposure, but all doses resulted in vitellogenin expression after 8 weeks. In both exposures, *o,p'*-DDT resulted in a female-skewed sex ratio at the two highest doses. Two fish exposed for 8 weeks at 1.94 μg/L exhibited ovotestes. Both the 2 and 8 week exposures significantly affected fertility and in particular, hatching success at all concentrations. Because vitellogenin expression was not as sensitive an indicator as the other parameters measured, Cheek *et al.* (2001) concluded that the absence of vitellogenin expression should not be used to indicate a lack of reproductive endocrine disruption.

Khan and Thomas (1998) also evaluated the effect of *o,p'*-DDT, however, looking at the release of gonadotropin hormone (GTH) in Atlantic croaker. Fish were exposed to *o,p'*-DDT through the diet (0.02 and 0.1 μg DDT/g body weight per day) for 3 and 7 weeks during gonadal recrudescence. The *o,p'*-DDT resulted in a significant increase in plasma GTH after both the 3 and 7 week exposures. The increase in GTH was accompanied by a slight increase in the relative size of the ovary as indicated by an increase in the GSI (Khan and Thomas, 1998).

Knudson and Pottinger (1999) assessed the binding affinity of the pesticides toxaphene and dieldrin to both the $E_2$ receptor (ER) and the testosterone receptor (TR) in rainbow trout, using a competitive binding assay with 1 pmol radiolabeled $E_2$ or testosterone. Concentrations of toxaphene and dieldrin up to 50,000 times that of $E_2$ or testosterone failed to dislodge the endogenous hormones, indicating a low affinity of these two pesticides for rainbow trout ER and TR.

*Phthalate Esters.* Certain phthalates appear to be weakly estrogenic in whole fish. Jobling *et al.* (1995) investigated the estrogenicity of a variety of phthalate esters including di(2-ethyhexyl) phthalate (DEHP), butylbenzyl phthalate (BBP), and di-n-butylphthalate (DBP), in an assay to assess the ability of the compounds to inhibit binding of $E_2$ to the ER in rainbow trout liver. DEHP showed the lowest affinity for trout ER, with an affinity of approximately 1:100,000 that of $E_2$ for ER.

BBP showed an affinity of 1:10,000, however, DBP showed an affinity approaching 1:10 that of $E_2$. Jobling *et al.* (1995) noted that phthalates are the most ubiquitous man made contaminants in the environment, and that thousands of tons of plastics containing phthalates are disposed of annually in landfill sites, enabling these compounds to migrate into surface and groundwaters. Jobling *et al.* (1995) also stated that the presence of these compounds in the aqueous environment is well known in river, waste, and drinking water as well as in fish and sediments.

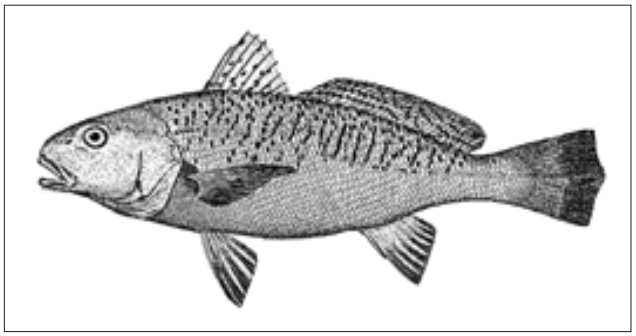
Christiansen *et al.* (1998) injected immature rainbow trout with 500 mg/kg of either BBP or DBP. A pre-exposure blood sample was taken on Day 0, followed by a blood sample and sacrifice on Day 9. Vitellogenin was measured using a direct sandwich ELISA technique. BBP increased vitellogenin by a factor of approximately three, while the DBP did not increase the vitellogenin concentration above the detection limit (Christiansen *et al.*, 1998). It is interesting to note here that while Christiansen *et al.* (1998) did not detect an increase in vitellogenin production in hepatocytes exposed to DBP, this same compound showed a fairly strong affinity for rainbow trout liver ER (Jobling *et al.*, 1995), possibly indicating an antagonistic potential for DBP.

*Metals.* In general, a number of trace elements have been shown to have a negative effect on endocrine function in fish. Ruby *et al.* (2000) investigated the effects of lead exposure on sexually maturing female rainbow trout. Fish exposed to 10 μg/L of waterborne lead ($Pb(NO_3)_2$) during the period of recrudescence had significantly lower GSIs and oocyte diameters than control fish on day 12. In addition, Ruby *et al.* (2000) assessed the effect of lead on the pituitary gland, the source of GTH. They found that the mean number of GTH producing granular pituitary basophils was lower in lead exposed females than in controls, suggesting the site of action of lead may be the pituitary.

Thomas (1988) administered lead to Atlantic croaker in the diet (1.34 mg/70g fish/day). After 30 days, the GSI in the croaker was only 32 percent of controls. The exposure to lead also resulted in lower circulat-

ing $E_2$ levels. However, *in vitro* experiments showed that the steroidogenic capacity of the ovarian tissue was not decreased by exposure to lead.

Thomas (1989) looked at the effects of cadmium on Atlantic croaker. Females at an early stage of gonadal recrudescence were exposed to cadmium for 30 days in 30‰ seawater at a concentration of 1 mg/L. In contrast to lead, cadmium caused an accelerated rate of ovarian growth when compared to controls. In addition, croaker exposed to cadmium had elevated levels of plasma $E_2$, leading Thomas (1989) to suggest that vitellogenesis was enhanced by exposure to cadmium. *In vitro* experiments revealed increased GTH secretion by the hemipituitaries in cadmium treated fish.



*Cadmium has been associated with elevated levels of $E_2$ and enhanced gonadal growth in female Atlantic croaker (Micropogonias undulatus). Image courtesy of NOAA/NMFS.*

Ricard *et al.* (1998) investigated the effects of $CdCl_2$ in rainbow trout. Adults were exposed to 10 and 25 µg Cd/L. Exposure to cadmium tended to decrease (though not significantly), concentrations of the thyroid hormones $T_3$ (triiodothyronine) and $T_4$ (thyroxine), which are believed to be involved in the adaptation of fish to environmental changes (e.g., temperature or salinity). In addition, exposure significantly increased concentrations of cortisol, a hormone involved in stress response in trout and other fish.

*β-Sitosterol.* Pulp and paper mill effluents can impact reproductive development in fish (Tremblay and Van Der Kraak, 1998; McMaster *et al.*, 1991). β-Sitosterol is one of the major plant sterols discharged from pulp and paper mills. MacLatchy *et al.* (1997) exposed goldfish to 75, 300, 600, and 1,200 µg/L of β-sitosterol, typical of concentrations from bleached kraft mill effluent. The experiment was run for 12 days using a static system renewed every two days. In general, plasma reproductive steroid levels in both males and females were decreased as a result of the treatment. MacLatchy *et al.* (1997) noted that gonadal cholesterol levels in fish treated with the highest level of β-sitosterol were also decreased, while gonadal cholesterol levels were unchanged in $E_2$ treated fish. This led the authors to suggest that decreases in plasma steroids could be related to the availability of cholesterol to the P450 enzymes (e.g., P-$450_{scc}$ and P-$450_{17\alpha}$) involved in the conversion of cholesterol to the androgens and estrogens.

Tremblay and Van Der Kraak (1998) investigated the effects of β-sitosterol on rainbow trout. β-Sitosterol induced the production of vitellogenin in immature rainbow trout during a 3 week exposure, although the induction appeared to be somewhat lower than that produced by 4-nonylphenol. At higher levels of β-sitosterol (75 and 100 µg/L), the levels of plasma testosterone fell below the detection limit of the radioimmunoassay (Tremblay and Van Der Kraak, 1998), similar to what McLatchy *et al.* (1997) found in goldfish.

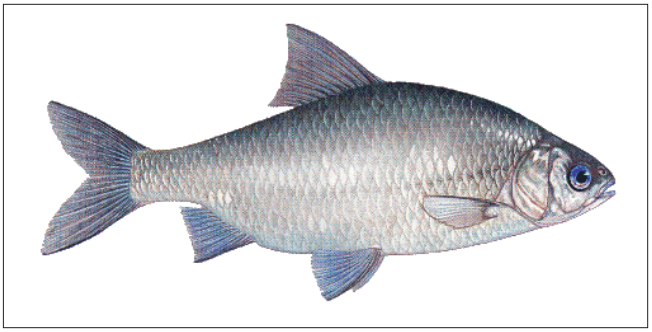
*Synthetic Estrogens.* As noted earlier, the synthetic estrogen $EE_2$ used in oral contraceptives can also have a significant impact on the endocrine system in fish at very low concentrations. Scholz and Gutzeit (2000) investigated long term effects of $EE_2$ exposure to Japanese medaka. Freshly hatched males (XY) were exposed in a semi-static system to 100 ng/L $EE_2$ for 2 months, followed by a 6 week recovery period. At this concentration, all medaka were sex reversed, having developed an ovary. In females (XX), a reduced production of eggs was noted and was reflected in the GSI at 10 and 100 ng/L. At a 10 ng/L concentration, no alterations of testicular structure or function was detected.

Lange *et al.* (2001) assessed the effects of $EE_2$ on the life cycle of fathead minnows. Fish were exposed in a continuous flow-through system to concentrations of 0.2, 1.0, 4.0, 16, and 64 ng/L (nominal concentrations) for 305 days. Male fish exposed to ≥ 4.0 ng/L $EE_2$ failed to develop normal secondary sexual characteristics. Histological analysis at 56 days posthatch revealed the female/male ratio in the 0.2 and 1.0 ng/L exposures to be approximately 50:50. In the 4.0 ng/L exposure, the sex ratio was 84:5, with ovotestes contained in 11 percent of the fish. In fish exposed to 4.0 ng/L for 152 days, no testicular tissue was observed at all. Lange *et al.* (2001) calculated an overall no observed adverse effect $EE_2$ concentration of 1.0 ng/L.

Metcalfe *et al.* (2001) assessed the histologic effects of $EE_2$ on Japanese medaka. Fish were exposed in a static renewal system for 85-110 days post hatch. Experiments were begun one day after hatch, and fish were exposed in a static renewal system to 0, 0.1, 1.0, 10, 100, and 1,000 ng/L $EE_2$ (nominal concentration). The highest concentration was lethal to most of the fish, but those that did survive contained eosinophilic fluid in the organs and body cavity. In the 100 ng/L exposure, 91 percent of the fish were identified as female, significantly different from the controls. Ovotestes were observed in males from all treatments except the 100 ng/L.

## FRESHWATER FIELD STUDIES

The next two sections contain summaries of some of the endocrine disrupter-related field work completed on fish in fresh and saltwater systems. Much of the work to date on environmental endocrine disruption has occurred in Europe. Studies reviewed in both sections fell into three broad categories and included investigations adjacent to STPs, pulp and paper mills, and general surveys that covered a defined geographic area rather than specific point source discharge types. Investigations occurring near these activties have produced some of the most significant findings. Table 6 contains a summary of results from a number of the freshwater investigations reviewed in this report.



*The discovery of hermaphrodite specimens of the roach Rutilus rutilus in rivers close to sewage outfalls in the U.K. helped spur investigations of endocrine disruption in the U.K. Image courtesy of FishBase.org.*

***Sewage Treatment Plants.*** Some of the most dramatic examples of endocrine disruption in fish have been observed near the outfalls of STPs. All of the effects appear to be estrogenic. Recent work indicates that natural and synthetic estrogens are probably responsible for much of the estrogenicity in STP effluents and effects seen in fish, although the APEs may also be contributing to observed effects under certain influent characteristics. A number of studies that have attempted to characterize the estrogenic components of STP effluents are also included in this section.

*United Kingdom.* Part of the impetus for research into endocrine disruption in fish, can be traced to casual observations by anglers in the mid-1980s of hermaphrodite roach (*Rutilus rutilus*) inhabiting STP lagoons (Routledge et al., 1998; Purdom *et al.*, 1994). In one of the earlier studies of the association between STP effluents and estrogenic effects, Purdom *et al.* (1994) carried out a number of field exposures with rainbow trout (*Oncorhynchus mykiss*) at 15 STPs throughout England and Wales. Controls consisted of maintaining fish in well or spring water. Male and female fish were exposed in or near the effluent discharges from 1 to 3 weeks. The original hypothesis was that the synthetic estrogen $EE_2$ found in oral contraceptives contributed to the estrogenic effect of the STP effluents.

At each site, Purdom *et al.* (1994) found clear evidence of increased plasma vitellogenin in all fish, both male and female. Vitellogenin in male fish at the control sites ranged from 0.05-1.80 µg/ml, and in females from 4.5-88.3 µg/ml. In males exposed to effluents from STPs, plasma vitellogenin values ranged from 23-147,000 µg/ml, and in females from 470-112,000 µg/ml. In most cases, the effect of the effluent was so obvious that tests of statistical significance were deemed inappropriate (Purdom *et al.*, 1994). From these results, the authors concluded there was a nationwide distribution of estrogens in STP effluents.

Although one of the goals of the research had been to determine whether $EE_2$ contributed to the effect, attempts to measure levels in the effluents using GC/MS as well as radioimmunoassay were unsuccessful. Purdom *et al.* (1994), however, felt that $EE_2$, and possibly the APEs were likely candidates for the observed effects.

Sumpter and Jobling (1995) also conducted a nationwide survey in England and Wales, exposing caged male rainbow trout to effluents from 28 STPs covering all 10 Water Authority areas, in order to determine the extent of estrogenic effects. They noted that many of the compounds identified as endocrine

disrupters inevitably find their way into rivers via STPs. At 13 of the 28 sites, trout were not able to survive the 3 week experiment. At the 15 sites where fish did survive, there was a pronounced increase in plasma vitellogenin in all fish, compared to controls (Figure 10). Variability in the response ranged from 500 to over 50,000 fold at the different sites, which they attributed to a number of factors including the composition of the effluent (i.e., which compounds were present and at what concentrations), water temperature, and the age of the fish.

