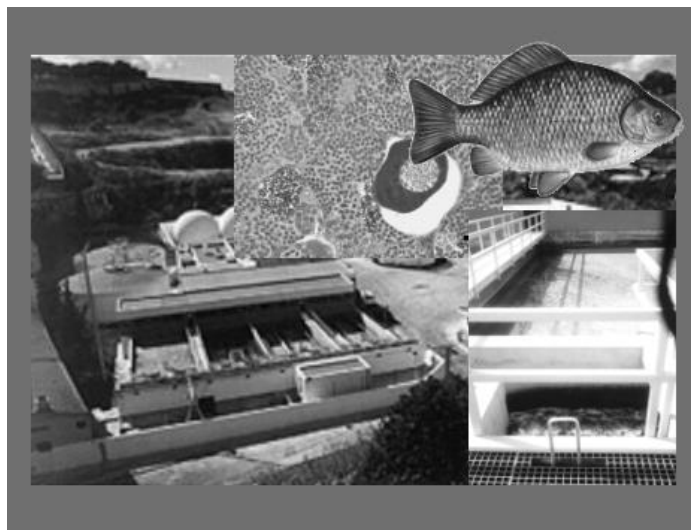


MÁRIO EMANUEL CAMPOS DE SOUSA DINIZ

**CONTRIBUTION TO THE STUDY OF ENDOCRINE DISRUPTOR
COMPOUNDS IN CYPRINIDS: ASSESSMENT OF EFFECTS ON FISH
EXPOSED TO FINAL EFFLUENT OF URBAN WASTEWATER
TREATMENT**



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Philosophy in Environmental Engineering

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2005**

Author's declaration

The author states that he afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation, according to the *nº 2 of art. 8º do Decreto –Lei 388/70*.

Mário Emanuel Campos de Sousa Diniz

“Doubt, the essential preliminary of all improvement and discovery, must accompany the stages of man's onward progress. The faculty of doubting and questioning, without which those of comparison and judgment would be useless, is itself a divine prerogative of the reason”.

Albert Pike

"Water is the driving force of all nature."

Leonardo Da Vinci

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A special thank to my wife and to my parents.

ABSTRACT

Since the early 1990s, there has been growing concern about a group of chemical compounds with endocrine activity which are present in the aquatic environment and can have adverse effects on reproduction in humans and wildlife species. Many reported effects have been related to several classes of chemical compounds that are present in wastewater effluent, such as pesticides, aromatic compounds, alkylphenols, phytoestrogens and natural estrogens. In addition, the efficiency to remove these chemicals by the sewage treatment plants (STPs) has been questioned since, in some cases, they generate compounds with a higher toxicity than the parent compounds. Therefore, freshwater systems are particularly vulnerable to the presence of endocrine disruptor compounds (EDCs) since the proximity to the sources of pollution and the low dilution factor of these waters makes the inhabiting fauna highly exposed.

The main objective of this study was to evaluate the estrogenic potency of a STP effluent and the adverse effects to fish by applying a combination of chemical analysis and various bioanalytical tools.

A STP located in Chelas, a district of Lisbon (Portugal), that receives large volumes of urban wastewater and variable amounts of industrial effluent, was selected as model to carry out studies on the endocrine disruption issue. Thus, some EDCs were identified and quantified in the STP effluent using analytical instrumentation (ELISA, LC-MS-MS, HPLC) and biological assays were carried out under laboratory and in semi-field conditions.

Experiments were carried out by exposing different species of cyprinids (*Cyprinus carpio*, *Carassius carassius* and *Carassius auratus*) to selected EDCs (17 β - estradiol, 17 α -ethynylestradiol, Bisphenol A, Nonylphenol and Octylphenol) in laboratory experiments, using different EDCs separately and in mixture, for different concentrations of the treated sewage effluent in semi-field assays.

To assess the estrogenic effects in fish several endpoints were used such as induction of vitellogenin (Vtg), gonadosomatic index (GSI), histopathology (used to evaluate effects of exposure to EDCs at tissue level), detection of intersex condition and circulating steroid hormone (E2 and 11-KT) levels.

Results from the semi-field experiments, showed a consistent increase in Vtg induction in

male exposed to the treated STP effluent. A significant reduction of the gonadosomatic index was also found and moderate to severe histopathological effects were observed in testes. Spermatogenesis was progressively reduced and in some cases total inhibition occurred, mainly in fish exposed to 100% effluent. One of the most pronounced effects observed was the development of oocytes within the testes of male exposed to the treated effluent. Moreover, steroid concentrations revealed an inverse correlation between 11-Ketotestosterone and E2 in males and elevated levels of E2 were also detected in exposed female. Seasonal variations were also observed in estrogenic responses and were attributed to weather influences on sewage dilution. In addition, size/age sensitivity was assessed and histological examination and condition indices suggested that smaller fish showed stronger estrogenic adverse effects (e.g. development of oocytes and spermatogenesis inhibition in most fish).

The results from the laboratory assays agreed to semi-field experiments, showing Vtg induction and a decrease in the GSI. The histological findings were also similar but the results suggested that the strongest estrogenic responses concerning the assessed endpoints were caused by the tested steroids.

As a global conclusion, the instrumental analysis and the biological endpoints provided evidence that sewage effluent contains chemical compounds capable of affecting the endocrine system of fish. As a consequence it can also be concluded that treated domestic from the STP that was used to carry out these studies effluent possess estrogenic activity.

Additionally, the treated effluent is discharged into the River Tagus estuary and considering that there are various river and estuarine organisms of commercial value that may bio-accumulate EDCs and be consumed by humans, and that river water is also used in the production of drinking water, public health problems may arise from the exposure to estrogenic chemicals.

RESUMO

Desde o início da década de 90, tem existido um interesse crescente relativamente a um grupo dos compostos químicos com actividade estrogénica, que se encontram no ambiente aquático e podem ter efeitos adversos ao nível da reprodução nos seres humanos e nos organismos vivos do meio natural. Muitos destes efeitos encontram-se relacionados com diversas classes de compostos químicos, que normalmente se encontram nos efluentes de ETARs (Estações de Tratamento de Águas Residuais), tais como pesticidas, compostos aromaticos, alquilfenóis, fitoestrogénios e estrogénios naturais. Além disso, a eficiência de remoção destes compostos químicos nas ETARs tem sido questionada uma vez que, em alguns casos, geram compostos com uma toxicidade mais elevada do que os compostos que lhes deram origem. Consequentemente, os sistemas de água doce encontram-se particularmente vulneráveis à presença de compostos desreguladores endócrinos (EDCs), dado que a proximidade às fontes de poluição e o baixo factor de diluição de algumas destas massas de água tem como consequência um nível de exposição elevado para o biota.

O objetivo principal deste estudo foi avaliar o potencial estrogénico do efluente tratado de uma ETAR previamente seleccionada e avaliar os seus efeitos adversos, através da utilização combinada de análises químicas e diversos ensaios bioanalíticos. Assim, uma ETAR localizada em Chelas, distrito de Lisboa (Portugal), que recebe volumes elevados de águas residuais urbanas, assim como quantidades variáveis de efluentes industriais, foi seleccionada para o estudo da desregulação endócrina em peixes. Deste modo, foram identificados e quantificados no efluente da ETAR alguns compostos EDCs alvo, utilizando para o efeito diversa instrumentação analítica (ELISA, LC-MS-MS, HPLC) e ensaios biológicos realizados em laboratório e na ETAR.

Os ensaios foram realizados através da exposição de diferentes espécies de ciprinídeos (*Cyprinus carpio*, *Carassius carassius* e *Carassius auratus*) a EDCs previamente seleccionados (17 α - etinilestradiol, 17 β - estradiol, Bisphenol A, Nonilfenol e Octilfenol) em laboratório, expondo os peixes aos EDCs individualmente e em mistura. Na ETAR os peixes foram expostos a diferentes concentrações do efluente tratado. Por forma, a avaliar os efeitos estrogénicos nos peixes foram usados diversos biomarcadores, como a indução de vitelogenina (Vtg), níveis plasmáticos de esteroides circulantes 17 β - estradiol (E2) e 11-Ketotestosterona (11-KT), histopatologia, detecção da condição de intersexo e diversos

índices somáticos e de condição, como indicadores complementares, na avaliação dos efeitos ao nível do crescimento e desenvolvimento das gónadas.

Os resultados dos ensaios na ETAR mostraram um aumento consistente na indução de Vtg nos machos expostos ao efluente tratado da ETAR. Foram ainda observados, uma redução significativa do GSI e efeitos histopatológicos moderados a severos nas gónadas dos peixes, como a redução progressiva da espermatogénese e, nalguns casos, a inibição total da mesma, principalmente nos peixes expostos a 100% do efluente tratado. Um dos efeitos mais pronunciados foi o desenvolvimento de ovócitos no interior dos testículos dos machos expostos ao efluente tratado.

Para além disso, as concentrações plasmáticas de esteroídes nos machos revelaram uma correlação inversa entre a 11-KT e E2. Níveis elevados de E2 foram também detectados nas fêmeas expostas. As variações sazonais observadas na resposta estrogénica parecem estar associadas a factores climáticos que por sua vez influenciam a diluição do efluente. A sensibilidade dos peixes em função do tamanho/idade foi também avaliada e o GSI e a avaliação histológica parecem sugerir que os peixes de menor tamanho se encontram mais susceptíveis à exposição a EDCs.

Os resultados dos ensaios de laboratório estão em concordância com os que foram obtidos nos ensaios realizados na ETAR, mostrando de igual modo a indução de Vtg e a diminuição do GSI nos machos expostos. Os resultados da avaliação histológica são também similares aos observados nos peixes expostos ao efluente tratado, embora os resultados sugiram que as respostas estrogénicas mais fortes foram causadas pelos esteroídes testados.

Como conclusão global, os resultados da análise instrumental e os ensaios biológicos evidenciam que o efluente tratado da ETAR contém EDCs com capacidade para afectar negativamente o sistema endócrino dos peixes, podendo ainda concluir-se que o efluente tratado da ETAR utilizado para a realização destes estudos possui actividade estrogénica. Adicionalmente, como o efluente tratado da ETAR é descarregado no estuário do Rio Tejo e, considerando que existem diversos organismos estuarinos de elevado valor comercial e que podem bioacumular EDCs e ser consumidos pelo Homem, e ainda que a água do rio é também utilizada para a produção de água de consumo, é de admitir que possam advir problemas de saúde pública devido a uma eventual exposição a EDCs.