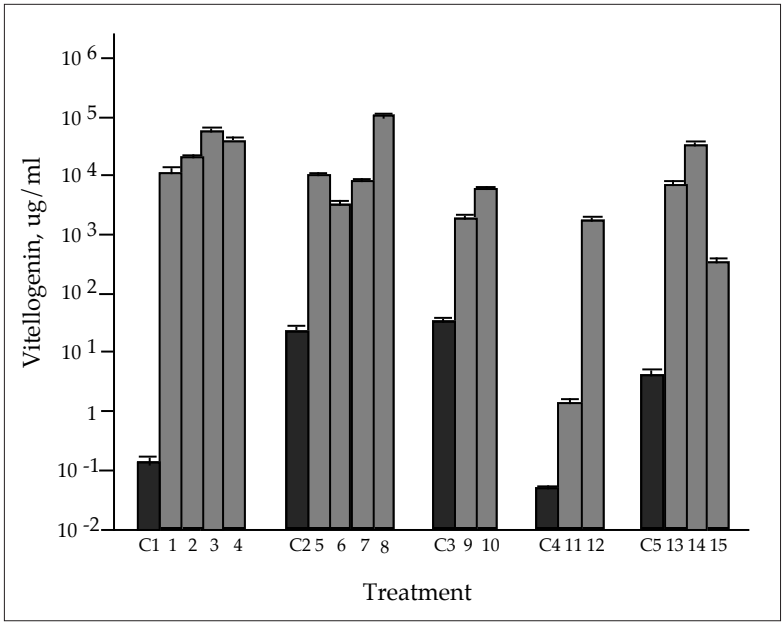



Figure 10. Results of a survey to assess estrogenicity of effluents from sewage treatment plants to rainbow trout in England and Wales. Numbers represent the 15 STPs where fish survived; C1-C5 were control sites. Mean ±SEM. From Sumpter and Jobling, 1995. Reprinted by permission.

Sumpter and Jobling (1995) concluded that effluents from all STPs in the U.K. were probably estrogenic to fish. They also noted it was possible that some synthetic estrogens, particularly $EE_2$, may be a contaminant in the aquatic environment.

Harries *et al.* (1996) completed a series of experiments in the U.K. with male rainbow trout placed in cages at the point of discharge and varying distances downstream of five sewage treatment outfalls on the River Lea, which flows past the northeastern portion of London. During the summer months, effluent from STPs comprise as much as 82 percent of the total flow of the upper portion of the river (Harries *et al.*, 1996). After 3 weeks at the majority of sites, fish held up to 15 kilometers downstream of the inputs showed an increase in plasma vitellogenin, with statistically significant elevations up to 4.5 kilometers downstream.

Levels of vitellogenin in the controls (reservoir and laboratory) were approximately 0.01 μg vitellogenin/ml plasma. Levels of vitellogenin in male fish exposed to River Lea water ranged from approximately 0.1 to 1,000 μg/ml vitellogenin/ml plasma during the July/August timeframe. Field exposures repeated during November resulted in lower responses. Harries *et al.* (1996) attributed this to an overall increase in dilution resulting from additional rainfall during that time of year. They also concluded that the elevated levels of vitellogenin were the result of treated sewage effluent in the river, as there was a general pattern of decline in vitellogenin levels with distance downstream from each outfall.

Although the compounds responsible for induction were not identified, the authors speculated that the alkylphenols or the parent APEs, along with the synthetic estrogen $EE_2$ might have contributed to the observed estrogenicity. Harries *et al.* (1996) noted, however, that no gross abnormalities have been reported in the population structure of rainbow trout in the River Lea, but that the stocking of fish in the river would tend to reduce the importance of natural reproduction.

In another study by Harries (Harries *et al.*, 1997), an assessment was made of vitellogenesis in caged male rainbow trout placed at various distances downstream of STPs in five other rivers in England (Great Stour, Arun, Chelmur, Stour, and Aire). In four cases where the cages were placed directly in the effluent, there were rapid and very significant increases in plasma vitellogenin in all fish, ranging from 0.03 to almost 52 μg vitellogenin/ml plasma, demonstrating the effluent was estrogenic. Initial vitellogenin levels in the males were around 0.01 μg/ml. The GSIs of the exposed fish were also significantly reduced.

Table 6. Field studies of endocrine disruption in freshwater species of fish.

| Fish/Species | Sex | Land Use | Location/ Study Site | Caged/wild fish | Vtg | I | T | E2 | GSI | GD | EM | Possible Cause(s) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Roach *(Rutilus rutilus)* | M/F | Sewage treatment plants | 15 sites in England | caged; 1-3 weeks | + | | | | | | | 17α-ethinylestradiol | Purdom *et al.*, 1994 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plants | 15 sites in England and Wales | caged; 3 weeks | + | | | | | | | 17α-ethinylestradiol | Sumpter and Jobling, 1995 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plants | 5 sites on River Lea in U.K. | caged; 3 weeks | + | | | | | | | 17α-ethinylestradiol and alkylphenols | Harries *et al.*, 1996 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plants | 5 sites on River Lea in U.K. | caged; 3 weeks | + | | | | – | | | Estrogens and nonylphenol | Harries *et al.*, 1997 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plants | Berlin STP | 6 months | + | | | | | | | Estrogens and nonylphenol | Hansen *et al.*, 1998 |
| Roach *(Rutilus rutilus)* | M | Sewage treatment plants | 8 rivers in the U.K. | wild | + | + | | | – | | | Estrogens and alkylphenols | Jobling *et al.*, 1998 |
| Roach *(Rutilus rutilus)* | M | Sewage treatment plants | Chelmsford STP, U.K. | caged; 1 month | + | + | | | | | | Estrogens and alkylphenols | Rodgers-Gray *et al.*, 2000 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plants | River Aire, U.K. | caged; 3 weeks | + | | | | | | | Alkylphenols from wool scouring plant | Sheahan *et al.*, 2002a |
| Gudgeon *(Gobio gobio)* | M | Sewage treatment plants | River Aire, U.K. | wild | + | + | | | | | | Estrogens and alkylphenols | van Aerle *et al.*, 2001 |
| Rainbow trout *(Oncorhynchus mykiss)* | M | Sewage treatment plant | Western Sweden | caged; 2 weeks | + | | | | | | | Estrogens and alkylphenols | Larsson *et al.*, 1999 |
| Mosquitofish *(Gambusia a. holbrooki,* | M | Sewage treatment plant | Hawkesbury River, Australia | wild | | | | | | | – | Estrogens and alkylphenols | Batty and Lim, 1999 |
| Common carp *(Cyprinus carpio)* | M | Sewage treatment plants | St. Paul, Minnesota | wild | + | | NE | | | | | Estrogens | Folmar *et al.*, 1996 |
| Walleye *(Stizostedion vitreum)* | M | Sewage treatment plants | St. Paul, Minnesota | wild | + | | – | + | | | – | Estrogens and alkylphenols | Folmar *et al.*, 2001 |
| Walleye *(Stizostedion vitreum)* | F | Sewage treatment plants | St. Paul, Minnesota | wild | + | | | + | | – | | Estrogens and alkylphenols | Folmar *et al.*, 2001 |

M/F, male and female fish collected in study and effects same in both sexes; NE, no effect; Vtg, vitellogenin; I, intersex condition; T, testosterone; E2, estradiol; GSI, gonadosomatic index; GD, gonadal development; EM effect on external male/female morphology

Table 6. Field studies of endocrine disruption in freshwater species of fish (continued).

| Fish/Species | Sex | Land Use | Location/ Study Site | Caged/wild fish | Vtg | I | T | E2 | GSI | GD | EM | Possible Cause(s) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Largemouth bass (*Micropterus salmoides floridanus*) | M | Pulp and paper mill | St. Johns River | wild | | | – | | | | | β-Sitosterol | Sepúlveda *et al.*, 2002 |
| Largemouth bass (*Micropterus salmoides floridanus*) | F | Pulp and paper mill | St. Johns River | wild | – | | | | – | | | β-Sitosterol | Sepúlveda *et al.*, 2002 |
| Whitefish (*Coregonus lavaretus*) | M/F | Pulp and paper mill | Lake Saimaa, Finland | caged; 1 month | + | | | | | | | β-Sitosterol | Soimasuo *et al.*, 1998 |
| Perch (*Perca fluviatilis L.*) | F | Pulp and paper mill | Lake Saimaa, Finland | wild | | | | | – | | | β-Sitosterol | Karels *et al.*, 2001 |
| White sucker (*Catostomus commerson* | M/F | Pulp and paper mill | St. Maurice River, Quebec | wild | | | – | – | | | | β-Sitosterol | Gagnon *et al.*, 1994 |
| White sucker (*Catostomus commerson* | M/F | Pulp and paper mill | Lake Superior, Canada | wild | | | – | – | – | | | β-Sitosterol | McMaster *et al.*, 1991 |
| Mosquitofish (*Gambusia holbrooki*) | F | Pulp and paper mill | St. John's River, Florida | wild | | | | | | | – | β-Sitosterol | Bortone and Cody, 1999 |
| Mosquitofish (*Gambusia holbrooki*) | F | Pulp and paper mill | Perdido Bay, Florida | wild | | | | | | | – | β-Sitosterol | Cody and Bortone, 1997 |
| Mosquitofish (*Gambusia holbrooki*) | F | Pulp and paper mill | Fenholloway River, Florida | wild | | | | | | | – | β-Sitosterol | Jenkins *et al.*, 2001 |
| Channel catfish (*Ictalurus punctatus*) | F | Sugar beet processing plant | Red River, North Dakota | wild | | | | | | | – | β-Sitosterol | Hegrenes, 1999 |
| Largemouth bass (*Micropterus salmoides*, | M/F | Power plant, chemical manufacture | Escambia/Blackwater River, Florida | wild | + | | – | | | | | Contaminants | Orlando *et al.*, 1999 |
| Common carp (*Cyprinus carpio*) | M | General survey | Various locations in U.S. | wild | +/NE | | –/NE | | | –/NE | | Contaminants | Goodbred *et al.*, 1997 |
| Common carp (*Cyprinus carpio*) | F | General survey | Various locations in U.S. | wild | | | | –/NE | | | | Contaminants | Goodbred *et al.*, 1997 |
| Fathead minnow (*Pimphales promelas*) | M/F | General survey | Mississippi River | wild | NE | | | | | | | None | Parks *et al.*, 1997 |

M/F, male and female fish collected in study and effects same in both sexes; NE, no effect; Vtg, vitellogenin; I, intersex condition; T, testosterone; E2, 17β-estradiol; GSI, gonadosomatic index; GD, gonadal development; EM effect on external male/female morphology

On the River Aire, the effluent was extremely estrogenic. Vitellogenin levels at the sites downstream of the Marley STP in Keighley on the River Aire ranged from approximately 1 - 10,000 μg vitellogenin/ml plasma. Harries *et al.* (1997) noted there are a number of wool scouring mills in the basin that use alkylphenol polyethoxylate detergents to wash the fleece. An analysis of water samples near the Marley STP confirmed the presence of 4-nonylphenol, with concentrations as high as 330 μg/L, compared with concentrations of 2.9 and 0.9 μg/L near other STPs. Harries *et al.* (1997) remarked that the estrogenicity at other sites could be due to additional contaminants such as dieldrin, lindane, and phthalates.

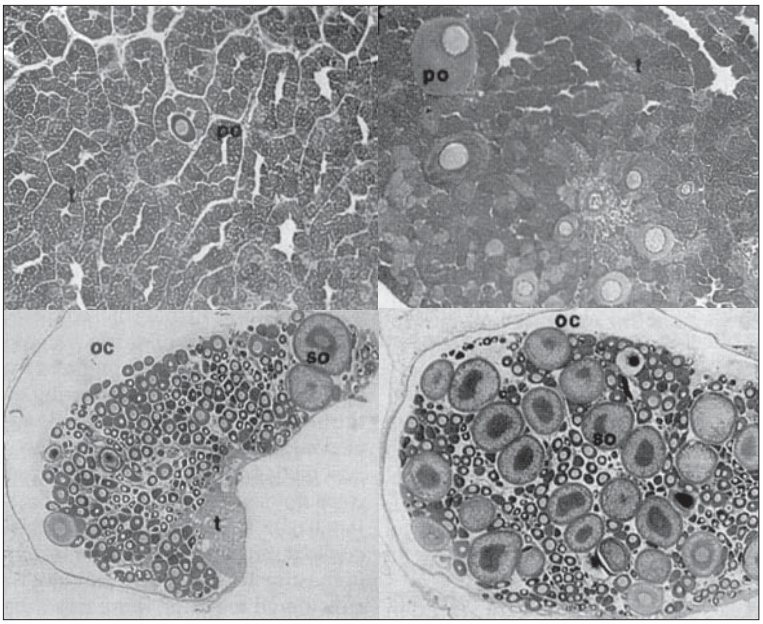



Figure 11. Gradations of ovotestes discovered in roach fish (*Rutilus rutilus*) in the U.K. The degree of intersexuality ranged from normal testes to testes with both spermatogenic tissue (t) and occasional primary oocytes (po) to gonads that contained primary and secondary oocytes (so) and an ovarian cavity (oc). From Jobling *et al.* (1998). Reprinted by permission.

Sheahan *et al.* (2002a) conducted a followup study at the Marley STP in Keighley in an effort to identify the estrogenic contaminants in the effluent. High performance liquid chromatography fractionation paired with recombinant yeast assays revealed the estrogenic activity of the effluent was primarily due to the alkylphenolic compounds 4-nonylphenol, nonylphenol-1-ethoxylate, and nonylphenol-2-ethoxylate, along with the natural estrogens $E_2$ and estrone. Studies with caged male rainbow trout (1 year old) exposed for 3 weeks to the effluent resulted in vitellogenin levels as high as 10,000 μg/ml. Sheahan *et al.* (2002a) noted that the high use of APEs by the wool scouring mills which discharge to the STP created an unusual situation, one in which the alkylphenolic chemicals may potentially have been responsible for up to 90 percent of the vitellogenin induction observed in caged male trout held in the final effluent discharge from the STP. Sheahan *et al.* (2002a) noted, however, that given the differential uptake, metabolism and bioconcentration of the alkylphenolic chemicals versus the estrogens in fish, it was very difficult to assess which of these two groups contributed most to the effects seen in the fish.

Efforts have been underway since the early 1990s at the Marley STP in Keighley to tighten the discharge limits in an effort to improve downstream water quality, including reducing the discharge of APEs. Sheahan *et al.* (2002b) monitored the concentration of the alkylphenolic chemicals in the effluent as well as the effect on caged rainbow trout from 1994 to 1997. Reductions in the concentration of the APEs as a result of improved treatment processes, and a subsequent reduction in vitellogenin production and an increase in the GSI in caged male rainbow trout has been observed. Concentrations are likely to continue to decrease as the major wool scouring mill that discharged to the STP has since relocated to another basin (Sheahan *et al.*, 2002b).