ABREVIATIONS

AchE - Acetylcholinesterase

ANOVA – Analysis of Variance

APEs – Alkylphenol Poly Ethoxylates

BKME – Bleach Kraft Mill Effluent

BOD5 – Biochemical Oxygen Demand

BPA - Bisphenol A

BSA – Bovine Serum Albumine

COD – Chemical Oxygen Demand

CSTEE - European Union Scientific Committee of Toxicity, Ecotoxicity, and the Environment

CYP1A - cytochrome P450 1A

DDE - 4, 4-dichloro-diphenyl-dichloroethene

DDT - 4, 4-dichloro-diphenyl-trichloroethane

DES - Diethylstilbestrol

E1 - Estrone

E2 – 17 β - estradiol

E3 - Estriol

EE2 – 17 α - ethynylestradiol

EDC – Endocrine Disruptor Compounds

EIA – Enzyme Immuno Assay

ELISA – Enzyme Linked Immuno Assay

ER – Estrogen receptor

EU – European Union

GC – Gaseous Chromatography

GC – MS – Gaseous Chromatography coupled to Mass Spectrometry

GSI – Gonadosomatic index

H&E – Hematoxylin and Eosin

HPLC – High Performance Liquid Chromatography

HSI – Hepatosomatic index

INE – Instituto Nacional de Estadística

INM – Instituto Nacional de Meteorología

K – Total condition factor

KI - Somatic condition index

LC – Liquid Chromatography
LC – MS – MS - Liquid Chromatography / Mass Spectrometry / Mass Spectrometry
LIM/ESE - Laboratory of Immunology and Microbiology/
N – Nitrogen
NP - Nonylphenol
OP - Octylphenol
P - Phosphorous
PAH - polycyclic aromatic hydrocarbons
PBS - phosphate buffered saline
PBS Tween – phosphate buffered saline with Tween
PCB - polychlorinated biphenyls
PGC – Promordial Germ Cell
PNPP - p-nitro-phenylphosphate
SPE – Solid Phase Extraction
TSS – Total Suspended Solids
VSS – Volatil Suspended Solids
STP – Sewage Treatment Plant
T – Testosterone
TIU – Trypsin Inhibitor Unit
U.K. – United Kingdom
Vtg – Vitellogenin
WWTP – WasteWater Treatment Plant
USA - United States of America
11 – KT – 11-Ketotestosterone

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PART I

CHAPTER 1 – GENERAL INTRODUCTION

1. Endocrine Disruption

1.1. Historical Background

Since the early 1990s, there has been growing interest in the phenomenon of endocrine disruption (ED) and in recent years there has been much concern about environmental chemicals, which interact with the endocrine system, because of their possible adverse effects on the health of humans and wildlife (Tyler et al., 1998; Vos et al., 2000; Kinnberg and Toft, 2003). However, ED effects are not surprising since in the 1930s Dodds and Lawson had already explored the structural basis of oestrogenic activity and shown that a wide range of chemicals synthesised to closely approximate or 'look like' oestradiol, e.g. some biphenolic chemicals and alkylphenols, possessed weak oestrogenic activity. Furthermore, research published over 20 years ago showed very conclusively that some species of birds, particularly those that eat fish, were adversely affected by agricultural chemicals and industrial wastes. A variety of effects on both embryos and adults were reported, including impaired differentiation of the reproductive system, eggshell thinning, and impaired incubation and chick rearing behaviours (Sumpter, 1998).

It was Rachel Carson who, with her work on DDT in the 1950's and 60's, first highlighted the subtle impacts that chemicals in the environment have on animals. Since her publication "*Silent Spring*" (Carson, 1962), there has been increasing awareness that chemicals in the environment can exert profound and deleterious effects on wildlife populations and that human health is inextricably linked to the health of the environment.

However, it was only with the publication of the book "*Our Stolen Future: Are We Threatening Our Fertility, Intelligence and Survival?*" that Colborn et al. (1996) drew public attention to this pollution. That event was followed by numerous research programmes, reviews, workshops and conferences (e.g. Colborn et al., 1993; IEH, 1995; Toppari et al., 1995; European Commission, 1996; Kavlock et al., 1996; Tattersfield et al., 1997; Kendall et al., 1998).

Thus, for the past few decades, an increasing effort has been made to elucidate the endocrine disrupting properties of both natural and man-made compounds with regard to hormonal activity (Tyler et al., 1998; Lindholm et al., 2001). A growing number of reports describes alterations to the endocrine system in numerous species. Moreover, a number of

environmentally persistent chemicals have been shown to be able to mimic endogenous hormones and, according to reports, can disrupt the reproductive and endocrine system of humans and wildlife (Minier et al., 2000).

1.2. Definition of Endocrine Disruptor Compound (EDC)

The existence of environmentally significant levels of compounds with the capacity to act as endocrine disruptors (EDCs) and the consequences that they may have on both wildlife and the human population have led Institutional and Government working bodies to propose various definitions of an EDC, though there is lack of consensus as to an appropriate definition. For example, during the Weybridge Conference (1996), the European Union Scientific Committee on Toxicity, Ecotoxicity, and the Environment (CSTEE) Working Group on Endocrine Disrupters agreed on the International Programme on Chemical Safety (IPCS) definition of an endocrine disrupter as “*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population*”. It was also agreed that “*a potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism*”. (European Commission, 1996; Solé et al., 2001; Baker, 2001).

Moreover, the Office of Research and Development in the U.S. held a workshop in 1995 where an environmental endocrine disruptor was broadly defined as “*an exogenous agent that interferes 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*”. This reflected a growing awareness that the issue of endocrine disruption in the environment extends considerably beyond that of exogenous estrogens and includes antiandrogens and agents that act on other components of the endocrine system (Kavlock et al., 1996).

In May 1997, the U.S. Environmental Protection Agency (EPA) task force on endocrine disruption (EDSTAC) agreed on the following operational definition: “*An **endocrine disruptor** is an exogenous chemical substance or mixture that alters the functions(s) of the endocrine system and thereby causes adverse effects to an organism, its progeny, or (sub) population*”. The inclusion of the word “adverse” effect was controversially discussed. In order to achieve consensus, the EDSTAC finally agreed to the following general description:

*“The EDSTAC describes an **endocrine disruptor** as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, the populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle” (Lintelmann et al., 2003).*

The Canadian Environmental Protection Act (1999) also defined a hormone disrupting substance: *“Hormone disrupting substance” means a substance has the ability to disrupt the synthesis, secretion, transport, binding, action, or elimination of hormones in an organism, or its progeny, that is responsible for the maintenance of homeostasis, reproduction, development or behavior of an organism.* In Britain, ED effects are now a high priority for evaluation by the Environment Agency, which has recently issued a document setting out its policy for tackling the issue (Allen et al., 1999).

However, there is a on-going debate about whether any effect on the endocrine system (adverse or otherwise) should be described as disruption. This is akin to the wider argument about whether small changes in biochemical or cellular markers of pollution, because they may predict long-term damage, can rightfully be described as adverse in themselves when they may lie entirely within an organism normal homeostatic range. Clearly, modulations of the endocrine system are occurring all the time due to entirely natural environmental factors, so small perturbations caused by anthropogenic chemicals may not push the system into the pathological range. The key questions are therefore whether biochemical systems, and the cells which contain them, can adapt successfully to chemical exposure, and if not, to what extent there will be knockon consequences for individuals and populations (Matthiessen et al., 1998).

1.3. Development of appropriate test systems and test guidelines

In November 1996, the 25th Joint Meeting of the OECD Environmental Health and Safety Programme established a Special Activity on Endocrine Disruptors in order to ensure testing harmonization among countries, which is the reflection that no existing OECD Test Guidelines are currently sufficient for the detection of EDCs in both mammalian and non-mammalian species. In the meeting a number of possible enhancements were identified which could be added to the relevant existing OECD Test Guidelines. No specific recommendations were made for *in vitro* and *in vivo* screening but promising tests were identified. It was also

supported the revising of existing OECD Test Guidelines to take into account adverse effects caused by endocrine disrupters rather than developing new Test Guidelines. In the case of new Test Guidelines, the most commonly identified needs were reproductive effects in aquatic wildlife species and effects on sex-linked hormones in both mammalian and aquatic species (Huet, 2000).

Nevertheless, the choice and development of appropriate test systems to identify and characterize chemicals that may disrupt the endocrine system is complicated by a number of factors. For example, receptor-mediated endocrine disrupters do not obey the normal rules of toxicity because endogenous levels of hormones are present in the body. Endocrine disrupters may also act via a number of different mechanisms producing a wide range of responses, possibly at very low doses. In addition, although there are a large number of potential assays available, most of which are unvalidated and based on a range of different mechanisms, at present there are no specific test guidelines in place for the detection of endocrine disrupting chemicals.

1.4. Endocrine Disruptors in the aquatic environment

Endocrine disruptor chemicals, as well as other pollutants, have a variety of sources. These sources may have implications for human exposure, effects on wildlife and effects or accumulation within the environment (Birkett, 2003). Generally, sources of pollution fall into two main categories: point sources and nonpoint sources (Table 1.1).

Table 1.1: Point and nonpoint sources (*from* Birkett, 2003).

Point Sources	Nonpoint sources
Discharges from sewage treatment works to rivers	Runoff and underdrainage from agricultural land into rivers
Discharges of industrial wastewaters to rivers	General contamination of recharge rainfall to outcropping aquifers
Discharges of farm effluents to rivers	Septik tank soakaways into permeable strata
Discharges from small domestic sewage treatment plants to rivers	Washoff of litter, dust, and dry fallout from urban roads to rivers
Discharges by means of wells or boreholes into underground strata	General entry of sporadic and widespread losses of contaminant to rivers
Discharges of collected landfill leachate to rivers	Seepage of landfill leachate to underground strata and rivers

Freshwater systems are particularly vulnerable to the presence of EDs since the proximity to the sources of pollution and the low dilution factor of these waters means that the inhabiting fauna suffers a high degree of exposure. Moreover, the purification role of sewage treatment plants (STPs) has been questioned since, in some cases, they generate compounds with a higher toxicity than the parent compounds. This is the case with non-ionic surfactants such as alkylphenol polyethoxylates (APEs), which degrade to nonylphenol (NP), and with estrogens and progestogens, which are deconjugated by microbial action that leads, in turn, to the biologically active form (Solé et al., 2001).

One of the key questions on this topic has been whether the endocrine-disrupting compounds are present in the environment at sufficiently high concentrations to elicit adverse reproductive effects in fish. A second key question is whether these substances have an ecological impact on wildlife. To date, the right answer to these questions remains unclear. There are examples of reproductive disruptions in fish but there is still no clear evidence that this has led to reduced spawning success (Lye et al., 1997; Matthiessen et al., 1998; Bjerselius et al., 2001).

1.4.1. Types of Endocrine Disruptor Chemicals in the aquatic environment

According to some authors, about 50 to 70 different compounds have been identified as possible ‘endocrine disrupting chemicals’ (EDCs) (Matthiessen et al., 1996; Ishibashi et al., 2001). However, as more chemicals are investigated, the number of those shown to possess estrogenic activity is likely to increase. This makes it difficult to make an informed guess as to which of the chemical(s) on the list are likely to be responsible for the estrogenic activity of effluent (Sumpter, 1995).

A potential route of exposure of terrestrial and aquatic wild life is by contact with contaminated surface waters. EDC can enter surface water by a variety of mechanisms including discharges of both point (sewage treatment, pulp mill and industrial effluent) and non-point (urban and agricultural runoff and overland flow after rainfall) sources (Folmar et al., 2002; Barceló, 2004).

To date, the best studied and probably the most common group of EDCs are environmental estrogens or xenoestrogens, estrogen mimics, which are exogenous chemicals that elicit similar effects to endogenous female sex steroids (such as 17 β -estradiol). The mechanism

typically used is interaction with the estrogen receptor, which can disrupt an animal's endocrine system (Nimrod and Benson, 1998; Rodgers-Gray et al., 2000).

Furthermore, endocrine disrupting compounds can interfere with the endocrine system at multiple levels and exert their effect through numerous modes of action (Barton and Andersen, 1998). They include both natural and synthetic steroid estrogens, as well as a variety of estrogen-mimicking chemicals (Jobling et al., 2003), which occur as complex mixtures, whereby certain compounds might have either additive or antagonistic effects upon the toxicological action or biological response of other chemicals (Calabrese, 1997; Arukwe et al., 2001).

Thus, many naturally occurring compounds (lignans, coumestrans, isoflavones, mycotoxins), synthetic estrogens (e.g. 17 α -ethynylestradiol (EE2) or Diethylstilbestrol (DES)) and natural estrogens (E2, estrone (E1), and estriol (E3)) are known to be estrogenic and present in the aquatic environment (discharged by sewage effluents). In addition, numerous man-made chemicals, such as dioxins and furans (persistent organochlorines that are fat soluble and tend to bioaccumulate in tissues), the organochlorine pesticides (such as methoxychlor, *o,p*-DDT, and its derivatives or metabolites and chlordane), some phthalates (predominantly used as plasticisers in a wide variety of applications including PVC) and industrial chemicals such as alkylphenolic detergent components, bisphenol A, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) have been identified as environmental estrogens based on their estrogen-like effects (Sumpter, 1995; Gimeno et al., 1995, 1996; Jobling et al., 1996; Routledge et al., 1998; Nimrod and Benson, 1998; Miles-Richardson et al., 1999; Arukwe et al., 2001cd; Baker, 2001; Van den Belt et al., 2001).

Heavy metals such as cadmium, lead and mercury have no inherent hormonal activity, but in higher doses are toxic to gonadal cells (Davis et al., 1999). The major classes of environmental estrogens are summarized in Table 1.2 (Arukwe, 2001d). Furthermore, some of these EDC could act also as antiestrogens (e.g. chlorinated dioxins, furanes, PCB-congeners, PAH), as androgens (e.g. tributyltin), or as antiandrogens, (e.g. p,p-DDE, vinclozolin) (Gutendorf and Westendorf, 2001; Bayley et al., 2002).

Table 1.2: Major classes of environmental estrogens (*from* Arukwe, 2001d).

Natural Products	Environmental Pollutants	Industrial Chemicals	Pharmaceutical Chemicals	Complex Mixtures
Genistein	DDT	Brominated flame retardants	Ethinylestradiol	Sewage effluents
Naringenin	Dioxins	BPA	Diethylstilbestro	Industrial effluents
Coumestrol	Kepone	Nonionic surfactants	Norgestrel	Air particulates
Zearalenone	PCBs	Endosulfan	Gestodene	Sediment extracts
Equol	PAHs	Phthalate esters	Contraceptives	Tissue extracts
Phloretin		Insecticides		
Enterlactone		Parabens		
β -sitosterol				
Daidzen				
Kaempferol				

Among the cited compounds, the natural steroids (estrone and 17β -estradiol) and the synthetic steroid 17α -ethinylestradiol (EE2) deserve particular attention for displaying the highest estrogenic capacities. Females excrete primarily conjugated estrogens (McKerns 1969) but, as presence of free and hence biologically active compounds is reported to be more relevant at the vicinity of sewage treatment plants (STPs), deconjugation probably occurs by bacteria during water treatment. EE2 is the main component of contraceptive pills, and it is also more resistant to breakdown than natural estrogens; its principal source into the aquatic environment is through urban sewage water (Solé et al., 2000).

1.4.2. Presence of Endocrine Disruptor Compounds in Sewage Treatment Plants

In developed countries, with a high density of people, the volume of sewage entering STPs, and thus effluent leaving, can be considerable and contribute significantly to the overall flow of some rivers, particularly those in urban areas (Sumpter, 1997). Obviously the amount of effluent entering a river, the size of the receiving river, the quality of the effluent and the weather conditions at the time, plus, in all probability, certain other factors, will all influence whether or not a river is estrogenic and, if so, to what degree. Nevertheless, it is not unusual for the effluent from an STP to constitute a significant proportion of the flow of a river. In some cases, particularly when slow-flowing rivers pass through urban areas (where there are often a number of large STPs), the effluent can contribute the greater part of the flow; figures as high as 80% or 90% of the water in a river passing an STP are reached in periods of low rainfall. This is what led to the suggestion that not only neat effluent but also certain river sections might be estrogenic (Sumpter, 1995).

In many countries municipal STP effluents have been shown to contain natural and pharmaceutical estrogens from human waste, as well as other household and industrial chemicals capable of eliciting an estrogenic response in aquatic animals (Purdom et al., 1994; Sumpter and Jobling, 1995; Desbrow et al., 1998; Lye et al., 1997; Harries et al., 1999; Folmar et al., 2001). However, when it is realized that over 80,000 man-made chemicals are in routine use and a thousand new ones are added each year, all of which degrade to produce very many more chemicals, it is obvious that effluents will be highly complex mixtures of chemicals (Sumpter, 1997). This fact illustrates the difficulty of studying the endocrine disruption issue.

1.4.2.1. Identification of Endocrine Disruptor Compounds

Several studies have shown that endogenous and synthetic estrogen hormones are present in sewage treatment plant (STP) effluents and that these compounds may be an important source of estrogenic activity (Desbrow et al., 1998; Ternes et al., 1999; Baronti et al., 2000). In addition to these steroids, the weakly estrogenic NP has been found in STP effluents, river systems, estuaries, sediments and tissues (Ahel et al., 1994; Blackburn and Waldock, 1995; Blackburn et al., 1999; Lye et al., 1999), and may already have reached the status of ubiquity (Kuch and Ballschmiter, 2001; Ackermann et al., 2002)

Some effluents (such as from a single industry) may be relatively uncomplicated, and of known composition, but others (such as from STPs) will be extremely complex and of unknown composition. It is important to realize that not only are effluents usually complex mixtures of chemicals, but that the constituents making up the mixtures might vary considerably from one type of effluent to another. Thus, for example, BKME contains high concentrations of plant-derived chemicals, including phytosterols (Mellanen et al., 1996), whereas effluent from STPs does not; instead, it would contain primarily chemicals which are used domestically, and their degradation products. Effluents from industrial sources would be expected to vary considerably, depending on which type of industry produced the effluent. All these effluents enter the aquatic environment. The only way to determine which endocrine-disrupting chemicals are present in such effluents, and at what concentrations, is to study each effluent individually, using some sort of fractionation procedure linked to a method of determining the endocrine activity of the fractions (Sumpter, 1997).

Individual potencies differ considerably between natural or synthetic hormones being quite potent at low concentrations and phytoestrogens and xenoestrogens being more weakly estrogenic but typically found at higher concentrations (Desbrow et al., 1998; Islinger et al., 1999; Lindholm et al., 2000; MacLachy et al., 1997; Mazur and Adlercreutz, 1998; Routledge et al., 1998; Servos, 1999). To compound the issue, the presence of these various estrogenic analytes in wastewater effluents is highly variable (Desbrow et al., 1998; Routledge et al., 1998; Spengler et al., 2001; Sheahan et al., 2002a). Research in the United Kingdom showed strong temporal (Rodgers-Grey et al., 2000) and spatial (Harries et al., 1997; Jobling et al., 1998) variation in wastewater effluent estrogenicity.

1.4.2.1.1. Analytical instrumentation and bioassays used to quantify EDCs

Endocrine disruptors are usually present in the environment at low concentrations, so pre-concentration techniques are usually needed to determine them. Solid-phase extraction (SPE) followed by chromatographic techniques is one of the most common techniques. Analytical tools based on conventional techniques such as gas or liquid chromatography (GC and LC) coupled to sensitive detection systems such as mass spectrometry (MS) or MS-MS have been applied to detect EDCs. Unfortunately, most of these techniques are time-consuming and have elevated cost due to the fact that extensive cleanup and sample treatment methods that often involve derivatization procedures are required (Goda et al., 2000; Castillo et al., 2000).

Alternatively, immunochemical techniques offers a large number of advantages, the most relevant being the selectivity and sensitivity shown by the specific antibodies for the target analyte, the use of small sample volumes, the low cost, and the simplicity of the methodologies. However, immunochemical methods developed are usually focused on families or groups of chemicals, but not on a large number of compounds with estrogenic activity, which usually belong to different families, in order to control their presence in real water samples. A variety of immunochemical methods against those hormones have also been reported and several antibodies both monoclonal and polyclonal) and immunoassay kits are commercially available (Estévez-Alberola and Marco, 2004).

Various types of short-term estrogenicity tests can be used, such as binding assay to estrogen receptor (ER), proliferation of MCF-7 cells (E-screen assay), transient reporter gene expression in MCF-7 cells, reporter gene expression in yeast strains stably transfected with

the human ER (yeast estrogen screen), an estrogen-responsive reporter gene and more recently the development of specific biosensors (Goda et al., 2000; Matsui et al., 2000).

1.5. Endocrine disruption in wildlife

A primary concern associated with estrogenic and anti-androgenic anthropogenic chemicals is interference with reproduction and development. While evidence suggests that EDCs in the aquatic environment can negatively affect fish reproduction, the extent to which most natural fish populations are impacted by EDCs remains unknown. Fish, like other oviparous vertebrates, may be especially sensitive to exogenous estrogens (Mills et al., 2001; Zaroogian et al., 2001).