Jobling *et al.* (1998) demonstrated the widespread occurrence (Figure 11) of intersex roach *Rutilus rutilus*, a cyprinid fish in U.K. rivers, and that it was associated with exposure to effluents from STPs. Wild populations of roach were sampled upstream and downstream of STPs in eight rivers (Wreake/Eye, Ouse, Lea, Arun, Nene, Trent, Rea, and Aire), representing a range in water quality from very good to poor (Jobling *et al.*, 1998). Upstream sites were separated from downstream sites by one or more physical barriers, and upstream sites were usually separated from downstream sites by several kilometers. Upstream sites were still subject to exposure from sewage effluents from smaller treatment plants even further upstream. Because no riverine sites

could be located that were not impacted by STPs, controls consisted of lakes and canals.

Although the level of treatment at the various STPs was not provided, Jobling *et al.* (1998) characterized the fish capture sites in terms of the strength of the influent or load coming into each plant, and the diluting ability of the receiving waters. The level of vitellogenin in male and female fish were characterized using a radioimmunoassay, and the gonads of the fish were examined histologically for gonadal anomalies.

When examined externally, all fish collected appeared to be either male or female. Histological examination of the fish, however, revealed a large proportion of the males examined were in fact intersex. Specifically, intersex males were characterized by the presence of ovotestes and/or an ovarian cavity in the testicular tissue (Figure 11).

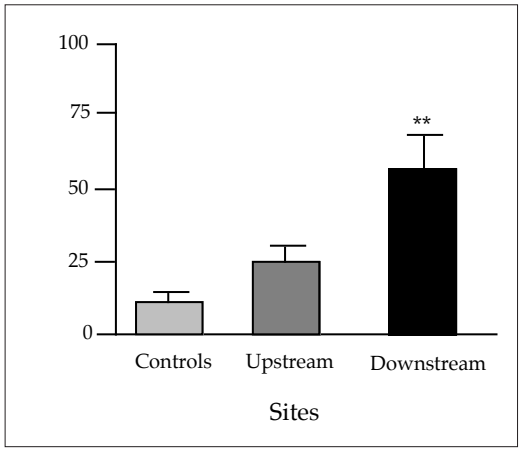



Figure 12. Comparison of the overall incidence of intersex roach in control, upstream and downstream sites. From Jobling *et al.*, 1998. Reprinted by permission.

The incidence of intersexuality in males (Figure 12) ranged from 16 to 100 percent at sites downstream of the STPs, and from 12 to 44 percent at upstream sites. In some males, the degree of intersexuality was slight, while in some fish, more than 50 percent of the gonadal tissue was ovarian. The degree of intersexuality was also significant at downstream sites. Intersexuality at the control sites ranged from 4 to 18 percent. Jobling *et al.* (1998) noted that while intersexuality is considered rare in roach, rates of up to 5 percent have been observed in the common carp *(Cyprinus carpio)*, a cyprinid fish related to the roach. The authors stated, however, that even though a low level of intersexuality in roach may be "natural", the results of the survey suggested the incidence of intersexuality in U.K. rivers was considerably higher than what might be expected, and that the condition was strongly associated with discharges from STPs.

An evaluation of plasma vitellogenin in male roach also provided evidence that some populations were exposed to estrogenic contaminants. Jobling *et al.* (1998) noted that the concentration of vitellogenin found in intersex fish was intermediate between female roach and males (Figure 13). There was also a positive correlation between the degree of intersexuality and plasma vitellogenin concentrations, and a negative correlation between vitellogenin levels in males and the GSI. A second species of fish, the gudgeon (*Gobio gobio*) from several rivers exhibited similar anomalies, indicating the effects were unlikely to be species specific.

Jobling *et al.* (1998) discussed possible causes for the observed effects. There was a significant correlation between the proportion of intersex fish and the strength of the effluent from the STPs, leading the authors to conclude that estrogenic constituents of the sewage effluents were responsible for the occurrence of intersexuality in the fish. While the authors did not investigate the chemicals responsible, they noted that natural ($E_2$ and estrone) and synthetic ($EE_2$) estrogens have been reported in STP effluents, including some of the effluents that impact the sites sampled in the study. In addition, they noted that the concentrations of estrogens reported in STP effluents were high enough to cause vitellogenin synthesis in males in laboratory studies. They cautioned, however, that other chemicals, namely the alkylphenolic compounds, are also present in many sewage effluents, and thus the role of these types of chemicals should not be underestimated.

In a followup study, van Aerle *et al.* (2001) assessed the extent of reproductive endocrine disruption in gudgeon in the River Aire. Fish were collected at a series of locations near STPs, as well as control (lake) sites. Vitellogenin was measured using an ELISA. The GSI was calculated and the gonads were examined histologically for gonadal anomalies. Intersex gudgeon were found at all sites, with frequencies as high as 15 percent. Fish from Thwaite Weir on the River Aire had the greatest severity of intersexuality, with over 40 percent of the fish collected having greater than 50 percent ovarian tissue containing both primary and secondary oocytes interspersed within the testicular tissue.

Interestingly, while no intersex fish were found at a site downstream from the Marley STP at Keighley, 97 percent of the gudgeon collected from that site were female. Although they noted that monosex populations may be natural for this species, because this phenomenon was not observed at any other locations on the River Aire, van Aerle *et al.* (2001) stated they could not rule out the possibility that the almost exclusively female population of gudgeon was the result of complete sex reversal in males downstream of the Marley STP. At all locations, unlike with roach, there were no differences in the GSI between sites. Although intersex gudgeon were found at the sites sampled, the incidence was not as high as that found by Jobling *et al.* (1998) for roach. van Aerle *et al.* (2001) stated this was somewhat surprising as gudgeon are a more demersal species, frequently in contact with sediments, which would seem likely to increase their exposure to estrogenic compounds. As a possible explanation, they noted that most of the gudgeon collected were from areas with gravel beds as opposed to mud, and also that species likely differ in terms of their susceptibility to endocrine disruption. van Aerle *et al.* (2001) concluded, as did Jobling *et al.* (1998), that the occurence of intersex fish was not species specific.

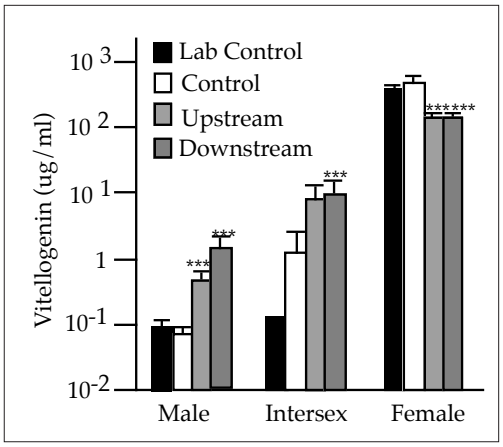



Figure 13. Plasma vitellogenin in roach from various rivers, upstream and downstream of STP effluents. Asterisks denote differences at 0.001 level of significance from field control sites. From Jobling *et al.* (1998). Reprinted by permission.

Desbrow *et al.* (1998) reported on the results of a chemical fractionation and screening technique to isolate estrogenic components from seven domestic STP effluents in the U.K., using a recombinant yeast assay. Three sterols were isolated from the estrogenic fractions of the sewage extracts, and were identified as the natural hormones $E_2$ and estrone, and the synthetic hormone $EE_2$. Concentrations of $E_2$ and estrone ranged from 1 ng/L to almost 50 and 80 ng/L, respectively. The concentration of $EE_2$ was generally below the detection limit but in three of the effluent samples was found at concentrations ranging from 0.2-7.0 ng/L. They suggested that natural and synthetic hormones might be responsible for the observed induction of vitellogenin in males placed downstream of discharges from STPs that received mainly domestic wastes (Desbrow *et al.*, 1998).

In a follow-up paper, Routledge *et al.* (1998) conducted a series of *in-vivo* experiments with adult male rainbow trout and adult roach for 21 days using similar, environmentally relevant concentrations of $E_2$, and estrone (1, 10, and 100 ng/L). They found that both male rainbow trout and roach were very sensitive to these concentrations of $E_2$ and estrone, producing high levels of plasma vitellogenin. The threshold $E_2$ concentration in the trout was estimated to be between 1 and 10 ng/L. Male rainbow trout seemed more sensitive to $E_2$ than male roach, which they suggested could be due to the smaller egg size and subsequently lower vitellogenin requirements of the roach. They also noted that the synthetic estrogen $EE_2$ is not only a potent estrogen but is also persistent due to the 17α-ethinyl group which acts to reduce its rate of metabolism, and that when present could be a major contributor to any estrogenic response. Routledge *et al.* (1998) concluded that the steroidal estrogens identified in the domestic sewage effluent were present in sufficient amounts to cause the synthesis of vitellogenin *in vivo*.

Rodgers-Gray *et al.* (2000) investigated the estrogenicity of effluent from the Chelmsford STP in the U.K., using a toxicity identification and evaluation (TIE) method. Estrogenic fractions of the effluent from a particular STP were identified using a estrogen-responsive recombinant yeast screen followed by identification of the estrogenic components using gas chromatography/mass spectrometry. Rodgers-Gray *et al.* (2000) were able to show that the natural estrogens $E_2$ and estrone were the major contributors to the estrogenic activity of the effluent. The synthetic hormone $EE_2$ was also detected occasionally along with 4-nonylphenol and nonylphenol ethoxylates, although the APEs were not identified as contributing to the estrogenicity of the samples of STP effluent.

Male roach were also exposed to various concentrations of the effluent. After only 1 month in the 100 percent effluent experiment, the concentration of plasma vitellogenin in exposed male roach increased to 109 μg/ml. The concentrations of $E_2$ (7-88 ng/ml) and estrone (15-220 ng/ml) measured in the effluent at the time of the exposures suggested to Rodgers-Gray *et al.* (2000) that the natural steroids alone were probably capable of producing the observed induction of vitellogenin. In a little over 1 percent of the males exposed to the effluent, primary oocytes were occasionally detected in testicular tissue, although the intersexuality was not correlated with effluent concentration or duration of exposure.

*Western Sweden*. Larsson *et al.* (1999) investigated the estrogenicity of an effluent from a small STP in western Sweden receiving primarily domestic wastewater. They exposed juvenile rainbow trout in cages downstream of the STP for a period of 2 weeks and found a very significant induction of vitellogenin (1.5 mg/ml) in all eight exposed fish.

Gas chromatography/mass spectrometry revealed the presence of the synthetic estrogen $EE_2$(4.5 ng/L), the natural estrogens estrone (5.8 ng/L), and $E_2$ (1.1 ng/L), along with 4-nonylphenol (840 ng/L) and bisphenol-A (490 ng/L). They noted that because the estrogenicity of 4-nonylphenol and bisphenol-A is typically $10^{-3}$ to $10^{-4}$ that of natural estrogens, the relative importance of these phenolic compounds in the effluent was probably minor. On the other hand, the level of $EE_2$ exceeded levels shown to be estrogenic in fish by 45 times. They concluded that levels of $EE_2$ found in the effluent were sufficient to explain a major part of the observed estrogenicity, and that this compound poses a potential hazard to fish and possibly other aquatic organisms.

*Germany*. Hansen *et al.* (1998) investigated the estrogenicity of effluents from a Berlin STP to male rainbow trout exposed to varying concentrations of effluent (10, 20, 30, and 40 percent), and found there was a marked increase in vitellogenin in males exposed to 20 percent and greater STP effluent.

In southwestern Germany, Korner *et al.* (2001) analyzed effluents from 16 municipal STPs in the state of Baden-Württemberg using the E-screen (MCF-7) assay to detect estrogenic activity. Cell number was assessd by the measurement of total protein using sulforhodamine B and extinction at 550 nm. Effluents from all the STPs contained concentration dependent estogenic activity or EEQ in the range of 0.2 - 7.8 ng/L, with the natural estrogens $E_2$, $EE_2$, and, to a lesser extent, estrone contributing 90 percent or more of the $E_2$ equivalent concentration.

*New South Wales, Australia.* Batty and Lim (1999) investigated the effects of sewage effluent on the morphology of male mosquitofish (*Gambusia a. holbrooki*) in a river in New South Wales. In mosquitofish, the modified anal fin, or gonopodium, is formed under the influence of testosterone, and is critical for sperm transfer. Batty and Lim (1999) looked at differences in gonopodium length in fish inhabiting reference sites versus males inhabiting sites downstream of the STP. The gonopodium in males inhabiting the site downstream of the STP was significantly reduced in size compared to the reference site. They also established a site that received agricultural runoff (fertilizers and pesticides), but found no differences between the fish at this site versus the reference site.

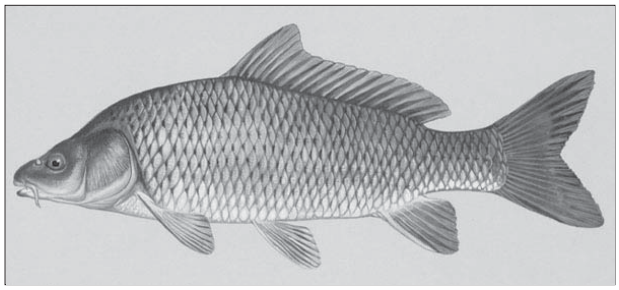
*Minnesota/Mississippi Rivers.* Folmar *et al.* (1996) investigated the levels of vitellogenin in common carp collected at five riverine locations around St. Paul, Minnesota. Vitellogenin was identified using Western blot analysis and quantified using a capture ELISA assay.

Seven of 10 male fish collected from an effluent channel below the St. Paul, Minnesota metropolitan STP plant had detectable levels of vitellogenin; six males had vitellogenin approaching those of females captured at the same site (Folmar *et al.*,1996). Interestingly, males from the same location had plasma testosterone concentrations comparable to carp taken from the St. Croix River reference site. Folmar *et al.* (1996) also noted that male fish collected on the Minnesota River, a tributary that receives extensive agricultural runoff, did not show any induction of vitellogenin. As noted earlier, certain pesticides or components of pesticide formulations may be endocrine disrupters.