Numerous reports in the scientific and popular literature have described the potential effects of environmental estrogens (usually termed endocrine disrupting chemicals, EDCs) on humans and wildlife species and emphasize the need to better understand both estrogenic presence and persistence (Harries et al., 1996; Jobling et al., 1998; Sumpter, 1995; Folmar et al., 2001). Many of these chemicals have estrogenic activity and can disrupt normal functions of sex steroids by causing feminization phenomena in all classes of vertebrates (Ashby et al., 1997; Panter et al., 1998; Kloas et al., 1999). Furthermore, recent studies indicate that abnormalities in the male reproductive system may also be induced by antiandrogens (Vos et al. 2000; Kinnberg and Toft, 2003). However a main question remains: do environmentally relevant concentrations of these xenobiotics affect individual reproduction and thereby potentially impact populations? (Koger et al., 2000b). In humans, for example, there is an increasing body of evidence suggesting a possible link between environmental chemical pollution and adverse effects on both male and female reproductive systems. Several field and laboratory studies on a range of different animal species have demonstrated estrogenic and anti-estrogenic effects of chemicals in the environment (Arukwe et al., 2000; Metcalfe et al., 2000; Bowerman et al., 2000; Dreze et al., 2000). However, the importance of these effects on the whole ecosystems is not known and it is not possible to make assumptions about the relevance to humans of even such well documented cases (Arukwe et al. 2001c).

1.5.1 Effects in wildlife

Information on the impacts of EDCs on wildlife populations is limited to a few species in several vertebrate and invertebrate taxa (Tyler et al., 1998). In the aquatic environment,

effects have been observed in mammals, birds, reptiles, fish, and mollusks from Europe, North America, and other areas. The observed abnormalities vary from subtle changes to permanent alterations, including disturbed sex differentiation with feminized or masculinized sex organs, changed sexual behavior, and altered immune function. Crucial in establishing causal evidence for chemical-induced wildlife effects appeared semifield or laboratory studies using the wildlife species of concern. Impaired reproduction and development causally linked to endocrine-disrupting chemicals are well documented in a number of species and have resulted in local or regional population changes (Vos et al., 2000).

Among vertebrates, fish are particularly sensitive to the influence of biotic or abiotic factors of the aquatic medium. This sensitivity can be related to their permanent presence in this medium, to their poikilothermy (body temperature dependent on the ambient temperature) and to the importance of the exchanges (respiration, osmoregulation, nutrition, etc.) with an aquatic ecosystem able to dissolve a great number of natural or synthetic molecules, and to transform and/or to concentrate them in various physical or biological compartments. Due to their singular situation in the phylogenetic tree and of the great diversity of their adaptation strategies, fish species share many common physiological features with other vertebrates, higher vertebrates among others, thus, offering as sensitive indicators of the quality of aquatic environments, and useful models to understand by which mechanisms external perturbations may disrupt essential biological functions in other vertebrates (Jalabert et al., 2000).

The responses and relative sensitivities of different animal species to EDCs have not been comprehensively compared and may vary, both within and between taxa. Consequently, there are fundamental shortfalls in our knowledge of the relative importance of endocrine disruption in different wildlife taxa, and of the level of the problem of endocrine disruption in wildlife relative to other environmental stressors. Most of the evidence for endocrine disruption in wildlife has come from studies on species living in, or closely associated with, the aquatic environment, which is perhaps not surprising given the fact that our rivers and oceans act as repositories for the discharge of tens of thousands of chemicals in large volumes (Jobling et al., 2003). Additionally, to date, very few studies have compared the effects of estrogens and estrogenic effluents in different vertebrates in the same taxon (fish or otherwise), or between different taxa (Thompson et al., 2000).

1.5.1.1 Invertebrates

Few studies have examined the effects of EDCs on invertebrates, mainly due to the general lack of knowledge of their basic endocrine physiology. Some of the strongest data from field studies to demonstrate causes and effects of EDCs are derived from prosobranch molluscs in which organotin compounds were reported to cause imposex and intersex, resulting in a virilization of female snails in over 150 species of molluscs (Matthiessen and Gibbs, 1998; Barroso et al., 2002; Evans et al., 2001). These effects have been reported in mollusc populations worldwide and are usually attributed to TBT (which is used as antifoulant by naval industry) and ship traffic (Tyler et al., 1998). For example, The earliest known example of endocrine disruption in UK wildlife concerns the effects of the anti fouling compound tributyltin TBT on marine molluscs first observed in the early 1970s. (Matthiessen and Gibbs, 1998). Mechanistic studies on the induction of imposex and intersex in prosobranch molluscs indicate that steroids (particularly testosterone) may play an important role in the manifestation of these abnormalities (Bettin et al., 1996; Gooding and LeBlanc, 2001). Moreover, exposure of two prosobranch snails (*Marisa cornuarietis*; a tropical freshwater snail, and *Nucella lapillus*; a marine species) to the environmental estrogens BPA and 4-tert octylphenol has been shown to affect egg production, suggesting that these invertebrates are sensitive to the effects of estrogens (Jobling et al., 2003).

1.5.1.2. Fish

More than twenty years ago, acting on casual observations by anglers, Thames Water Authority staff found a low incidence (around 5%) of intersex, or hermaphroditism, in populations of wild roach (*Rutilus rutilus*, a common lowland freshwater cyprinid fish) living in a sewage effluent settlement lagoon, just downstream of an STP discharge point, in the River Lea catchment area (South-East England) (Purdom et al., 1994; Sumpter, 1998; Tyler and Routledge, 1998). High incidences of intersex gonads and induction of vitellogenin have been observed in caged male rainbow trout (*Oncorhynchus mykiss*) placed in the lagoons of sewage treatment works and in roach collected from a variety of British rivers (Purdom et al., 1994; Jobling et al., 1997). These findings led to the suggestion that sewage effluent contained a chemical, or mixture of chemicals, that might be estrogenic to fish (Sumpter, 1995; Sumpter and Jobling 1995). The effluents from many STWs, distributed throughout the country, were investigated and all proved strongly oestrogenic to both trout and carp (Purdom et al., 1994).

Subsequent work has focused on rivers receiving effluent, where Harries et al. (1995, 1996, 1997), using caged rainbow trout, have shown that some UK rivers downstream of discharges from sewage treatment works are oestrogenic for several kilometres, although the effect usually declines rapidly with distance on account of dilution and other processes.

An extensive field study in wild roach (*Rutilus rutilus*) by Jobling et al. (1998) throughout the U.K., has shown that this oestrogenic exposure is accompanied in most instances by the presence of intersex conditions. In some cases, 100% of the male fish contain oocytes in their testes (ovotestis), and there was a strong correlation between these findings and the proximity of these fish to STP effluent discharges, particularly downstream (Allen et al., 1999; Minier et al., 2000). Further, not only were plasma vitellogenin concentrations extremely high, but the testes of the caged male trout were much smaller than those of control fish (Harries et al., 1996, 1997; Sumpter, 1998). Whereas in other cases, even though the effluent entering was oestrogenic, no effects were observed in caged fish placed at varying distances downstream of where the effluent entered; presumably the active chemicals in the effluent were either degraded rapidly, or diluted below an effective concentration (Harries et al., 1997, Sumpter, 1997). Furthermore, a very recent study has reported feminization in a second species of wild fish, the gudgeon (*Gobio gobio*), in these same rivers. In the studies on wild roach and gudgeon, indicators of feminisation included induction of vitellogenin (a female specific yolk protein precursor) in male fish and intersexual gonads (containing both male and female tissue) and/or aberrant reproductive ducts (female-like) (Van Aerle et al., 2001).

Although the composition of effluents differs, depending on the industrial and domestic inputs they receive, the same studies developed in USA, Germany and France produced similar results (Folmar et al., 1996; Tyler et al., 1998). For example, in the extensive waterways of Berlin, Germany, 70% of the fish population were reported as female. (Hansen et al., 1998). Several other reports have also described elevated blood levels of vitellogenin feral male carp (*Cyprinus carpio*), and native flounder (*Platichthys flesus*) collected near sewage treatment works in both the United Kingdom and the United States (Purdom et al., 1994; Sumpter and Jobling 1995; Folmar et al., 1996; Harries et al., 1996, 1997; Jobling et al., 1998; Lye et al., 1998; Allen et al., 1999a, 1999b). Contrary to these observations, Nichols et al. (1999) observed no Vtg production in male, or altered sex steroid concentrations in either male or female fathead minnows (*Pimephales promelas*) caged in the effluent from a central Michigan wastewater treatment plant. (Folmar et al., 2001).

Elevated vitellogenin levels and testis abnormalities were also found in male flounders (*Platichthys flesus*), a marine fish living in estuaries (Tyne estuary) receiving effluent from sewage-treatment works and other industrial discharges, compared with those caught at an uncontaminated reference site (Lye et al., 1997; Christiansen et al., 1998). In further studies, elevated vitellogenin concentrations were detected in male founder caught from a number of UK estuaries as well as sites in the North and Irish seas (Allen et al., 1999; Matthiessen et al., 1999). In the Tyne and Mersey estuaries 7 and 9%, respectively, of male founder contained ovo-testes (Allen et al., 1999, Matthiessen et al., 1999). In addition to estrogenic effects observed in natural aquatic environments, laboratory studies have revealed various endocrine disrupting properties of the compounds in question (Christiansen et al., 1998; Panter et al., 1998; Lindholm et al., 2001).

1.5.1.2.1. Laboratory evidence

Laboratory studies indicated that some chemicals in the environment, both natural and synthetic, have the potential to disrupt the endocrine system and that these may, at least theoretically, be partly responsible for the observed changes. The chemicals thus identified include (besides the naturally occurring steroid hormones) phyto- and myco-estrogens, and anthropogenic chemicals such as synthetic hormones, Bisphenol-A or alkylphenols (Furhacker et al., 2000).

In controlled laboratory studies, several chemicals have been shown to be estrogenic to fish, inducing Vtg synthesis in males and also intersexuality or sex reversal in young fish (Rodgers-Gray et al., 2000), though interspecies differences clearly exist (Laenge et al., 1997). For example, Gimeno et al. (1996) showed that, for carp (*Cyprinus carpio*) exposure to 17 β -estradiol, genetically male fish developed female sexual characteristics resulting in total phenotypic sex reversal in the exposed fish. The induction of vitellogenin in male rainbow trout has been associated with retarded testicular growth (Jobling et al., 1996). Furthermore, it has been demonstrated that the unusual production of vitellogenin in male fish can result in kidney damage (Wester and Canton, 1986) and it has been suggested that it may reduce metabolic expenditure on growth and spermatogenesis (Herman and Kincaid, 1988). However, a laboratory study investigating the effect of an estrogenic STP effluent on gonadal sex differentiation in maturing adult roach revealed no effluent exposure-related abnormalities. But in a subsequent study with fry (50 days post hatch, dph), disruption in

gonadal duct development due to exposure to an estrogenic STP effluent was demonstrated, whereas no effect on germ cell development was observed (Rodgers-Gray et al., 2001).

Many other exposure experiments have shown to induce intersex gonads (ovo-testes) in a variety of fish species (e.g. swordtail, Japanese medaka, loach, mosquitofish) following injections or implants of estrogens or dosing via the water. In guppies, medaka, salmonids and carp it was also shown the induction of intersex gonads by exposing juvenile fish to sex steroids during the critical period of sexual differentiation. Similarly, ovotestes have been induced in some juvenile carp exposed during the period of sexual differentiation to the model pseudo-estrogen, 4-*tert*-pentylphenol (TPP), an industrial chemical intermediate and also in medaka treated as eggs for up to three months with the pseudo-estrogens, *b*-hexachlorocyclohexane or 4-nonylphenol. Additionally, 4-nonylphenol induced a shift in the sex ratio (female: male) towards the female direction (Gimeno et al., 1998b). Laboratory evidence of estrogenicity caused by alkylphenol ethoxylates (APEs) or to natural and synthetic hormones was also shown in cyprinid fish (Gimeno et al., 1997; Folmar et al., 2000) and in male Japanese medaka (*Oryzias latipes*), which developed oocytes in the gonad (testis-ova) (Gray and Metcalfe, 1997). Furthermore, low levels of E2 have been shown to induce vitellogenesis in male and juvenile rainbow trout (*Oncorhynchus mykiss*) experimentally exposed to these compounds (Svenson et al., 2003).

1.5.1.3. Reptiles and amphibians

Endocrine disruption in reptiles come from extensive studies on Florida alligators after a spill with dicofol and sulfuric acid (1980) in lake Apopka. The decline of juvenile alligators and high levels of plasma estradiol was observed. Moreover, smaller penis size, poorly organised testes and lower plasma testosterone levels have been reported and related with dicofol metabolites (DDD, DDE, and chloro-DDT) detected in the eggs of demasculinized alligators, supporting the hypothesis that the gonads of alligators had been permanently modified *in ovo* by DDE, altering steroidogenesis and inhibiting normal sexual maturation (Guillette et al., 1994; Tyler et al., 1998). In snapping turtles (*Chelydra serpentina*) it was also suggested feminization in males from the Great Lakes site (Fox, 2001).

It has been shown that known EDCs, can alter Vtg expression in a model amphibian species, *Xenopus laevis*. A study reported skin lesions in salamander (*Ambystoma tigrinum*) living in a lagoon receiving STP effluent and it was suggested that chemicals from sewage caused such

effects. However studies are scarce, despite the fact that many amphibian populations are declining throughout the world, and not conclusive. Consequently, this class of vertebrates need more attention with more field and laboratory studies (Vos et al., 2000).

1.5.1.4. Birds

Several examples of pollutant-induced toxicity in wild bird populations, predominantly fish-eating birds, and reproductive or development toxicity following exposure to chlorinated pesticides, PCBs, PCDFs, and PCDDs have been reported (for a review see Vos et al., 2000). Reproductive impairment in these birds is characterized by high embryonic and chick mortality, edema, retardation of growth, and developmental deformities and thereby, significantly depress subsequent reproductive success (Colborn et al., 1993; Colborn, 1994; Miles-Richardson et al., 1999). Reproductive failure of western gulls in the California Channel Islands was also linked to DDT contamination of eggs in the early 1970's (Fry and Toone, 1981). A female-biased sex ratio in adult herring gulls and feminization of herring gull embryos were found in Lake Ontario in the 1970s. Abnormalities in gonadal histology occurred in gull chicks in the late 1990s (Fox, 2001).

1.5.1.5. Mammals

Impaired reproduction in the male Florida panther (*Felis concolor cory*), living in an area contaminated with PCBs, have been suggested to be the result of xenobiotic exposure, but, as yet, no cause effect data are available. In Canada, masculinization of female black bears and grizzly bears has been suggested the consequence of exposure to endocrine disruptors. Recently, a similar case was reported in polar bears in Europe. In top predators such as the European otter (*Lutra lutra*) and mink (*Mustela vison*) high concentrations of PCBs were reported and proposed to be responsible for decline in population by affecting reproductive capacity (Vos et al., 2000). Reproductive failure in Baltic sea seals has also been linked to endocrine disruptors. A high prevalence of uterine occlusions has been correlated with high body burdens of organochlorines and particularly PCBs. The data, however, are limited to a very few animals, there is no cause/effect information and how the exposures might have occurred is unclear (Tyler et al., 1998).

1.6. Endocrine disruption in Humans

During the last couple of decades, increases in several pathological disorders in the human male reproductive system, including cryptorchidism (mal-descent of the testis), falling sperm counts, decreased semen quality and testicular cancer, have been observed (Sharpe and Skakkeboek, 1993; Toppari et al., 1995). Recently, it has been suggested that the increasing incidence of male reproductive abnormalities may be a result of environmental pollution by man-made chemicals that have oestrogenic effects. Such reproductive abnormalities not only impair the individual, but may also threaten populations as a whole (Colborn et al., 1993; Toppari et al., 1995).

It is also important to consider sensitive groups, such as the elderly, children, the embryo, the sick, and pregnant women, who may be affected at much lower doses than others. Chemical pollutants that may affect reproductive health and new-born children include certain metals (e.g. lead and methyl mercury), pesticides (e.g. DDT), industrial chemicals (e.g. PCBs), solvents and other substances (Foster, 1998).

In addition, the widespread presence of small amounts of many chemicals is causing increasing concern, because alone, or in combination with other agents, they may contribute to cancer, allergies (UCB, 1997), impacts on reproduction and the immune response system, and neurotoxic effects (NRC, 1992; Kilburn, 1998). The timing of exposure to some chemicals is important, particularly for endocrine disrupting substances, where exposures during the first 30 days after conception seem to be critical. Although exposure to chemicals may be very low for most people (i.e. in parts per million or trillion of air, water or food), some chemicals at such low doses can be potent. For example, estradiol, operates at concentrations of parts per trillion - equivalent to one drop of water in 660 "rail tankers" (Brekin, 1997).

However, clear scientific evidence for many impacts of manufactured chemicals on human health (except for some occupational exposures) is also complex and difficult to identify. This is partly because people are exposed to many different substances and their breakdown products via indoor and outdoor pollution from several pathways, including air, water, food and passage through the skin (Wallace, 1993).

Therefore, no clear connection has been found between disorders in the human reproductive system and exposure to environmental oestrogens (for a review see Toppari et al., 1995).

1.7. Biomarkers of endocrine disruption in fish

Several biomarker systems for the detection and assessment of the estrogenic potential of xenobiotics or wastewaters have been developed. In fish, as important indicator organisms for pollution of aquatic environments, parameters measured *in vivo* include plasma steroid concentrations, alterations in secondary sex and induction of vitellogenin (Islinger et al., 1999).

1.7.1 Vitellogenin

The Vtg assay is one of the most frequently used *in vivo* biomarkers for estrogenicity in oviparous vertebrates (Sumpter and Jobling, 1995; Heppell et al., 1995; Tyler et al., 1996). Vtg is an egg yolk precursor protein synthesised by the female fish liver under estrogen stimulation (Sumpter, 1995; Denslow et al., 1999; Brion et al., 2000; Schmid et al., 2002) and is present in the serum of many oviparous vertebrates (e.g. fish, amphibians, reptiles, birds) undergoing vitellogenesis. Since the production of Vtg is estrogen-dependent, it is limited to females and little if any Vtg can be detected in males, juveniles or sexually immature females. Consequently, male fish normally do not synthesize measurable quantities of this protein, because of the very low, or even undetectable, concentrations of circulating estrogens. However, male fish do carry the Vtg gene and exposure (by injection or aquatic exposure) to xenoestrogens can trigger its expression by the same way that females do. Therefore the measurement of male plasma Vtg concentration has been used as a sensitive biomarker of exposure to estrogenic chemicals in aquatic environments (Sumpter, 1995; Sumpter and Jobling, 1995; Allner et al., 1999; Ishibashi et al., 2001; Folmar et al., 2001; Solé et al., 2001).

Consequently, several ELISAs (Enzyme Linked Immuno Assays) for measuring Vtg concentrations have been developed in a variety of fish species (e.g. *Zoarces viviparus*, *Salvelinus alpinus*, *Pimephales promelas*, *Acanthopagrus schlegeli*, *Salmo trutta*, *Solea vulgaris*, *Morone saxatilis*, *Oncorhynchus mykiss*; *Oryzias latipes*, *Cyprinus carpio*). But because there are some Vtg segments highly conserved among species it is possible to develop antibodies with wide cross-reactivity (Hansen et al., 1998; Denslow et al., 1999).

1.7.2. Proteins of *Zona radiata*

Teleostean eggs are enclosed by an extracellular envelope that protect the developing embryo from the environment. Commonly used terms to describe this eggshell structure are zona radiata (ZR) - *radiata* because of its striated appearance under the light microscope, zona pellucida (ZP), chorion, choriogenin and the vitelline envelope. The eggshell in teleosts consists of minor outer ZP and a major inner ZR. ZP is composed of complex proteoglycans, while ZR in fish consists mainly of filamentous proteins. Both proteins are physiologically induced by estrogens, synthesized in the liver and transported to the ovary during oogenesis in several fish species. The ZR of a number of fish species consists of 2 to 4 protein monomers. ZR-proteins in Atlantic salmon (*Salmo salar*), cod (*Gadus morhua*), and rainbow trout (*Oncorhynchus mykiss*) exist as 3 monomers known as α , β , and γ . The ZR-gene from several fish species has been identified. Among these, two different Zr genes corresponding to the mammalian ZP2 and ZP3 genes have been identified in carp and medaka (Celius et al., 2000).

Consequently, the induction of Zr-protein in male and juvenile oviparous vertebrates can be used also as an effective and sensitive biomarker for xenoestrogens. There are commercially available (Biosense laboratories, Norway) polyclonal antibodies that binds with high affinity to the three *zona radiata* proteins (Zrp), α , β , and γ in plasma samples from Atlantic salmon (*Salmo salar*). The antibodies also cross-reacts with Zrp from a variety of species including rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), flounder (*Platichthys flesus*) and halibut (*Hippoglossus hippoglossus*). More recently monoclonal antibodies (MAbs) to Zrp have also become available. The antibodies also works well in both ELISA and western blot for a number of species (Arukwe, 2003).