In a followup study, Folmar *et al.* (2001) assessed altered sex steroid levels and vitellogenin in walleye (*Stizostedion vitreum*) at two sites near the same STP in St. Paul, Minnesota and at a reference site on the Snake River. All fish collected, both males and females, from the STP effluent channel contained measurable levels of vitellogenin. Vitellogenin in males ranged as high as 3.2 mg/ml near the STP; no vitellogenin was detected in males from the downstream or reference site. Males from the effluent channel as well as a downstream site also had

significantly depressed levels of testosterone, and in the effluent channel, significantly elevated levels of $E_2$. Females taken adjacent to the STP also had significantly elevated levels of $E_2$ as compared to those from the downstream or reference sites. Finally, spermatogenesis appeared somewhat reduced as a number of fish taken near the STP contained spermatogonia with no evidence of spermatocytes/spermatids. Folmar *et al.* (2001) noted, however, that there was no correlation between $E_2$ concentration and vitellogenin in males which they suggested might be due to the presence of other natural and synthetic estrogens, and possibly to 4-nonylphenol and 4-nonyphenol ethoxylates.

*Lake Mead, Nevada.* Snyder *et al.* (2001) used the E-Screen with an estrogen receptor controlled luciferase report gene construct (MCF-7-luc) to identify and quantify estrogen receptor agonists in STP effluents at five sites in Lake Mead. Samples of effluent were fractionated (HPLC) to assess the contribution of PAHs, PCBs, and organochlorine pesticides, nonyl and octylphenols, or $E_2$ and $EE_2$. Results of the E-screen assays for the Lake Mead samples indicated that $E_2$ and $EE_2$ were the dominant environmental estrogens, accounting for over 99 percent of the observed estrogencity in the samples.



*The common carp, Cyprinus carpio has been used in a number of endocrine disrupter field studies. Image courtesy of USDOI/FWS.*

***Pulp and Paper Mills.***
Studies adjacent to pulp and paper mill discharges in Europe and in North America have revealed a number of effects, including depressed levels of reproductive hormones, decreased GSIs in both male and female fish, and external masculinization of females.

*Lake Saimaa, Finland*. Soimasuo *et al.* (1998) studied the effects of pulp and paper mill effluents on whitefish (*Coregonus lavaretus*) in Lake Saimaa in Southeast Finland. Immature hatchery-reared fish were exposed for 1 month at a series of sites on the lake. Interestingly, there were no significant changes in $E_2$ or testosterone concentrations in juvenile whitefish at any of the sites. There was expression of the vitellogenin gene in the vicinity of the pulp and paper mill discharging the highest concentrations of wood derived compounds (including β-sitosterol).

More recently, Karels *et al.* (2001) found lowered levels of $E_2$ and testosterone in female and male perch (*Perca fluviatilis* L.) in Lake Saimaa at sites downstream of two pulp and paper mills. Roach collected at the same sites, however, showed no significant differences in these steroid hormones. This may be an indication of differences in susceptibility between species. The GSI and fecundity in female perch was significantly lower at the sites adjacent to the pulp and paper mills, but similar in roach.

*St. Maurice River, Quebec*. Gagnon *et al.* (1994) investigated the effects of bleached kraft mill effluents on white sucker (*Catostomus commersoni*) in the St. Maurice River which empties into the St. Lawrence River. The site was chosen as there are no other industries or towns within 100 kilometers of the mill, which Gagnon *et al.* (1994) felt offered a unique opportunity to study the effects of effluents from the pulp and paper mill. Fish were sampled at three sites, one 3 kilometers above the mill (reference site), a second 2 kilometers downstream, and a third 95 kilometers further downstream. Fish were captured both during and after the spawning periods. In females, levels of $E_2$ and testosterone were significantly lower at the two downstream sites compared to the reference site.

In males, 11-ketotestosterone was significantly lower in fish at the two downstream sites. MacLatchy *et al.* (1997) found a similar reduction in plasma steroids in both males and females exposed in the laboratory to β-sitosterol, one of the major byproducts of the wood pulp delignification process (Cody and Bortone, 1997).

Although plasma steroid hormone levels in both male and female white sucker were depressed, the GSI was similar at all sites, and fecundity (number of eggs) in females was variable. The authors concluded that the parameters measured did not allow them to clearly relate pertubations in plasma steroid hormone levels to impaired reproduction as measured by gonad weight and fecundity.

*Lake Superior, Canada*. McMaster *et al.* (1991) investigated the effects of effluents from a bleached kraft pulp mill on white sucker. Male and female fish were collected from two areas on Lake Superior, spawning streams in Jackfish Bay, which is subject to paper mill effluents, and Mountain Bay, which receives no pulp mill effluents. Neither sampling area received municipal or industrial effluents. Fish of both sexes exposed to the pulp mill effluent exhibited significantly reduced GSIs, increased hepatosomatic indices or HSIs (liver weight/body weight X 100), and increased age to maturity. In addition, there were significant reductions in 11-ketotestosterone in males and $E_2$ in females.

*St. John's River, Florida.* Bortone and Cody (1999) investigated the phenomenon of arrhenoidy (masculinization) in female mosquitofish (*Gambusia holbrooki*) from Rice Creek and Etonia Creek, tributaries of the St. Johns River. Fish were collected at locations upstream and downstream of a large paper mill on Rice Creek, as well as a reference site (Etonia Creek). The parameter investigated was the transformation of the anal fin of females into the gonopodium-like structures typical of developing males. A simple measurement of anal fin length (AL) divided by standard body length (SL) was used to assess the degree of arrhenoidy in females. Females collected at the site immediately downstream of the mill on Rice Creek had significantly larger AL/SL values compared to the Etonia Creek reference site, indicating a masculinization, or elongation of the anal fin in females. Sites further downstream showed what appeared to be a distance/dose related response to the effluent exposure. Although not abundant enough to allow between site comparisons, Bortone and Cody (1999) noted gonopodial development in females of two other species, the least killifish (*Heterandria formosa*) and the sailfin molly (*Poecilia latipinna*) in Rice Creek.

*Pulp and paper mills can be a source of endocrine disrupting compounds. Image courtesy of USDOI/NPS.*

Sepúlveda *et al.* (2002) evaluated biomarkers of reproductive function in Florida largemouth bass *(Micropterus salmoides floridanus)*, at a number of sites in the St. Johns River, including one in close proximity to the paper mill on Rice Creek. Parameters measured included GSI, plasma vitellogenin, $E_2$, 11-ketotestosterone, and a histological assessment of the gonads. Both male and female fish were collected.

Female bass collected near the paper mill during spawning season had vitellogenin levels approximately an order of magnitude lower than those fish collected at the reference site. $E_2$ levels were also significantly lower. The GSI in both female and male largemouth bass during the spawning season were lower at the site near the pulp and paper mill compared to the reference site. Levels of 11-ketotestosterone were also significantly lower in males collected near the paper mill. Sepúlveda *et al.* (2002) suggested that lower levels of vitellogenin in females, and lowered GSIs in females and males near the mill, may have been the result of altered levels of $E_2$ and 11-ketotestosterone in these fish. Fish collected from a site 40 kilometers downstream of the paper mill also exhibited evidence (significantly lower GSI and vitellogenin in females, significantly lower 11-ketotestosterone in males) of endocrine disruption. Sepúlveda *et al.* (2002) suggested though that elevated levels of PAHs and PCBs in the tissues of the fish may have been responsible, and were a result of contamination associated with marine shipping near this site and not from the paper mill upstream.

*Perdido Bay, Florida*. In the late 1970s, Howell *et al*. (1980) had reported on the presence of arrhenoidy in mosquitofish in Elevenmile Creek which receives pulp and paper mill effluent. More recently, Cody and Bortone (1997) completed a followup investigation in Elevenmile Creek after process changes had been made at the mill. These changes included the reclamation of 97 percent of tall oils and terpenes, by-products of the pulping process. The phytosterol β-sitosterol is a major component of the tall oils discharged. The reference or control site in this study was Eightmile Creek, which receives no paper mill effluent.

Female fish collected at the two sites on Elevenmile Creek, both below the effluent discharge point for the mill, exhibited significant elongation of the anal fins, compared with the Eightmile Creek reference site. Cody and Bortone (1997) concluded that masculinization seen among female mosquitofish from Elevenmile Creek exposed to effluents from the pulp mill prior to the initiation of effluent treatment improvements had persisted. However, the lower frequency of highly masculinized females originally seen by Howell *et al.* (1980) suggested that an overall reduction in the degree of masculinization was associated with tall oil reclamation efforts.

*Female eastern mosquitofish, Gambusia holbrooki have been affected by effluents from certain pulp and paper mills. Image courtesy of USDOI/ USGS - Chris Appleby.*

*Fenholloway River, Florida.* Jenkins *et al.* (2001) assessed arrhenoidy in female mosquitofish from the Fenholloway River, and also analyzed water samples in an effort to identify possible androgenic components. The sampling site on the Fenhollway River was approximately 4 kilometers downstream from the settling ponds of a large paper mill. All female mosquitofish taken from the Fenholloway River had significantly elongated anal fins as compared to the Spring Creek reference site (Jenkins et al., 2001).

In the laboratory, water samples from the Fenholloway River were eluted through solid-phase extraction cartridges. Androgen agonist activity of the fractions was then tested using androgen-receptor transcription assays. Those fractions with androgen receptor agonist activity were analyzed using high performance liquid chromatography-mass spectrometry. Jenkins *et al.* (2001) identified one of the androgenic components in the effluent as androstendione, a precursor in androgen formation. The authors noted that its presence could have been brought about as a result of the microbial modification of plant sterols, including β-sitosterol, or that the androstendione might be a natural component of the original wood. Jenkins *et al.* (2001) cautioned, however, that other unidentified compounds in the effluent could be acting as androgens or androgen precursors, or that compounds within the effluent, such as β-sitosterol, might also act to interfere with steroid biosynthesis or degradation.

*Red River, North Dakota*. Although not adjacent to a pulp and paper mill, Hegrenes (1999) examined channel catfish (*Ictalurus punctatus*) for indications of arrhenoidy on the Red River near a sugar beet processing plant. Fish were identified as being either male or female, and then a subsample of the almost 900 fish captured were examined internally to assess spawning readiness, and to confirm external sex identification. The internal evaluation revealed that 2 percent of the females identified through dissection exhibited external male features. The masculinized female catfish were collected 5 miles downstream of Fargo, North Dakota. Nearby point sources included the Moorhead sewage treatment ponds, as well as a sugar beet processing plant. Hegrenes (1999) noted that while the sewage effluent might be a logical source of estrogenic compounds, chemicals in the effluent associated with sugar beet processing such as β-sitosterol might be more likely to have induced the observed arrhenoidy.

***General Surveys.*** A number of general surveys, not tied to specific land use types were found for freshwater fish in North America. These types of studies are important as they provide an indicator of the general level of endocrine disruption in a larger geographic area.

*Atlantic Canada.* Fairchild *et al.* (1999) used historical records of the aerial application of the insecticide Matacil 1.8D and catch data for the Atlantic salmon (*Salmo salar*) to study the potential effects of 4-nonylphenol. Matacil 1.8D contains the carbamate insecticide aminocarb as the active ingredient and the EDC 4-nonylphenol as a primary solvent.

Between 1975 and 1985, Matacil 1.8D was applied to forests in Atlantic Canada to control damage caused by the spruce budworm *(Choristoneura fumiferana)*. The pesticide was applied around the time of salmon smoltification, the developmental process involving several hormonal systems that enable the salmon to adapt to life in seawater. The author used the ratio of aminocarb to 4-nonylphenol in the formulation to estimate the aqueous concentrations of 4-nonylphenol that occurred after application.

In the Restigouche River basin in 1977, the authors found a significant negative relationship between salmon return and the proportion of tributaries sprayed with Matacil 1.8D. Fairchild *et al.* (1999) also found that for 16 rivers in the region, a significant proportion of the lowest salmon catches between 1973 and 1990 coincided with application of the pesticide formulation. Significantly, no relationship was found between application and salmon catch for Matacil 1.8F, a flowable form of Matacil that does not contain 4-nonylphenol as a solvent. Following a large application of Matacil 1.8D in 1979, the catch per unit effort in 1984 of the 1979 year class was the lowest recorded for any catches between 1981 and 1987. The authors also found a decline in recruitment and the spraying of Matacil 1.8D for a second species of fish, the blueback herring, (*Alosa aestivalis*) in New Brunswick.

*There is evidence that Atlantic salmon (Salmo salar) may have been impacted by 4-nonylphenol contained in a pesticide formulation used in the forests of New Brunswick and Newfoundland. Image courtesy of USDOI/FWS - William Hartley.*

The authors hypothesized that the 4-nonylphenol contained in Matacil 1.8D was the causative agent in the decline of both the salmon and blueback herring. Fairchild *et al.* (1999) also stated that the estimated levels of 4-nonylphenol present after forest spraying would fall into the same range as those currently found in discharges from STPs, industrial effluents and agricultural wastes. Although 4-nonylphenol is estrogenic, its possible effects on the thyroid, particularly to $T_3$ and $T_4$ might be useful here.

*Escambia and Blackwater Rivers, Florida.* Orlando *et al.* (1999) examined the reproductive physiology of largemouth bass (*Micropterus salmoides*) collected from the Escambia and Blackwater Rivers which empty into Pensacola Bay in northwest Florida. In Escambia Bay, fish were collected downstream from a coal fired electric utility and a nylon fiber and chemical intermediates manufacturing plant. The Blackwater River was used as a reference site. In this study, parameters measured included GSI and the HSI, plasma concentrations of $E_2$ and testosterone, and vitellogenin.

There were no differences in the GSIs for males or females between sites. While there was no difference in HSI for females between sites, males from the Escambia Bay site had larger HSIs relative to males from the Blackwater River. Levels of $E_2$ did not differ in females between sites, however, males from the Escambia River had significantly lower levels of testosterone than males from the Blackwater River.

There was no difference in the number of females with detectable vitellogenin between sites. However, three out of 15 males from the Escambia River had detectable vitellogenin, while detectable vitellogenin levels were not found in any male fish from Blackwater River.