1.7.3. Steroid hormones

Sex steroid hormones play important roles at all stages of the reproductive cycle in vertebrates; several studies on their structures and roles in mediating various stages of gonadal development and maturation in bony fishes have been reported (Barannikova et al., 2002). For example, 17 β -estradiol (E2) controls synthesis of vitellogenin and vitelline envelope proteins in the liver (Tyler et al., 2000; Dahle et al., 2003). Plasma levels of 11-KT are significantly higher in males than in females for fish in general and can induce one or more of the male

reproductive traits, including spermatogenesis and spermiation, male secondary sexual characters, and reproductive behaviour. Consequently, several studies have used sex steroid concentrations as biomarkers. Whereby, the monitoring of sex steroid hormones, such as 17 β -estradiol (E2) and 11-ketotestosterone in fish has been used to assess biological effects and exposure to environmental contamination (Ishibashi et al., 2001; Rohr et al., 2001).

1.7.4. Application of histology

Histology has been used for many decades in health evaluations of wild fishes. Histopathological biomarkers or cellular changes in tissues such as gill, liver, kidney and spleen have received much attention in assessing the effects of environmental stress, however, much less attention has been focused on endocrine, neural and gonadal histology – all systems that influence reproduction. Therefore, histology can be a powerful tool, especially when used in conjunction with measurements of other biomarkers and morphological studies (Blaze, 2002).

The diagnostic power of histopathology may be further enhanced by employment of additional histological techniques such as immunohistochemistry. Additionally, histomorphometry may help to detect minute effects (and early changes) which are not discernable on visual inspection, but which may be relevant in view of relatively short exposure times in the experimental setting. Furthermore, an added value of histopathology is in its capability to analyse the mechanistic effects of endocrine disruptors and to characterise effects more specifically. By comparing the impact of known or unknown samples on indicative histological parameters with that of specific reference compounds, the nature and magnitude of the evoked effects can be determined (Wester et al., 2002).

1.8. Mechanisms of action in Endocrine Disruption

Endogenous hormones are synthesized by endocrine glands (e.g. hypothalamus, pituitary, thyroid, parathyroid, adrenal glands, the pineal body, gonads) and transported through the bloodstream to the target organs where they are used to invoke a natural response. These target cells are comprised of a binding receptor and an effector site (IPCS, 2002).

Some “free” hormone molecules will never reach the receptors and are inactivated prior to excretion, primarily by the liver and kidneys in a process called metabolic clearance.

However, although endogenous hormones are generally short lived in the body due to metabolic clearance, when EDCs are present, these mechanisms may not apply, leading to the persistence and bioaccumulation of these chemicals in the body, and may ultimately interact with the endocrine system. The receptor sites have a very high affinity for a specific hormone, so only very low concentrations are required to achieve a response. Thus, the hormones utilized by the cell can be said to have a high potency, which can be defined as the quantity of a substance required to produce a given effect. Despite their high affinity for hormones, these receptors are also capable of binding other chemical compounds. This means that any EDCs present in low concentrations may cause an effect and elicit a response (Birkett, 2003).

Mechanisms by which compounds disrupt endocrine function are complex and not completely understood. (Lister and van der Kraak, 2001). Endocrine disruption occurs when EDCs interact with the hormone receptors, altering the natural response patterns of the endocrine system. The types of processes involved are shown in Fig. 1.1. The chemical may bind to the receptor and activate a response, thus acting as a hormone mimic, both estrogens and androgens, by binding to hormone receptors or influencing cell signaling pathways (those that act like estrogen are called environmental estrogens; those that act like androgen are called environmental androgens). This is defined as an agonistic effect (Fig. 1.1b). If the chemical (a hormone blocker) binds to a receptor, but no response is produced, this prevents the natural hormone from interacting and is termed an antagonistic effect (Fig. 1.1 c). An example of a blocker is how DDE (a metabolic breakdown product of DDT) blocked action of testosterone, in male alligators in Lake Apopka, Florida, which led to undersized penises. As agonists and antagonists bind to the same receptor, subtle changes in the receptor conformation are made to account for the distinct biological activities of the chemicals concerned. However, it is also possible for EDCs to interfere with receptor protein synthesis itself, to affect the synthesis, metabolism, transport or excretion of hormones, to interfere with the feedback mechanisms operating through the hypothalamus and pituitary gland, or to damage endocrine organs directly. This third category is often termed as "triggers" because chemicals interfere by attaching to protein receptors and then trigger an abnormal response in the cell. The best known triggers are dioxin and dioxin-like chemicals (Birkett, 2003).

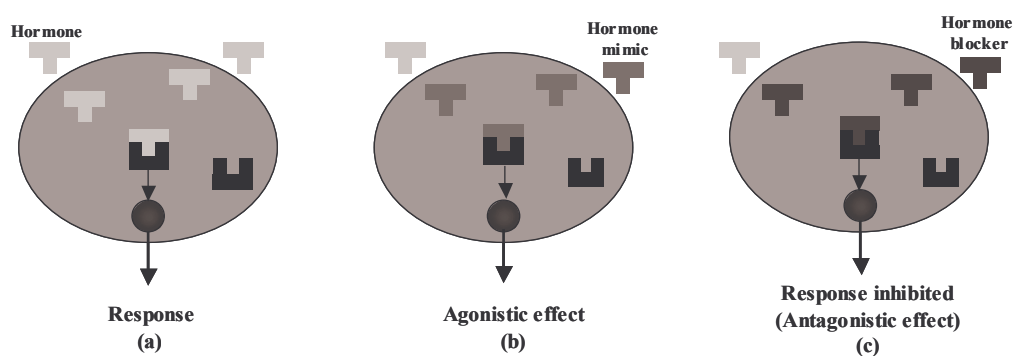


Figure 1.1: Endocrine disruption processes. (a) natural response; (b) agonistic effect; (c) antagonistic effect (Adapted *from* Birkett, 2003).

Most EDCs are small molecules and therefore mimic or antagonize small hormones such as steroid or thyroid hormones. They may eliminate the natural hormones responsible for regulating homeostasis and the development and reproductive processes (Birkett, 2003).

1.9. Fish Reproduction

Marine and freshwater aquatic environments support over 24,000 species of teleost (bony) fish. The diversity of fish species is manifested in a wide variety of reproductive strategies (Tyler and Sumpter, 1996). Fish, like all other vertebrates, reproduce sexually. In the vast majority of fish species, yolky eggs and spermatozoa are produced in separate individuals. The gametes are expelled into the external aquatic environment, where they subsequently fertilize the eggs activated (e.g. salmonids, cyprinids and tilapiines). Egg laying (or oviparity) involves fertilization and subsequent embryonic development outside of the maternal body cavity; juveniles hatch when the egg envelope is broken. The egg yolk provides the appropriate nutrients for developing embryos (Coward et al., 2002).

1.9.1. The Testes

1.9.1.1. Testicular morphology

In all vertebrates the testes are paired, except in the cyclostomes, where both gonads are fused, and in a few teleost species where only one testis develops. The testes tend to be uniformly oval or round, but in some vertebrates, such as various urodele amphibians and teleosts, they are composed of distinct lobes. Amongst teleosts, testes are elongate, white/cream coloured lobulate organs (Fig. 1.2), varying in size, and are generally a pair of organs attached to the dorsal wall of the body cavity (Hibiya and Sato, 1982; Coward et al., 2002).

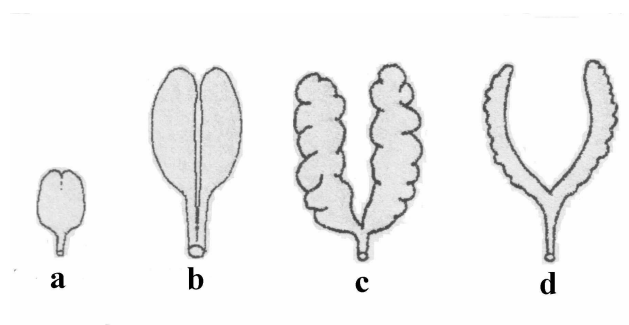


Figure 1.2: Schematic morphology of the testes from different fish. (a: mosquito-fish; b: rainbow-trout; c: carp; d: catfish).

Testes occupy the same position as the ovaries and are connected to the exterior by a duct, the vas deferens, which opens at the genital papilla (Scott, 1987). Although variable, testicular structure presents two basic types, “tubular” and “lobular”. Lobular testes are far more common amongst teleosts and consist of numerous lobules separated from each other by thin layers of connective tissue extending from the testicular capsule. The interstitium between lobules consists of interstitial cells, fibroblasts and blood and lymph vessels. The lobular component contains two cell types: germ cells and distinct somatic cells lining the periphery of the lobule. Originally referred to as lobule boundary cells, the somatic cells were considered homologues to mammalian Leydig cells. The term lobule boundary cell is considered acceptable, anatomically speaking, but functional homology to mammalian Leydig cells is doubtful (Nagahama, 1983). In some species, the lobule boundary cells are considered more likely to be homologous to Sertoli cells as cells are found in close proximity to spermatids/developing sperm (Coward et al., 2002).

In teleost germinal lobules or tubules, Sertoli cells and germ cells are organized in spermatocysts (or cysts) that represent the spermatogenic functional unit, in which one clone of germ cells develops synchronously (Jalabert et al., 2000). However, spermatogenesis tends to proceed asynchronously in the lobules, and therefore, provides batches of germ cells at different stages of differentiation. (Coward et al., 2002). In carp, there is no distinct cycle, and cysts containing spermatozoa and secondary spermatogonia can be found throughout the year (Scott, 1987).

1.9.1.2. Spermatogenesis

Spermatogenesis occurs by proliferation of spermatogonia through repeated mitotic divisions and growth (Hoar, 1969). Primary spermatogonia are found lying singly or in small groups at scattered sites along the tubule walls. These are stem cells which in most teleosts are present at all seasons; secondary spermatogonia proliferate from these stem cells by mitotic division. The secondary spermatogonia transform into primary spermatocytes. Meiotic division of the primary spermatocytes produces the secondary spermatocytes and these the spermatids (all still within the cyst membrane). The spermatids metamorphose into spermatozoa (spermiogenesis) and then the cyst ruptures, liberating the spermatozoa into the tubule lumen. At the time of spawning the testes become hydrated, and the sperm are ejected into the sperm duct (spermiation). After releasing the spermatozoa into the central lumen, the Sertoli cells

form a tight barrier along the walls of the tubules, so that there is no direct contact between the spermatozoa and the blood stream (Scott, 1987).