*National Survey (U.S.).* Goodbred *et al.* (1997) completed a survey covering a number of regions in the U.S. (Figure 14), to assess the potential for contaminant-induced endocrine disruption in the common carp *(Cyprinus carpio)*. The design of the project provided the opportunity to assess fish across a wide range of geographies and land use types. The goals of the study were to: 1) determine if endocrine disruption is widespread in the United States in areas inhabited by the carp, 2) evaluate any relationships between endocrine disruption and contaminants, and 3) aid in determining whether further studies are needed, as well as the type of investigation that would be most useful (Goodbred *et al.*, 1997). Twenty-five sites (23 streams and two impoundments) representing a wide range of geographic areas, land use types and contaminant levels were selected.

Between August and December 1994, during the period of gonadal maturation, approximately 650 male and female adult carp were sampled. Parameters measured included $E_2$, 11-ketotestosterone, vitellogenin, and gonadal histopathology. Because most of the sites sampled are part of the National Water Quality Assessment (NAWQA) Program, contaminant levels were already characterized. Data on organochlorine pesticides and PCBs in tissues, phthalates, phenols and PAHs in bed sediments, and dissolved concentrations of pesticides, were evaluated for correlations with the reproductive health of carp.

Goodbred *et al.* (1997) found no significant regional differences in steroid hormones for males, but steroid hormones in females from the Northern and Southern Midcontinent sampling region were significantly different from other regions of the country. Within all regions, there were significant differences in one or both hormones ($E_2$ and 11-ketotestosterone) for male and female fish between sites.

The lowest $E_2$ concentration in females occurred in Sanger, Texas north of Dallas. Conversely, the highest mean concentration of $E_2$ in both male and female fish occurred in a reservoir northeast of Modesto, California. The lowest mean 11-ketotestosterone concentration occurred in male carp from the Shenandoah River in West Virginia. Goodbred *et al.* (1997) also examined the ratio of $E_2$/11-ketotestosterone as a possible indicator of endocrine disruption. The lowest $E_2$ to 11-ketotestosterone ratio in both male and female carp occurred on the Platte River in Nebraska, which also had the highest dissolved pesticide concentration. Goodbred *et al.* (1997) found a significant negative correlation in male carp between 11-ketotestosterone and bed sediment concentrations of phenols. There was also a significant negative correlation between this ratio for both male and female carp and the concentration of dissolved pesticides.

There was substantial variability in the concentrations of $E_2$ and 11-ketotestosterone in individual male and female fish, even within a site. At most sites, concentrations of these hormones were within a factor of five of each other. Goodbred *et al.* (1997) cautioned that using levels of sex steroid hormones in fish, even within the same period of the reproductive cycle, is difficult because of natural variability. Perhaps more importantly, they concluded that subtle effects of endocrine disrupters are unlikely to be detected using differences in sex steroid hormones, and that only the strongest influences of contaminants are likely to be evident.

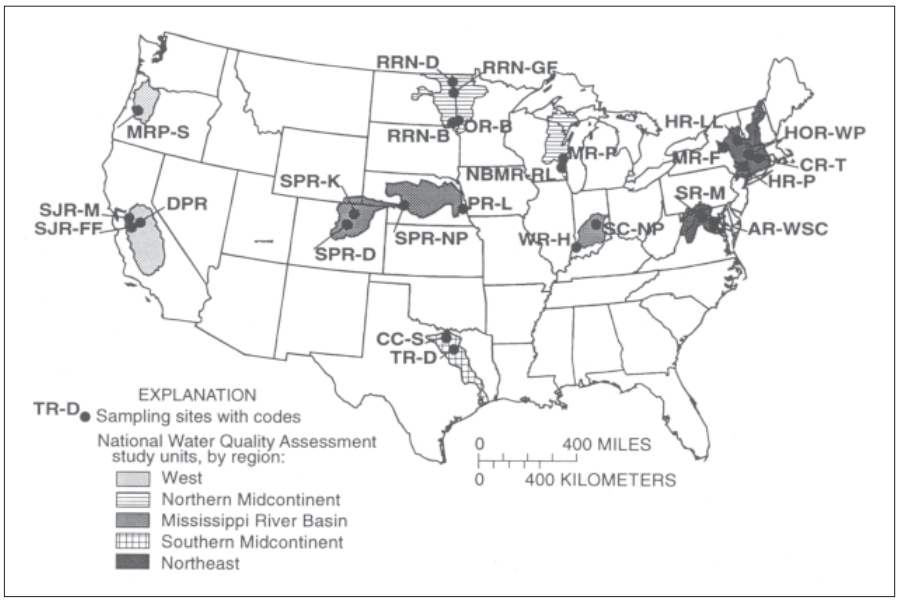



Figure 14. Regions and sampling sites in the USGS reconnaissance study. From Goodbred *et al.* (1997). Reprinted by permission.

Vitellogenin in carp was assessed using a capture ELISA technique and a monoclonal antibody specific for carp vitellogenin. The majority of male plasma samples, however, were negative for vitellogenin. Overall, approximately 16 percent of the male plasma samples contained detectable levels of vitellogenin.

The detection frequency of vitellogenin in male carp was not significantly correlated with any of the contaminant groups. In addition, the detection frequency of vitellogenin in male carp was not significantly correlated with concentrations of either 11-ketestosterone or $E_2$.

The highest frequency of vitellogenin detection at any site in male carp (approximately 53 percent) occurred in the Hudson River, New York, south of Lake Luzerne. Interestingly, Goodbred *et al.* (1997) reported that land use in this basin is primarily forest (91 percent).

Thirty percent of male fish caught in the Red River site near Drayton, North Dakota had detectable levels of vitellogenin, and on the White River at Hazleton, Indiana, vitellogenin was detected in 26 percent of male carp. The highest amount of vitellogenin detected in male carp at any site (0.83 mg/ml)

also occurred at the White River site. Earlier, Sumpter and Jobling (1995) found approximately 20 mg vitellogenin/ml of plasma in male rainbow trout downstream of STPs in the U.K. Goodbred *et al.* (1997) noted that levels of vitellogenin found in males in their study was far below the normal range found in females. They also noted that vitellogenin was detected in males at several minimally contaminated sites with no STP effluent sources, which might indicate that some male fish have low background levels of vitellogenin during a portion of the reproductive cycle.

Gonadal anomalies in the carp were also investigated. Out of 438 male and female carp examined, only one abnormality was found. This was the presence of a few basophilic cells found in a male carp from the Shenandoah River, which Goodbred *et al.* (1997) concluded were probably primary oocytes. This fish, however, contained no detectable vitellogenin in the plasma. This single abnormality can be compared with the nearly 25 percent of roach being classified as intersex above the STPs, and nearly 60 percent downstream, on eight rivers in the U.K (Jobling *et al.*, 1998). In addition, rates on intersexuality of up to 5 percent have been observed in carp (Jobling *et al.*, 1998).

*Mississippi River*. Parks *et al.* (1997) carried out a field study on carp from six sites along the Mississippi River in Tennessee, Mississippi and Louisiana. Five adult male and five adult female carp were taken at each site, and plasma vitellogenin was measured using a competitive ELISA employing a polyclonal antibody for fathead minnow (*Pimphales promelas*), that also recognized carp vitellogenin. Parks *et al.* (1997) found no indication of vitellogenin in male fish, nor was there any evidence of decreased vitellogenin in females between sites.

## ESTUARINE /MARINE STUDIES

This section summarizes some of the endocrine disrupter-related field work in estuarine and marine species of fish. As in the last section, studies were organized into three broad categories and included investigations adjacent to STPs, pulp and paper mills, and general surveys that covered a defined geographic area. Table 7 summarizes results of some of the studies reviewed.

***Sewage Treatment Plants.*** It appears that more endocrine disrupter work has been conducted in freshwater than in estuarine/marine systems, which is particularly true for studies adjacent to STPs. As in freshwater, estrogenic effects are detected near STPs, and range from the presence of vitellogenin in males, to gonadal anomalies and even intersex fish. Almost no investigations on the effects of sewage treatment effluents on estuarine/marine species in the U.S. were found.

*United Kingdom.* Lye *et al.* (1997) investigated abnormalities in reproductive health of the European flounder *(Platichthys flesus)* exposed to effluent from an STP in the U.K. Three sites were chosen, two on the Outer Tyne Estuary where a primary level sewage treatment plant is located, and one on the Solway Firth which drains a more sparsely populated basin. On the Tyne Estuary, one site was located adjacent to a large primary treatment STP that serves approximately 1.25 million people, the other site was located 2 kilometers upstream of the outfall.

Mature male and female flounder were collected in July, 1995. Parameters measured included plasma vitellogenin (expressed as a percentage of protein content compared to a control female), HSI, and macroscopic examination of the gonads.

Vitellogenin was found in the plasma of male flounders from all sites investigated. Sixty percent of the males found at the site adjacent to the STP on the Outer Tyne Estuary contained detectable levels of vitellogenin, compared with 20 percent of male fish from the Solway Firth. A similar pattern was found for female flounder; 73-80 percent from the Tyne Estuary had detectable levels of vitellogenin compared with 55 percent of females from the Solway Firth. Lye *et al.* (1997) also found that male flounder from the Tyne Estuary had higher levels of vitellogenin than males from the Solway Firth as did females, although the differences in both males and females were not statistically significant due to variability within the population.

Although not observed in any fish from Solway Firth, 53 percent of the males taken at the site adjacent to the STP contained malformed testes, consisting of truncated lobes and thick interstitial tissue. No severe abnormalities were observed in females from any of the sites. Lye *et al.* (1997) concluded that the presence of vitellogenin in at least 20 percent of the fish from all sites indicated this phenomenon may be widespread.

Table 7. Field studies of endocrine disruption in saltwater species of fish.

| Fish/Species | Sex | Land Use | Location/ Study Site | Caged/wild fish | Vtg | IS | E2 | GSI | EM | GD | S | Possible causes/ Contaminants present | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| European flounder *(Platichthys flesus)* | M | Sewage treatment plant | Outer Tyne Estuary, U.K. | wild | + | | | | | – | | Natural estrogens and organic contaminants | Lye *et al.*, 1997 |
| European flounder *(Platichthys flesus)* | F | Sewage treatment plant | Outer Tyne Estuary, U.K. | wild | + | | | | | | | Natural estrogens and organic contaminants | Lye *et al.*, 1997 |
| European flounder *(Platichthys flesus)* | M | Sewage treatment plant | Outer Tyne Estuary, U.K. | wild | + | | | + | | – | | Natural estrogens and organic contaminants | Lye *et al.*, 1998 |
| European flounder *(Platichthys flesus)* | F | Sewage treatment plant | Outer Tyne Estuary, U.K. | wild | + | | | + | | – | | Natural estrogens and organic contaminants | Lye *et al.*, 1998 |
| European flounder *(Platichthys flesus)* | M | Sewage treatment plant | Outer Tyne Estuary, U.K. | wild | + | | | | | | | Natural estrogens and nonylphenols | Lye *et al.*, 1999 |
| Burbot *(Lota lota)* | M | Sewage treatment and industrial facilities | Bothnian Bay, Finland | wild | | | | | | – | | Natural estrogens and organic contaminants | Pulliainen *et al.*, 1992 |
| Burbot *(Lota lota)* | F | Sewage treatment and industrial facilities | Bothnian Bay, Finland | wild | | | | | | – | | Natural estrogens and organic contaminants | Pulliainen *et al.*, 1992 |
| Winter flounder *(Pleuronectes americanus* | M | Pulp and paper mill | St. Georges Bay, Newfoundland | wild | | | | | + | | – | Chemicals in paper mill effluent | Barker *et al.*, 1994 |
| Winter flounder *(Pleuronectes americanus* | F | Pulp and paper mill | St. Georges Bay, Newfoundland | wild | | | | | + | | – | Chemicals in paper mill effluent | Barker *et al.*, 1994 |
| Killifish *(Fundulus heteroclitus)* | M | Pulp and paper mill | Miramachi Bay, New Brunswick | wild | | | | | | – | | Chemicals in paper mill effluent | Leblanc *et al.*, 1997 |
| Killifish *(Fundulus heteroclitus)* | F | Pulp and paper mill | Miramachi Bay, New Brunswick | wild | | | | | | – | | Chemicals in paper mill effluent | Leblanc *et al.*, 1997 |
| European flounder *(Platichthys flesus)* | F | Mesocosm study; contaminated sediments | Wadden Sea, Netherlands | wild | + | | + | NE | | – | | PCBs and PAHs | Janseen *et al.*, 1997 |
| European flounder *(Platichthys flesus)* | M | Mesocosm study; contaminated sediments | Wadden Sea, Netherlands | wild | NE | | | | | | | PCBs and PAHs | Janseen *et al.*, 1997 |

Abbreviations: NE, no effect; Vtg, vitellogenin; IS, intersex condition; T, testosterone; E2β-17-estradiol; GSI, gonadosomatic index; EM effect on external male/female morphology; S, effect on spawning

Table 7. Field studies of endocrine disruption in saltwater species of fish (continued).

| Fish/Species | Sex | Land Use | Location/ Study Site | Caged/wild fish | Vtg | IS | E2 | GSI | EM | GD | S | Possible causes/ Contaminants present | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| English sole *(Parophrys vetulus)* | F | Industrial, urban | Duwamish Waterway, Puget Sound | wild | – | | | | | – | | PCBs and PAHs | Johnson *et al.*, 1988 |
| English sole *(Parophrys vetulus)* | F | Industrial, urban | Hylebos Waterway, Puget Sound | wild | | | | | | ± | | PCBs, PAHs and other organic contaminants | Collier *et al.*, 1998 |
| Swordfish *(Xiphias gladius)* | M | General survey | Straits of Messina, Italy | wild | + | | | | | | | Unknown | Fossi *et al.*, 2001 |
| Flounder *(Pleuronectes yokohama* | M | General survey | Tokyo Bay | wild | + | | + | – | | | | Unknown | Hashimoto *et al.*, 2000 |
| European flounder *(Platichthys flesus)* | M | General survey | Five estuaries in the U.K. | wild | + | + | | NE | | | | Natural estrogens and organic contaminants | Allen *et al.*, 1999 |
| Winter flounder *(Pleuronectes americanus* | F | General survey | Long Island Sound | wild | + | | | | | | | PCBs, PAHs, metals | Pereira *et al.*, 1992 |
| Winter flounder *(Pleuronectes americanus* | F | General survey | Boston Harbor | wild | – | | | | | | | PCBs, PAHs, metals | Pereira *et al.*, 1992 |
| Winter flounder *(Pleuronectes americanus* | F | General survey | Boston Harbor | wild | | | | | | – | | PCBs and PAHs | Johnson *et al*., 1992 |
| White croaker *(Genyonemus lineatus)* | F | General survey | San Pedro Bay, California | wild | | | | | | – | – | DDT and PCBs | Hose *et al*., 1989 |
| Killifish *(Fundulus heteroclitus)* | M | General survey | Chesapeake Bay | wild | NE | | | –/NE | | NE | | Pristine to polluted areas (PAHs, PCBs, metals) | Pait and Nelson, in prep. |
| Killifish *(Fundulus heteroclitus)* | M | General survey | New York Harbor | wild | NE | | | | | | | No effects seen | McArdle *et al.*, 1998 |
| Killifish *(Fundulus heteroclitus)* | F | General survey | New York Harbor | wild | NE | | | | | | | No effects seen | McArdle *et al.*, 1998 |
| Sheepshead minnow *(Cyprinodon variegatus)* | M | General survey | Chesapeake Bay | wild | NE | | | | | | | PAHs and PCBs | Pait and Nelson, in prep. |

Abbreviations: NE, no effect; Vtg, vitellogenin; IS, intersex condition; T, testosterone; E2, 17β-estradiol; GSI, gonadosomatic index; EM effect on external male/female morphology; S, effect on spawning

Lye *et al.* (1998) completed a more intensive followup investigation on the seasonal reproductive health of European flounder at the same sites in the U.K. Male and female flounder were collected in the Tyne estuary, both upstream and downstream of the sewage treatment plant, and from the Solway Firth in July, September and December 1995, and April 1996. Lye *et al.* (1998) assessed relative levels of vitellogenin, gonadal histopathologies, GSI, and HSI in male and female flounder.