Although they are different from amniote, anamniote Sertoli cells carry out some functions similar to these of mammalian; i.e. they support and supply nutrients to germ cells, they have ionoregulatory functions, and phagocytose germinal elements. In various teleosts, Sertoli cells exhibit a phagocytic activity at the end of the reproductive season, and, together with invading macrophages, engulf degenerating germ cells and residual sperm cells (Jalabert et al., 2000). Investigations on the fine structure of somatic and Sertoli cells in many different species of vertebrates have demonstrated the presence of organelles associated with steroid synthesis. Such studies suggest these cells may produce steroid hormones necessary for the regulation of spermatogenesis. Furthermore, the histochemical localization of steroidogenic enzymes in these cells in various species strengthens this hypothesis (Pudney, 1987; Scott, 1987).

Despite differences detected in the current literature concerning cells nomenclature, in the present work it was considered that cells similar to Sertoli's, interstitial cells, and Leydig's are present in teleost testis, although they do not possess all amniote morphofunctional characteristics.

1.10. The Ovary

1.10.1. Ovarian morphology

The ovaries are paired elongated structures lying along the median line just ventral to the body cavity. Developing ovaries are elongate hollow organs consisting of numerous partial transverse septa projecting into the ovarian lumen. It is upon these septa that developing oocytes grow (Coward et al., 2002). Each ovary is covered with a thin layer of peritoneum with, under this, a thin connective tissue, the tunica albuginea (Gupta, 1975).

Ovarian development in most teleosts has been classified as synchronous or asynchronous, according to the growth pattern of the oocytes in the ovary at any one time (Tyler and Sumpter, 1996). In group synchronous ovulators, there are generally two populations of developing oocytes in the ovary at any one time, in asynchronous ovulators eggs are recruited from a heterogeneous population of developing oocytes and are subsequently ovulated in several batches during each spawning season (Tyler and Sumpter, 1996).

The ovaries contain the germinal components and endocrine elements the follicle cells responsible for the production of the female sex hormones. Follicle cells surround the germ cells, which are located in the ovarian stroma (consisted of connective tissue, nerves, and blood vessels). This stroma provides structural support for the germ cells as they develop and is also the source of the endocrine follicle cells (Hoar, 1969).

1.10.2. Oogenesis

In all teleosts studied to date, oocytes exhibit the same basic pattern of growth. Several staging systems for oocyte development have been proposed, which incorporate physiological, biochemical, morphological and histological criteria, including follicle size, somatic tissue types and yolk presence (Tyler and Sumpter, 1996). Although classification schemes tend to vary, the major developmental events that occur, however, can broadly be classified into six main phases or periods according to the state of oocyte growth (Figure 1.3): oogonial proliferation, oogenesis, primary oocyte growth and folliculogenesis, cortical alveolus stage, vitellogenesis, maturation and ovulation (Coward et al., 2002).

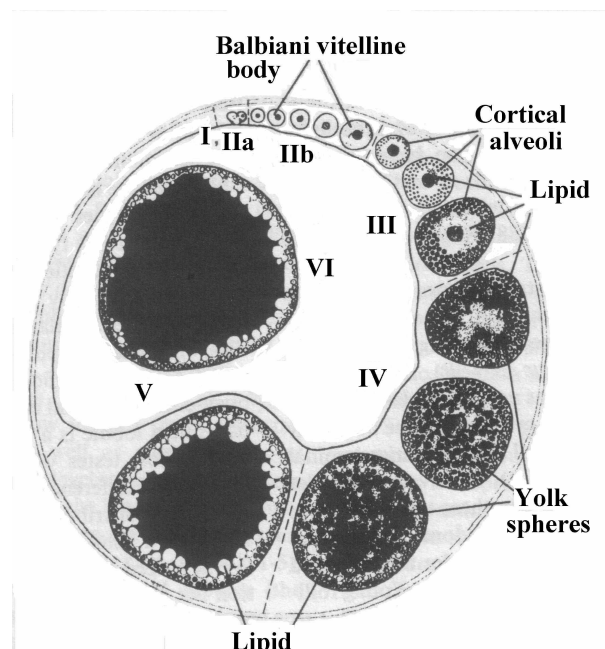


Figure 1.3: Diagram of the ovarian organization and phases of follicular development of pipefish. Phases of follicular growth: I, oogonia; IIa; chromatin-nucleolus oocytes; IIb, perinucleolar oocytes; III, cortical alveoli oocytes; IV, vitellogenic oocytes; V, maturing oocyte; VI, ovulated oocyte. (from Takashima and Hibiya, 1995).

1.10.2.1. Oogonial proliferation

Premeiotic sex cells (oogonia), develop from the primordial germ cells, and are generally found in small nests, appearing as small and rounded, with large nuclei each containing a single prominent nucleolus. The oogonia proliferate by mitotic division soon after the ovaries have differentiated, and following this, some of the oogonia are transformed into primary oocytes (Scott, 1987). In teleosts, reptiles and amphibians, however, unlike all other vertebrates, dividing oogonia persist in the adult ovary and may continue to divide by meiosis to form oocytes throughout the animal's life. These oogonia may be found individually or, as is quite often the case, lying in cell nests (Jalabert, et al., 2000)

1.10.2.2. Primary oocyte Growth Phase

At the beginning of the primary growth phase oocytes are very similar to the oogonia, but each oocyte becomes invested with a layer of follicle cells. In the course of oocyte growth, the nuclei increase in size and multiple nucleoli appear. The cytoplasm also increase in volume and becomes strongly chromophilic. The aggregates become surrounded by less densely stained cytoplasmic material to form the complexes known as "Balbiani bodies". The balbiani body is a complex composed of a heterogeneous population of cytoplasmic organelles, including mitochondria, multivesicular bodies, endoplasmic reticulum and Golgi elements, together with fibrogranular material (Tyler and Sumpter, 1996). Toward the end of the primary growth phase, the Balbiani bodies dissolve and the cytoplasm becomes clear again and a follicular layer begins to develop (Coward et al., 2002).

1.10.2.3. Folliculogenesis

Folliculogenesis begins during the meiotic transformation of oogonia and results in primary oocytes being drawn away from oogonial nests to become closely related with pre-follicle cells (Coward et al., 2002). Thus, on entering meiotic prophase the oocyte acquires an investment of epithelial cells derived from the ovarian stroma. This forms the ovarian follicle. The primordial follicle is enclosed by a single layer of flattened undifferentiated cells, the follicular epithelium. The plasma membranes of oocyte and follicle cells at this stage are relatively smooth and closely apposed to each other. As the oocyte grows, the follicular epithelium increases in size and in many vertebrates undergoes mitotic division to produce a

monolayer of somatic cells that develops in close contact around the primary oocyte, with numerous intercellular junctions and contacts between microvilli of somatic and germinal cells. That cell layer will constitute the membrana granulosa (Scott, 1987).

Enlargement of the granulosa is accompanied by the development of an heterogeneous outer layer of cells called the theca, which is added externally over the basement membrane, and remains essentially unchanged throughout follicular growth (Patino & Sullivan, 2002). As the follicle matures and the oocyte increases in size, a clear, highly polymerized membrane develops, called the zona pellucida in mammals or zona radiata in non mammals. This membrane separates the oocytes from the adjacent follicular cells. Following development of the zona pellucida/radiata the plasma membrane of the oocyte produces numerous microvilli which penetrate this membrane. At the same time irregular cytoplasmic projections are formed by adjacent granulosa cells, which also enter the membrane and establish close contact with each other (Fig. 1.4) (Pudney, 1987).

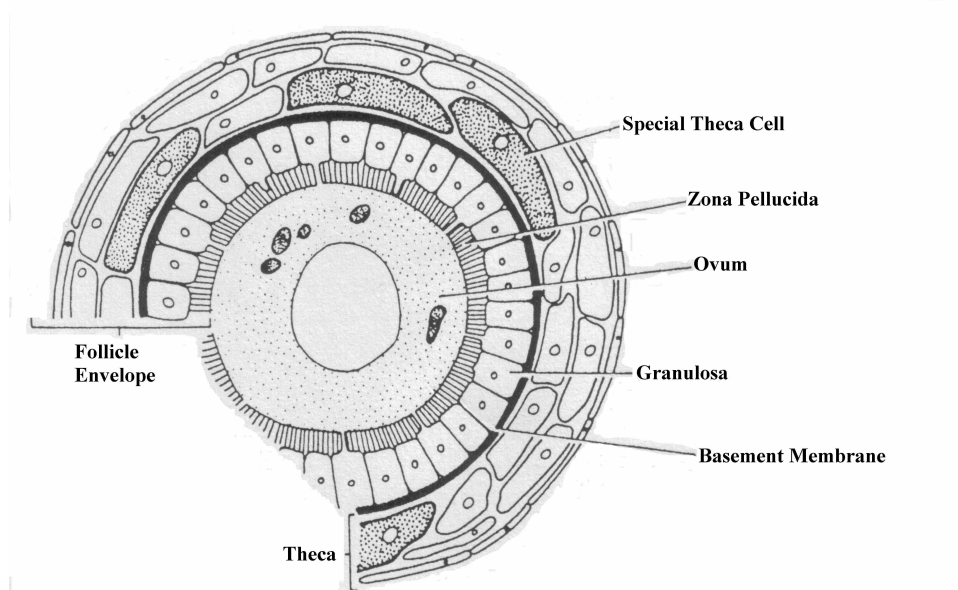


Figure 1.4: Diagram illustrating an ovarian follicle (Adapted from Tyler and Sumpter, 1996).

1.10.2.4. Cortical alveolar stage (previtellogenic growth)

Large amounts of glycoproteins (polysialoglycoproteins) also are synthesized by the oocyte during mid- to late previtellogenic growth. This material is incorporated into vesicles/alveoli newly formed at the oocyte's periphery. These are known as cortical alveoli because of their initial location, but their relative depth first increases as they grow in size and number and

then decreases during later vitellogenic growth as yolk accumulates centripetally in the oocyte. During the cortical reaction to fertilization, the contents of the cortical alveoli are discharged into the perivitelline space between oocyte and the overlying acellular vitelline envelope (VE), which is also sometimes referred to as zona radiata or chorion. (Patino & Sullivan, 2002).

1.10.2.5. Maturation

In a fully grown vitellogenic oocyte, the nucleus –with its chromossomes at the diplotene or dictyate stage-lies in the center of the oocyte. Maturation starts with the germinal vesicle migrating to the periphery at the animal pole. A large space filled with yolk-free cytoplasm forms at this site, and the nuclear membrane then disintegrates.

1.10.2.6. Ovulation

Ovulation is the release of a mature oocyte from the ovarian follicle into either the body cavity (e.g rainbow trout) or the lumen of the ovary (e.g. turbot). However, prior to the actual expulsion of the oocyte, several preparatory steps must occur including the disruption of the microvillar connections between the follicle wall and the oocyte (follicular separation) and the opening of the follicle wall (follicular rupture) (Goetz and Garczynski, 1997).