Vitellogenin was found in male fish in both waterways. In the Tyne, 64 to 95 percent of the male flounder caught contained detectable levels of plasma vitellogenin, while 27 to 50 percent of the male flounder from the Solway Firth contained plasma vitellogenin. While not quantified, levels of vitellogenin were compared to control females (Figure 15). Male fish from the Tyne Estuary sites had levels of vitellogenin between 60 and 155 percent of that found in control female flounders.

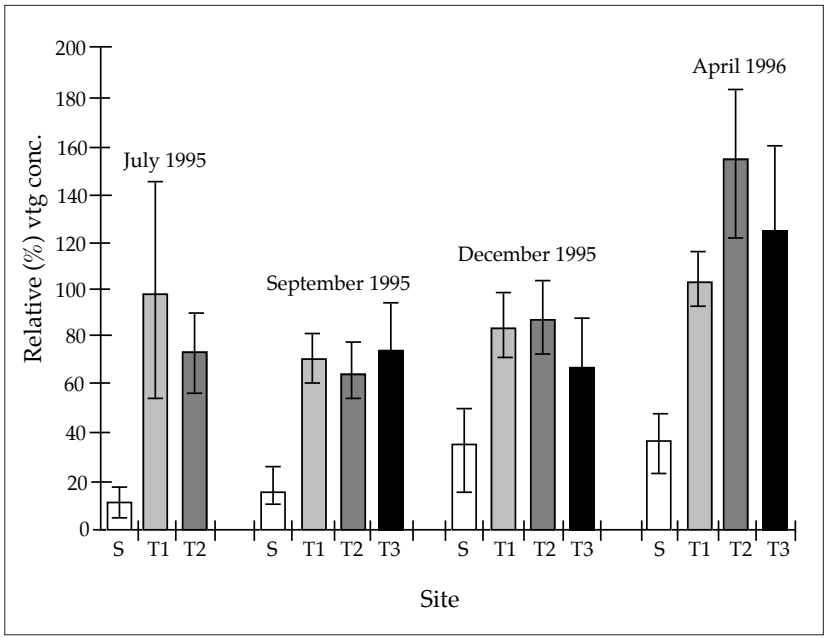



Figure 15. Vitellogenin (vtg) (mean ± SEM) in male flounder (*Platichthys flesus*) from the Tyne Estuary (T1, T2, and T3) and Solway Firth (S) in the United Kingdom. Values are expressed as a percentage of vitellogenin in control female. (From Lye *et al*., 1998). Reprinted by permission.

A substantial proportion of male fish (53 to 67 percent) from the Tyne estuary exhibited testicular anomalies, compared with a lower proportion in males from the Solway Firth (maximum of 12 percent). Male flounder having the highest levels of plasma vitellogenin also had significant pathological testicular abnormalities. These included disorganized and vacuolized lobules, connective tissue thickening, similar to that found by Lye *et al.* (1997), along with the reduction or even absence of germ cells within the testes. There were no indications, however, of intersex fish. Female flounders from the Tyne exhibited increased vitellogenin levels and a higher proportion of degenerated oocytes compared with females from the less polluted Solway Firth. Vitellogenin levels in both males and females varied somewhat by season for both sexes. In males, there were no differences in testicular abnormalities between seasons, while in females, gross ovarian abnormalities (e.g., large masses of aggregated and deformed eggs, atretic oocytes and ovarian tissues) were found in September, December and April, but were absent in July.

The GSI in females from the Tyne in September and April was significantly larger than those females from the Solway Firth. In male flounder, significantly larger GSIs in the Tyne were recorded only in April compared to those in Solway Firth. In addition, the HSIs for both female and male flounder from the Tyne were significantly higher than in Solway Firth.

Lye *et al.* (1998) concluded that in addition to most U.K. rivers, the data strongly suggested that at least some U.K. estuaries are experiencing contamination by estrogenic substances. They also noted that while the implications for reproduction and population are not yet known, there is concern that the observed effects may impact reproductive viability which could in turn have serious consequences at the population and community levels.

Lye *et al.* (1999) investigated tissue (muscle and liver) concentrations of alkylphenols in mature male European flounder in the Tyne and Tees estuaries in the U.K. 4-Nonylphenol (5-55 ng/g, wet weight), and nonylphenol monoethoxylate (190-940 ng/g wet weight) were found in tissues of mature male flounder. These fish also had detectable plasma vitelloge-

nin levels. These same compounds were also found in sediment samples from both estuaries. However, given that natural and synthetic estrogens had been found in domestic sewage, Lye *et al.* (1999) concluded the estrogenic responses seen in the flounder were unlikely to be accounted for solely by the presence of the alkylphenols detected in tissue and sediment.

***Pulp and Paper Mills.*** There have been relatively few investigations of endocrine disruption in estuarine and marine waters adjacent to pulp and paper mills. When detected, impacts tend to include depression or delay of the reproductive cycle in affected fish. A number of investigations have concentrated on flatfish.

*St. George's Bay, Newfoundland*. Barker *et al.* (1994) investigated the effects of pulp and paper mill effluents on a population of winter flounder *(Pleuronectes americanus)*. Male and female fish were collected from two sites in St. George's Bay, Newfoundland, one at Port Harmon adjacent to a pulp and paper mill, and at St. George's a reference site located 12 kilometers away from the pulp and paper mill. The mill at Port Harmon produces paper for newsprint, and uses sodium hydrosulphite for the bleaching process. The flounder (total of 400) were taken monthly by hook and line or captured by scuba divers from 1991 to 1992 during the spring (May) and summer (June-September) months of both years.

*Significant endocrine-related impacts have been found in burbot Lota lota at some locations in Finland/Sweden. Image courtesy of Idaho State University - Ernest Keeley.*

A number of parameters were measured in the fish, including GSI and the state of gonadal maturation. Surprisingly, the GSIs from male and female Port Harmon (mill site) fish were higher than those from the St. George's site (Barker *et al*., 1994). Fish taken from Port Harmon in the May-June time period contained mature gonads, while fish from the St. George's (reference) site had already spawned. In addition, of the fish captured during July and August, 12% of both males and females from Port Harmon contained fully developed gonads, while all fish from St. George's were spent. Barker *et al.* (1994) concluded that the higher percentage of postspawned females and significantly lower GSIs in the St. George's flounder indicated delayed spawning in fish from Port Harmon.

*Bothnian Bay, Finland/Sweden*. Pulliainen *et al.* (1992) investigated the effects of industrial loadings on burbot on the northern coast of the Bothnian Bay, between Finland and Sweden. Burbot (*Lota lota*) were caught over a 3 year period in gill nets at various locations on the northern coast of the Bay, known to be impacted by inputs from pulp and paper, municipal sewage, and steel industries. Pulliainen *et al.* (1992) reported that 87-98% (male and female) of the adult burbot taken off the coasts of the cities of Tornio and Kemi, Finland were sterile. Non-maturing burbot (87 percent of catch) were also found in the Tornio River approximately 10 kilometers upstream. Pulliainen *et al.* (1992) noted that typically only 10-15 percent of adult burbot fail to mature. The male/female ratio of the burbot was 0.83 in catches off Tornio, and 0.77 in catches off Kemi. The authors speculated the effects observed could be related to the presence of toxic chemicals, even though a clear cause and effect relationship could not be established.

*Miramachi Estuary, New Brunswick*. Leblanc *et al.* (1997) investigated the effect of bleached kraft pulp mill effluents on the reproductive period in the killifish *F. heteroclitus*. Mature male and female fish were collected using minnow traps at locations 4, 21, and 39 kilometers downstream of the pulp mill. Two reference sites were established on the Bouctouche Estuary. A variety of parameters were assessed, including GSI, percentage of mature fish, fecundity and egg size in *F. heteroclitus* collected during the spring/summer spawning period. Fish from Miramachi Estuary 4 kilometers and 21 kilometers downstream of the pulp mill exhibited delayed gonadal maturation and smaller egg size than fish from the site 39 kilometers downstream of the pulp mill. Gonadal parameters did not vary significantly between sites on the Bouctouche Estuary. Interestingly, fish from the site 4 kilometers downstream of

the pulp mill exhibited a higher fecundity (number of mature eggs) and GSI compared with the other sites. In addition, the condition factor was not reduced at the site closest to the pulp mill which suggested to Leblanc *et al.* (1997), that more nutrients were available to the fish collected near the mill than at the other sites.

***General Surveys.*** Investigations of endocrine disruption in fish not tied to specific land use types or adjacent to effluent discharges provide an opportunity to assess the ambient level of effects in a waterbody. A number of studies were located from Europe and North America.

*United Kingdom.* Allen *et al.* (1999) surveyed the Tyne, Thames, Crouch and Mersey estuaries and a number of offshore sites for evidence of endocrine disruption in European flounder. The estuarine portion of the River Alde was chosen as the control site. Between two and five sites were chosen in each estuary, and male fish were sampled two times in 1996, in September, and again in December. Importantly, sites were not specifically chosen with STP outfalls in mind, but rather where fish could be found (Allen, pers. comm.).

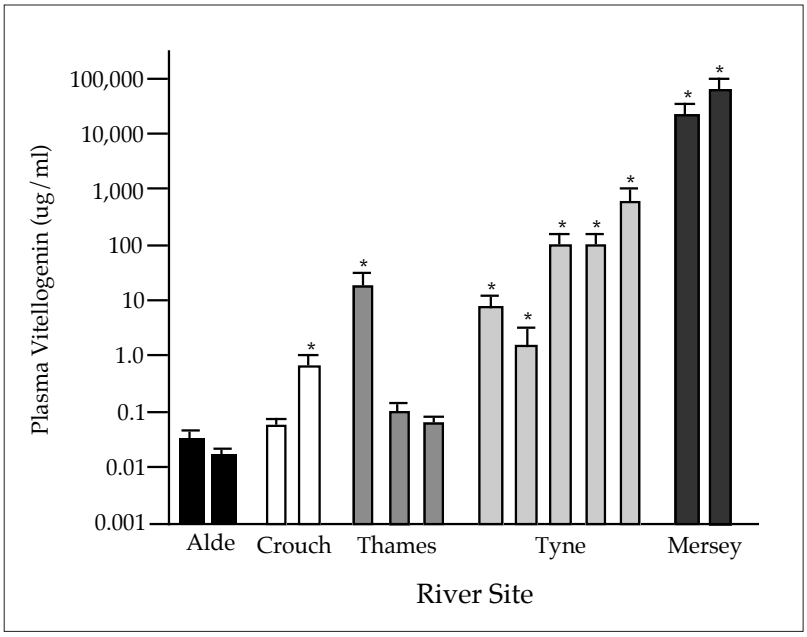



Figure 16. Vitellogenin in wild male flounder from five U.K. estuaries. Asterisks denote significantly elevated levels ($p$<0.05) compared with control estuary (Alde). From Allen *et al.* (1999). Reprinted by permission.

On the Tyne estuary, male fish collected at all five sites had significantly elevated plasma vitellogenin levels as compared with fish from the River Alde control site (Figure 16). On the Thames, males from one of three sites had significantly elevated plasma vitellogenin. Males from the two sites in the Mersey estuary had the highest levels of vitellogenin of all sites surveyed. Allen *et al.* (1999) noted that as expected, HSIs were elevated in fish with high induction, however, there was no significant effect on the GSIs. Male flounder collected from the North Sea and from the English Channel, and from most sites in the Irish Sea, had plasma vitellogenin levels higher than those fish taken from the River Alde.

Although no serious gonadal anomalies were seen in fish from the Tyne, Thames, or Crouch estuaries, approximately 20 percent of males from the Mersey estuary were intersex, (i.e., containing ovotestes). Higher levels of contamination or the possibility that the fish might breed within the estuary were offered as possible explanations for gonadal anomalies and high levels of vitellogenin in the Mersey fish. Allen *et al.* (1999) concluded that several U.K. estuaries are suffering contamination with estrogenic materials which is having adverse biological effects and may be damaging the ability of the fish to reproduce.

*Wadden Sea, Netherlands.* Janssen *et al.* (1997) exposed European flounder for up to 3 years in large (40m X 40m X 3m) mesocosms. The first mesocosm (A) contained relatively clean Wadden Sea sediment and water. The second (B) was indirectly contaminated with water from the third mesocosm. The third mesocosm (C) contained dredge spoil from the Rotterdam Harbor. Juvenile (1-year old) male and female flounder captured from a relatively clean site on the Wadden Sea were introduced into these self-contained mesocosms in 1990. The contaminants in the dredge spoil included PCBs and PAHs. A relatively clean reference site was also established on the Wadden Sea. Parameters included vitellogenin measured by gel electrophoresis and image analysis, $E_2$ and testosterone (radioimmunoassay) analyses, and gonadal histology.

Spawning in the flounder occurs in winter. In November 1992, no severe abnormalities were observed in ovarian structure, all ovaries were in early or advanced stages of vitellogenesis, characterized by smaller oocytes. In May 1993, all females from the Wadden Sea reference site as well as from mesocosms A and B were in the previtellogenic phase, which is natural for that time of year. Females from mesocosm C, however, contained not only previtellogenic oocytes, but also later or yolk granule stage eggs, more characteristic of fish in November, just before spawning. This asynchronous development of oocytes was not observed in fish from mesocosms A and B, or from the reference field site. There were no significant differences, however, in the GSIs between sites during the May sampling.

Relative measurements in November 1992 revealed that plasma vitellogenin in females in mesocosm C was significantly higher than females from mesocosm A (Janssen *et al.*, 1997). Likewise, measurements made in May 1993 revealed the same trend, with vitellogenin levels highest in females from mesocosm C (Figure 17). No plasma vitellogenin was detected in males during any phase of the reproductive cycle.

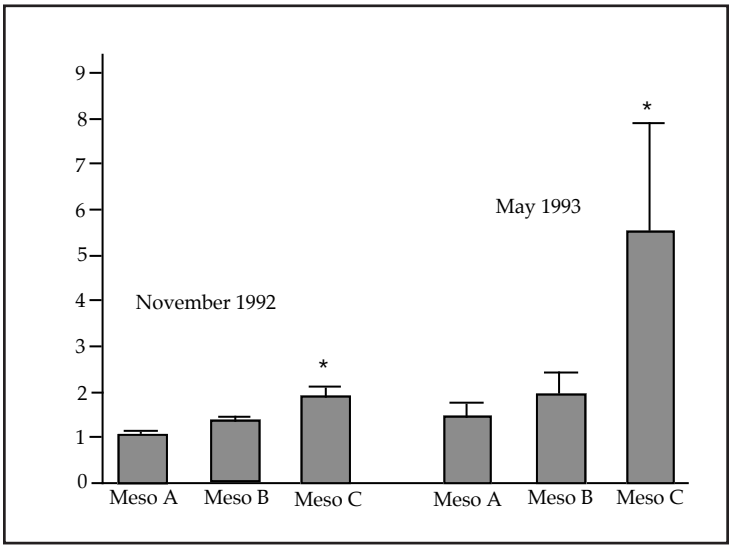



Figure 17. Relative plasma vitellogenin concentrations (mean ± SEM) of mesocosm female flounder, *Platichthys flesus*, in November 1992 and May 1993. Asterisks denote significant ($p < 0.05$) difference from the reference mesocosm (A). From Janssen et al. (1997). Reprinted by permission.

To better understand possible reasons for the increased vitellogenin in females from the contaminated mesocosm, Janssen *et al.* (1997) made measurements of plasma $E_2$ and testosterone in May 1993. They found that $E_2$ was significantly elevated in females in mesocosm C (264 pg/ml) as compared with the reference, mesocosm A (116 pg/ml). An accompanying set of *in vitro* experiments with ovarian tissue, however, indicated no significant difference in $E_2$ synthesis capability between the mesocosms.

Janssen *et al.* (1997) concluded that long term exposure to polluted water and sediments under semi-field conditions caused out-of-season plasma elevations of both $E_2$ and testosterone. Because the vitellogenin was prematurely elevated in females only, and the ovarian $E_2$ production capacity was unaltered, Janssen *et al.* (1997) concluded that the hormonal disruption was the result of a modification of $E_2$ clearance rates rather than the estrogenic action of pollutants.

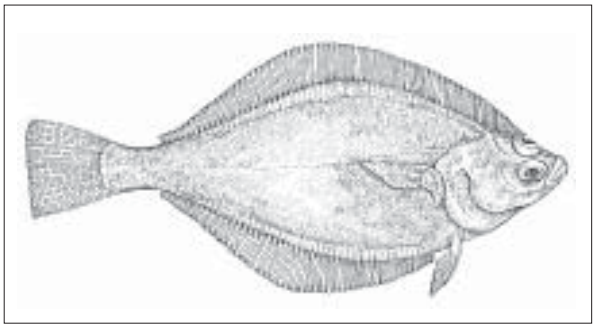
*Straits of Messina, Italy*. Fossi *et al.* (2001) assessed reproductive endocrine disruption in swordfish *(Xiphias gladius)* in Sicily. Three female and six male swordfish, ranging in ages from 1-5 years were collected. Relative amounts (absorbance at 492 nm) of vitellogenin and zona radiata proteins were measured using an indirect ELISA. Fossi *et al.* (2001) found significant induction of vitellogenin in the three older males, 4 to 9 years of age. Relative vitellogenin levels were not statistically different from the adult females tested. Similarly, zona radiata proteins were elevated in older males. They attributed the higher biomarker responses to the accumulation of EDCs over time in the fish. Fossi *et al.* (2001) also referred to unpublished work that male swordfish caught in the Atlantic (Azores) contained substantially lower levels of vitellogenin than those found in the Mediterranean. They concluded the results signal a warning of possible reproductive alterations in this top predator.

*Tokyo Bay*. Hashimoto *et al.* (2000) assessed elevated vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay. Mature male flounder, aged approximately 1-6 years were collected from two sites near Haneda and Yokohama in Tokyo Bay, and a reference site near

Hakkaido. Plasma vitellogenin was measured using an ELISA along with a fluorophore for the detection of vitellogenin.

Male flounder collected from the Tokyo Bay sites had significantly higher levels of plasma vitellogenin (0.025 - 2.2 μg/ml) than fish taken from the reference site (0.031 - 0.091 μg/ml). There were no significant correlations, however, between vitellogenin and the HSI, $E_2$ or testosterone levels, although Hashimoto *et al.* (2000) noted that lower GSIs were more often associated with males having higher levels of vitellogenin. In addition, 3 out of 20 males collected near Haneda were intersex although the degree of intersexuality was slight (Hashimoto *et al.*, 2000).

*German Baltic Coast.* Hansen *et al.* (1985) investigated the effects of contamination on Baltic herring (*Clupea harengus*) hatching success. Eggs stripped from ripe females in April were fertilized with sperm and incubated in clean seawater. Water exchange in the aerated incubation jars occurred every 48 hours, and then every day during the hatching period. Hatched larvae were counted and viable hatch (straight and healthy larvae) were determined as a function of contamination in parental tissues. Hansen *et al.* (1988) found that viable hatch was significantly affected by ovarian DDE concentrations higher than 18 ng/g, and PCB concentrations of more than 120 ng/g, both wet weight.



*Female English sole, Parophrys vetulus collected at several locations in Puget Sound in the past had lower levels of $E_2$ as well as delayed vitellogenesis. Image courtesy of NOAA/NMFS.*

*Puget Sound.* A significant amount of work on sole and flounder has occurred in the Puget Sound region of Washington. Much of this work has centered on the effects of organic contaminants on the overall health of fish, including reproductive success.

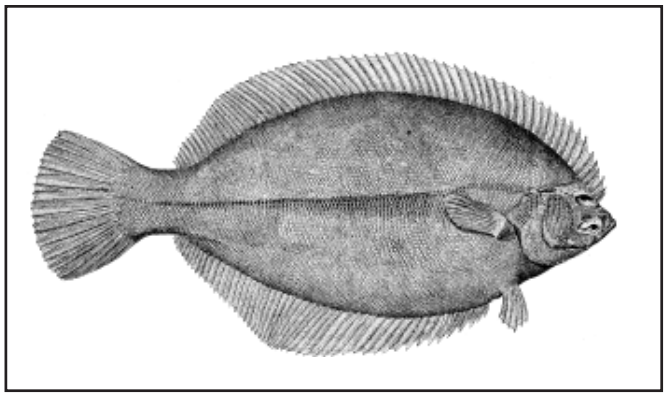
Johnson *et al.* (1988) conducted a study to assess whether exposure to contaminants was associated with altered ovarian development in adult female English sole (*Parophrys vetulus*) from Puget Sound. Fish were captured from four sites in the Puget Sound area, including three contaminated areas: Duwamish Waterway (high aromatic hydrocarbons and PCB concentrations), Eagle Harbor (high aromatic hydrocarbons and low PCB concentrations), Sinclair Inlet (low aromatic hydrocarbon concentrations and moderate concentrations of PCBs), and Port Susan, a minimally contaminated site. Parameters associated with ovarian maturation included GSI, plasma vitellogenin as estimated from alkaline-labile phosphate (ALP), plasma $E_2$, and ovarian developmental stage. Exposure to contaminants was assessed by measuring concentrations of fluorescent aromatic compounds in the bile, hepatic aryl hydrocarbon hydroxylase (AHH) activity, and hepatic concentrations of PCBs.

Johnson *et al.* (1988) found that female English sole from the Duwamish Waterway (2.53 μg/ml) and Eagle Harbor (1.89 μg/ml) had significantly lower levels of plasma $E_2$ than those from Port Susan (3.92 μg/ml). Interestingly, however, plasma $E_2$ levels in females from Sinclair Inlet, the moderately contaminated site, were significantly higher than in female fish from Port Susan.

The sampling of fish occurred during winter, the time of gonadal recrudescence, prior to spawning. There were a substantial number of females at all sites who had not entered vitellogenesis. The number of females who had not entered vitellogenesis was significantly higher, however, in fish from the Duwamish Waterway and Eagle Harbor, than from the Port Susan site. They also found that sole with higher AHH activity were less likely to undergo gonadal recrudescence than those fish from the less contaminated areas. Johnson *et al.* (1988) concluded that contaminant exposure may interfere with ovarian development in female English sole.

Beginning in 1994, a series of studies were conducted in the Hylebos Waterway in Puget Sound to determine the effect of contaminants on flatfish, including an assessment of reproductive function in fish inhabiting this severely contaminated area (Collier *et al.*, 1998). Contaminants in the Hylebos Waterway in central Puget Sound include PAHs, PCBs, hexachlorobutadiene, hexachlorobenzene, and a number of pesticides including DDTs and heptachlor.

As part of their investigation, gonadal development was assessed in female English sole. Collier *et al.* (1998) found precocious sexual maturation in 40-50 percent of the juvenile female sole from the Hylebos Waterway as measured by fish with developing eggs. Approximately 20-30 percent of the adult females had inhibited gonadal development. Collier *et al.* (1998) found that precocious sexual maturation was associated with exposure to both chlorinated hydrocarbons and PAHs, while inhibition in the adults was related to PAH exposure. They concluded that the reproductive injuries observed in Hylebos Waterway fish presumably would reduce the number of eggs and larvae contributed by these fish to the overall population of English sole.



*Winter flounder, Pleuronectes americanus collected from a number of sites in the northeast U.S., showed evidence of endocrine disruption. Image courtesy of NOAA/NMFS.*

The effects on the juvenile female English sole found by Collier *et al.* (1998) appear similar to the results obtained by Janssen *et al.* (1997) after exposing juvenile European flounder to PCBs and PAHs in mesocosms containing dredge spoil. In the mesocosm study, Janssen *et al.* (1997) attributed precocious oocyte development to higher levels of $E_2$ in females exposed to the dredge spoil, possibly brought on by lower clearance rates of endogenous estrogen.

*Boston Harbor and Raritan Bay.* Johnson *et al.* (1992) investigated the relationship between sediment contamination and a variety of reproductive parameters in prespawning winter flounder *(Pleuronectes americanus)*, including ovarian developmental stage, ovarian atresia, GSI, plasma $E_2$ levels, fecundity and egg weight from 11 sites in the northeast U.S. Sediment contaminant concentrations ranged from 20-50,000 ng/g dry weight for PAHs, and between 2 and 1,400 ng/g dry weight for PCBs. Exposure to contaminants was assessed by measuring concentrations of fluorescent aromatic compounds in the bile, hepatic aryl hydrocarbon hydroxylase and concentrations of PCBs in various tissues of the flounder, including the ovaries.

Decreased egg weights and an increase in atretic follicles were associated with high levels of PCBs or PAHs. However, no clear negative impact on gonadal recrudescence, GSI, plasma $E_2$, or fecundity in female winter flounder was found. Johnson *et al.* (1992) felt the apparent difference in effects in winter flounder compared with English sole (Johnson *et al.*, 1988), could be due to a number of factors. One possibility was interspecific differences in contaminant activation or detoxification. Another possibility was a noted difference in migration patterns. While English sole reside in the estuaries throughout the period of vitellogenesis before moving offshore to spawn, winter flounder remain offshore during early vitellogenesis, and then move into the estuaries prior to spawning. Johnson *et al.* (1992) hypothesized that winter flounder captured at the contaminated sites in Boston Harbor and Raritan Bay may not reside in these areas during the early stages of vitellogenesis, a time when contaminants could have a critical impact on steroidogenesis and subsequent ovarian development.

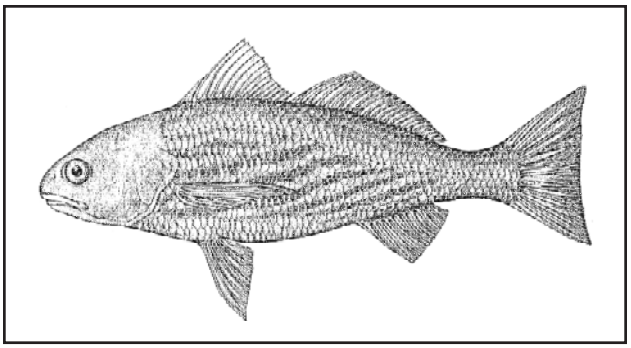
*Long Island Sound and Boston Harbor.* Pereira *et al.* (1992) studied relative levels of vitellogenin in female winter flounder during the reproductive season at several contaminated sites (including Black Rock and New Haven harbors, Connecticut) and a relatively clean site in Long Island, as well as in Boston Harbor. Vitellogenin levels were assessed in both adult and juvenile flounder indirectly by measurement of ALP.

Serum ALP values, indicative of vitellogenin, were actually higher in adult female flounder from the contaminated Black Rock Harbor (60.9 μg Pi/ml ) site than flounder from the relatively clean Shoreham (39.8 μg Pi/ml) site. There were no significant site differences in the juvenile females. Pereira *et al.* (1992) suggested that higher vitellogenin levels might be indicative of impeded uptake by the ovary, rather than higher $E_2$ levels, as higher levels of contamination have been associated with lower $E_2$ levels. They also suggested the effect was likely associated with contaminant burden, as younger fish which exhibited no differences in ALP levels would not have accumu-

lated the level of contaminants likely in the larger, older fish.

The focus of the work in Boston Harbor was to discover if the presence of tumors in female winter flounder affected the level of vitellogenin in the fish. Pereira *et al.* (1992) found that mean ALP values in tumor-bearing fish (10.7 μg Pi/ml) were significantly lower than nontumor-bearing fish (17.4 μg Pi/ml). They suggested it was not unreasonable that the potent mix of contaminants in Boston Harbor, including PCBs, PAHs and a variety of trace elements could have lowered vitellogenin levels in fish either directly by tumor-induced liver dysfunction or indirectly by interference with the production of $E_2$.

*San Pedro Bay, California*. Hose *et al*. (1989) investigated reproductive impairment in female white croaker (*Genyonemus lineatus*) inhabiting waters off the Southern California coast. Croaker were collected from two sites, one a highly contaminated area in San Pedro Bay, and the other a reference site, 80 kilometers to the south. Heavy contamination of sediments with DDT in this area occurred from 1950 to the 1970s and was the result of discharges from the Montrose Chemical Company via the Los Angeles County sewer system (Huh and Venkatesan, 1998). The authors noted that because of a temporal relationship between chlorinated hydrocarbon discharges and the decline of several major sport and commercial fish stocks in coastal waters, it had been suggested that contaminants contributed to reduced fecundity or larval survival of sport fish.



*White croaker, Genyonemus lineatus collected in San Pedro Bay, California exhibited lower fecundity, possibly related to ovarian DDT concentrations. Image courtesy of NOAA/NMFS.*

Mature female white croaker were collected from the sites, and following a 1 week acclimation period, were induced to spawn with injections (1 IU/g body weight) of human chorionic gonadotrophin (HCG). Females from San Pedro Bay produced significantly fewer eggs than fish from Dana Point, the reference site. Of the fish that did not spawn, examination of the ovaries revealed that spawning was imminent in all of the Dana Point females, but only in 16 percent of the San Pedro Bay females. The ovaries of the remaining San Pedro Bay fish contained only immature yolky oocytes that were unresponsive to injections of HCG. None of the fish that spawned contained ovarian DDT concentrations greater than 4 μg/g. In addition, fish from San Pedro Bay exhibited lower fecundity (32 percent), and fertility (14 percent), leading the authors to suggest that contaminants, DDT in particular, might partially explain the population declines observed for a number of southern California species of fish since the 1940s.

*Chesapeake Bay.* Pait and Nelson (in prep.) completed a project in the Chesapeake Bay to assess reproductive endocrine disruption in the killifish *F. heteroclitus*. Laboratory studies indicated that male *F. heteroclitus* were sensitive to 4-nonylphenol (Figure 6), as well as to $E_2$. Seven field sites representing a variety of land use activities ranging from relatively pristine sites, to industrialized areas near Baltimore were established in the Chesapeake Bay and tributaries.

Male and female fish were sampled during the fall months after reproduction had ceased, and again in the spring/summer, the peak reproductive period for this species, for a period of 2 years. All spring/summer sampling occurred within 2 days of the full moon, to coincide with the species' lunar spawning cycle. Parameters measured included plasma vitellogenin, GSI, HSI, and some gonadal histopathology. Vitellogenin was analyzed by direct ELISA using a monoclonal antibody prepared by the University of Florida.

The results of the plasma vitellogenin analysis in males is shown in Table 8. Eighty-six percent of the male fish sampled contained no detectable levels of vitellogenin as determined by the ELISA. In all cases except one, levels of vitellogenin were low, in the range of 0.01 - 0.09 mg/ml. In their study in freshwater locations around the U.S., Goodbred *et al.* (1997) detected vitellogenin in approximately 16 percent of male carp, similar to what was found for *F. heteroclitus*. In the U.K., others (Purdom *et al.*, 1994); Sumpter and Jobling, 1995), have found elevated levels of vitellogenin in all fish sampled, albeit close

to STPs. In the Chesapeake, there were also occasional positive detections of vitellogenin in males from the pristine sites. In their report, Goodbred *et al.* (1997) suggested that some male fish may contain low background levels of vitellogenin during some portion of the reproductive cycle.

Table 8. Vitellogenin in male *Fundulus heteroclitus* from Chesapeake Bay sites.

| Site/Characteristics | | Fall | | Spring | |
|---|---|---|---|---|---|
| Site | Land use | Vitellogenin (mg/ml) ±SEM (Range) | Number of samples | Vitellogenin (mg/ml) ±SEM (Range) | Number of samples |
| Annapolis | Urban/suburban | 0.00±0.00 (0.00 - 0.02) | 20 | 0.01±0.00 (0.00 - 0.07) | 20 |
| Back River | Sewage treatment | 0.00±0.00 (0.00 - 0.04) | 20 | 0.00±0.00 (0.00 - 0.03) | 20 |
| Beaverdam Creek | Reference | 0.00±0.00 (0.00 - 0.00) | 10 | 0.01±0.01 (0.00 - 0.09) | 20 |
| Choptank River | Agricultural | 0.00±0.00 (0.00 - 0.02) | 20 | 0.03±0.03 (0.00 - 0.58) | 20 |
| Fort Armistead | Industrial/sewage treatment | 0.01±0.00 (0.00 - 0.07) | 19 | 0.00±0.00 (0.00 - 0.04) | 20 |
| Point Lookout | Reference | 0.00±0.00 (0.00 - 0.00) | 10 | 0.00±0.02 (0.00 - 0.03) | 10 |
| Patapsco River | Industrial/urban | 0.00±0.00 (0.00 - 0.04) | 20 | 0.00±0.00 (0.00 - 0.03) | 20 |

Note - no significant differences found in vitellogenin concentration between sites or seasons. SEM, standard error of the mean. Pait and Nelson (in prep.).

A second species of fish, the sheepshead minnow *(Cyprinodon variegatus)* was also collected at three sites where *F. heteroclitus* was taken (reference and two contaminated sites). An analysis of vitellogenin in male sheepshead minnows revealed no significant difference from *F. heteroclitus*.

Although there were no apparent estrogenic effects, there was some evidence of negative effects at certain sites. The GSIs in male and female fish from Baltimore were significantly lower than fish from the reference site (Beaverdam Creek). Additionally, females from Baltimore had vitellogenin levels significantly lower than Beaverdam Creek. Data on sediment contaminant concentrations for the Baltimore locations as well as a reference site were obtained from published data bases. An analysis of the data revealed that lower GSIs in male *F. heteroclitus* were negatively and significantly correlated with sediment concentrations of PAHs and PCBs, and in females, lower vitellogenin levels and GSIs were negatively correlated with sediment PAHs. Histological examination of gonadal sections, however, did not reveal any ovotestes and also failed to reveal any major differences in the fish between sites.

*New York Harbor.* McArdle *et al.* (1998) also investigated endocrine disruption in *F. heteroclitus*. Parameters measured included liver microsomal vitellogenin, GSI and HSI.

Relative amounts of vitellogenin were assessed using Western blot and a polyclonal antibody for *F. heteroclitus*. The intensity of the vitellogenin band of each sample was compared with the microsomal protein band of a vitellogenic female from a control site (Flax Pond), loaded at three different concentrations (1, 5, and 10 μg) into the wells of the gel.

Adult male and female *F. heteroclitus* were collected by minnow trap in Newark Bay. None of the 22 adult males contained detectable levels of vitellogenin, while all five female *F. heteroclitus* contained levels of vitellogenin that were intermediate of the 1 and 5 μg bands.

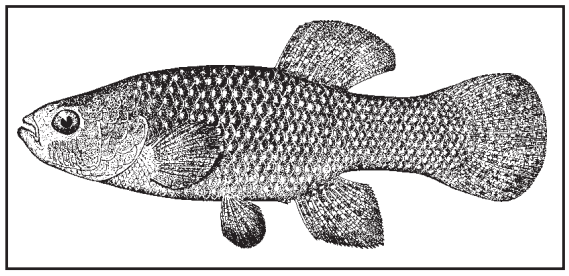
Zhou *et al.* (2000) investigated thyroid disfunction in *F. heteroclitus* in New Jersey. Fish were collected using minnow traps from two locations, Piles Creek, which is contaminated with petroleum hydrocarbons and heavy metals, and from a reference site near Tuckerton, New Jersey. They noted that fish from Piles Creek have reduced growth and longevity along with behavior deficits compared to the reference population.

The thyroid glands were processed for histologic examination, and concentrations of the hormones thyroxine and triidothyronine were analyzed using

radioimmunoassay. Zhou *et al.* (2000) found that fish collected from the contaminated Piles Creek site had abnormally large thyroid follicles with thickened epithelia. Although levels of triidothyronine were normal, levels of thyroxin were elevated. Zhou *et al.* (2000) noted the altered thyroid status may correlate with the sluggish behavior patterns observed in the Piles Creek fish, and that it was probably an example of environmentally related endocrine disruption.

## CONCLUSIONS

Endocrine disrupting compounds or EDCs have the potential to interfere with a variety of life processes controlled or influenced by the endocrine system. A number of synthetic and natural chemicals and chemical classes including synthetic estrogens (e.g., 17α-ethinylestradiol, $EE_2$), industrial intermediates (e.g., 4-nonylphenol, and bisphenol-A), PAHs (e.g., β-napthoflavone, benzo[a]pyrene, phenanthrene, and chrysene) pesticides (e.g., DDT, endosulfan, carbofuran and atrazine), metals (e.g., cadmium and lead), dioxins, and PCBs (e.g., Aroclor 1254 and Aroclor 1221) have been shown to adversely affect the endocrine system in fish. In addition, certain naturally occurring compounds, (e.g., β-sitosterol) can also interfere with normal endocrine function. The potency of most if not all EDCs is typically hundreds to thousands of times less than that of the endogenous hormones.



*The killifish, Fundulus heteroclitus, also known as the mummichog has been used in a number of laboratory and field investigations of endocrine disruption. From Goode, 1884.*

To date, most of the research on endocrine disruption in fish has concentrated on reproductive effects. Other areas of the endocrine system in fish and higher vertebrates are also at risk.

Significant environmental endocrine disruption has been seen in fish. Effects range from reduced levels of the reproductive hormones (i.e., estrogens and androgens), inhibited gonadal growth, the appearance of a female egg protein (vitellogenin) in male fish, to gonadal histopathologies and even intersex fish containing ovotestes.

Overt endocrine disruption in fish, however, does not appear to be a ubiquitous environmental phenomenon. When detected, endocrine disruption appears more likely to be associated with higher levels of contamination; fish from less polluted areas or areas downstream of pollutant discharges or spills typically are less affected using current indicators. It remains to be seen, however, whether subtle effects may be occurring at very low levels of contamination, a concern that has been raised by some laboratory studies.

Endocrine disruption in fish appears more frequently associated with three types of land use: sewage treatment plants (STPs) (estrogenic effects), pulp and paper mills (antiestrogenic and probably antiandrogenic effects), and areas of high industrial activity/chemical contamination (antiestrogenic and possibly antiandrogenic effects).

Some of the most significant examples of endocrine disruption in fish (e.g., ovotestes and degraded gonadal structure) have been found adjacent to STPs, particularly in the United Kingdom. Impacts such as these appear to be less common in the U.S. The reasons for this are still unclear, but may be related to higher population densities, smaller flow rivers in which effluents can be diluted, different treatment regimes, or perhaps simply a greater number of investigations in the U.K.

Compounds thought responsible for a significant portion of the estrogenic effects observed in fish near STPs include the natural estrogens $E_2$, its metabolite estrone, and the synthetic estrogen $EE_2$, which are not entirely removed or degraded by treatment processes. To a lesser extent, the degradation products of alkylphenol polyethoxylate surfactants (APEs) have also been implicated as contributing to observed effects. High use and discharge of APEs, as has been seen downstream of STPs that receive influent from certain textile (ie., wool processing) operations, may in some cases contribute very significantly to the observed estrogenicity in a river.

A second activity frequently associated with endocrine disruption in fish is the discharge of wastes from pulp and paper mills, which have been associated with both antiestrogenic and antiandrogenic

effects. One candidate compound that may be associated or contributing to the observed effects is the plant sterol, β-sitosterol, generated during the wood pulping process, although the possible role of the dioxins is still being debated. β-Sitosterol may contribute to observed effects by suppressing production of the reproductive hormones through inhibition of the enzyme systems involved in the conversion of cholesterol to estrogens and androgens. Effects include decreased levels of the steroid hormones, decreased gonadosomatic indices, and external masculinization of female fish. A number of studies in the U.S. and in northern Europe have detected these and other biochemical and morphologic effects on resident populations of fish.

Endocrine disruption has also been seen in areas of high industrial activity and chemical contamination. Although somewhat variable, effects typically include reduced levels of estrogens and inhibition of gonadal growth in female fish. More work is needed, however, to assess the effects of contaminants, particularly PAHs and PCBs on the endocrine system in male fish. Compounds thought responsible for observed effects include PCBs, PAHs and possibly dioxins. A number of studies in the Puget Sound area, and also in Europe have indicated some impacts in flatfish.

Although endocrine disruption in fish has been documented in a number of field studies, little is currently known regarding the effect of EDCs at the population level. Overall, it does not appear that EDCs are currently having serious effects on large populations of fish. However, several authors have noted that reproduction and population structure are almost certainly affected in areas where the greatest impacts on individual fish have been found, such as near certain STPs. To address this lack of information, the National Research Council (NRC, 1999) has recommended long term studies of populations subjected to EDCs in order to assess effects on population size, age structure and dynamics.

Detecting changes in population structure, however, will be difficult in part due to the mobility of many fish species and the possible recruitment of new individuals from outside the affected population. In addition, detection of subtle effects, such as differences in levels of endogenous hormones, is difficult due to natural hormonal variability between fish, and by a lack of knowledge regarding what is "normal" for each species, and for each species in a particular area.

As more laboratory and field research is conducted on the identity, presence, and effects of EDCs, a clearer picture will emerge of the risks to fish and higher vertebrates, as well as strategies to reduce or even eliminate the discharge and effects of these compounds.

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