Several species exhibit “arrest” of oocyte development, in many European freshwater species (e.g. cyprinids), oocytes may enter the yolk vesicle and exogenous vitellogenesis stages during the autumn and become arrested halfway through their development during the winter, only to resume development when water temperatures rise again in the spring. The ovary just after spawning is composed of many postovulatory follicles immature oocytes and mature eggs left unspawned, etc. The follicle cells in the postovulatory, hypertrophy and multiply, showing phagocytosis. Many of the oocytes become atretic in the course of maturation. Degeneration is first recognized in the yolk of peripheral portion of the oocytes. Follicle cells and wandering cells take part in the absorption of the denatured and unspawned eggs (Hibiya and Sato, 1982).

1.10.3. Some aspects of teleost vitellogenesis

Vitellogenesis is the principal event responsible for the enormous growth of oocytes and involves the uptake of a hepatically-derived glycolipophosphoprotein (vitellogenin, Vtg) by receptor-mediated endocytosis involving a specific receptor bound to the oocyte membrane (Tyler and Sumpter, 1996). Vtg is sequestered from the blood by growing oocytes to form an embryonic nutrient source. The nucleus (germinal vesicle) later migrates to the oocyte periphery to adopt a position at the animal pole immediately beneath the micropyle. Hydration during the final phases of development can account for up to 88% of final egg size in some teleosts (Coward et al., 2002).

1.10.3.1. Vitellogenins and their regulation

Vitellogenin is a dimeric glycolipophosphoprotein (containing about 20% lipid, mainly phospholipid, triglycerides and cholesterol) of high molecular mass (300 to 600 kDa native / 160 to 200 kDa subunit), transported via the blood stream to the ovary, and taken up by growing oocytes (Wallace, 1985; Mommsen and Walsh, 1988). Natural induction of hepatic vitellogenesis ultimately ensues from activation of the hypothalamuspituitary- gonad neuroendocrine axis in response to environmental and endogenous cues. Data obtained with some teleosts (e.g. salmonids) indicate that increased FSH levels in the blood induce follicular production of 17β -estradiol (E2), which in turn, stimulates hepatic vitellogenesis. Other estrogens, such as estrone, may contribute to induction of vitellogenesis by priming the liver to respond more strongly to E2 (Tyler et al., 1999).

1.10.3.2. Vitellogenic growth and its regulation

As shown by tracer studies (Selman and Wallace, 1989), Vtg enters the ovarian follicle through capillaries in the thecal layer. It then passes through the basement membrane separating the theca and granulosa cells, the intercellular spaces of the granulosa cell layer, and finally through the pore canals of the VE alongside the oocyte microvilli until it makes contact with the oocyte surface (oolemma). Vtg binds to a specific receptor (VgR) on the oocyte surface. Internalisation of the Vtg-VgR complex occurs in specialized areas of the membrane called 'coated pits'. Coated pits pinch off into the peripheral cytoplasm and give rise to coated vesicles that fuse with lysosome-like multivesicular bodies (Busson-Mabillot, 1984). Proteolytic cleavage of Vtg into yolk proteins occurs in these multivesicular bodies.

Depending on the species, yolk proteins are stored in yolk globules or platelets throughout the ooplasm (Wallace and Selman, 1990; Blazer, 2002).

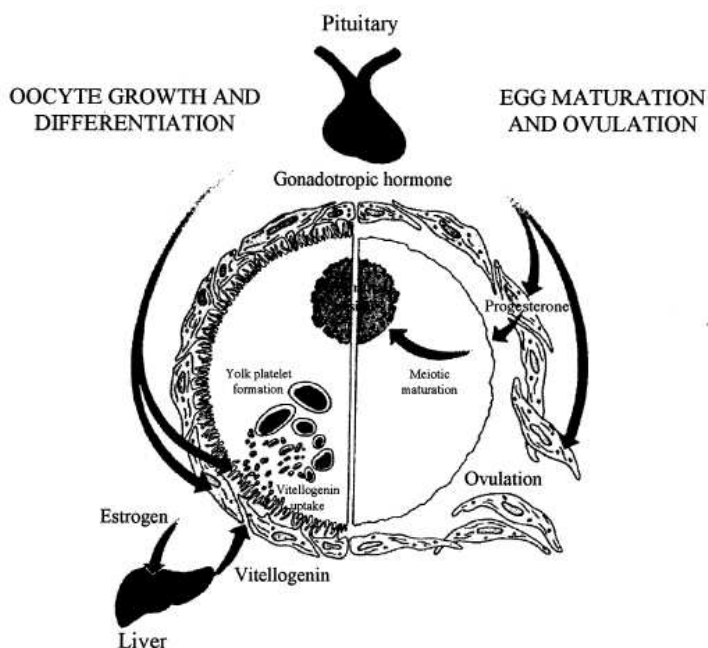


Figure 1.5: Example of hormonal regulation of oocyte growth and differentiation (left) and egg maturation and ovulation (right) (from Browder et al., 1991).

1.11. The Steroids – Structure and Mechanisms of Action

Estrogens and androgens are steroidal molecules whose structure is based on a phenantrene ring. Estrogens are constituted by C18 steroids, which includes estradiol and estrone and are secreted by follicle cells surrounding the oocytes of the ovary, while androgens are C19 steroids and include testosterone, dihydrotestosterone, and 11-ketotestosterone secreted mainly by the interstitial Leydig cells of the testes, however, ovaries and adrenals may also be responsible for male sexual behavior and secondary sexual characteristics and may play an as yet undefined role in female reproduction in nonmammalian vertebrates (Tyler et al., 1998).

In the mammals steroid biosynthesis, after a large number of steps, acetate is converted to cholesterol, which may be considered the precursor of all the steroid hormones. Although these hormones relate generally to the distinct endocrine glands of testis, ovary, and adrenal cortex, all of these glands have to some extent the possibility of secreting some, at least, of those generally formed by other glands, which is not surprising when the close embryological

origin of the gland is considered. For example, the testicular steroid, testosterone, is the precursor of the ovarian estrogens and is secreted in large amounts by the teleost ovary, and in female mammals the adrenal is one source of androgens. The interrelationships of the different steroids are shown in Fig.1.6 (Kime, 1987).

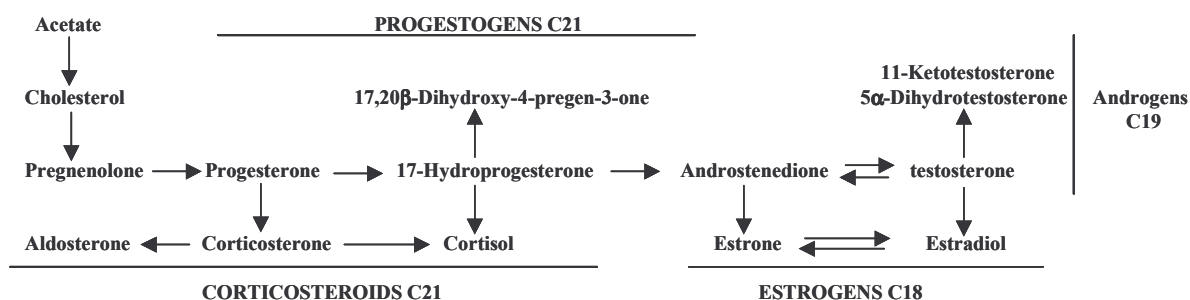


Figure 1.6: Interrelationship of the steroid hormones (Adapted *from* Kime, 1987).

Most steroids are only very slightly soluble in water, but conjugates are extremely water soluble. Conjugation both deactivates the steroids and converts them into more readily excretable forms. In mammals glucoronides are formed almost entirely by the liver, and their function is generally considered to be solely to aid excretion, but in some nonmammalian vertebrates they are also formed by endocrine tissues indicating that in such species they may perform some other function (Kime, 1998).

1.11.1. Teleost steroids

1.11.1.1. Steroids of the ovary

There are three major steroids produced by teleost ovary, estradiol, testosterone and a progestogene (e.g. 17,20 β -dihydroxy-4-pregnen-3-one) with plasmatic peak levels appearing at different stages of gametogenesis, indicating that they each affect different stages of the reproductive process. The role of female fish testosterone is not clear, but it may play an essential role in oocyte maturation. Like in other vertebrates, estrone and estradiol are major estrogens. Vitellogenin synthesis is mainly induced by estradiol, in the liver, however, oocyte uptake is stimulated by gonadotropin rather than steroids. Therefore, early stages of oogenesis require gonadotropin both for initiation of estrogen synthesis, which in turn stimulates vitellogenesis, and for uptake of vitellogenin into the oocyte (Scott, 1987).

During the seasonal reproductive cycle changes occur in the ovary, which involve a complex interplay between the ovary, the pituitary (which modulates environmental stimuli), the liver, and the interrenal (Scott, 1987).

1.11.1.2. Steroids of the testis

Androgens are formed in the thecal cells under the stimulus of gonadotropin and are then converted to estrogen by the granulosa cells. As in testicular steroidogenesis, temperature may have a marked effect on ovarian steroid production. In tilapia, temperature-stimulated estrogen production precedes significant increases in gonadal weight, and in female carp ovarian steroid production in vitro can increase as much as 20 times between 20 and 24°C. Besides FSH, androgenic sex steroids are the most important hormones regulating spermatogenesis, their production being stimulated in fish by both, FSH and LH. In fish, the quantitatively dominating steroids synthesized in the testis are 11-oxygenated androgens, such as 11-ketotestosterone (Borg, 1994); T is produced as well while DHT is rarely found in fish. In some species, the testes produce 11-oxygenated precursors of 11-KT, which are then converted to 11-KT by enzymes in different tissues outside the testis (Schulz & Miura, 2002).

The teleost testis differs from that of most other vertebrates in that it can synthesize derivatives of androstenedione and testosterone with either a hydroxyl or keto group at the 11 position in the steroid nucleus. These androgens are responsible for the induction of male secondary sexual characteristics and stimulation of spermatogenesis in teleosts. Although, 11-KT is the major androgen in teleosts, 11 β -hydroxy testosterone predominates in ambisexual teleosts. Another unusual feature of the teleost testis is its extremely high capacity for steroid glucuronide formation. In mammals, steroid glucuronidation is limited to the liver and is generally considered to be a means of facilitating the deactivation and excretion of steroid hormones (Scott, 1987).

Testosterone is not the single or most potent androgen produced by teleosts. Some have active 11 β -hydroxylase and 11 β -hydroxy- and 11-ketotestosterone, the latter steroid being 10 times more androgenic than testosterone. Early work on male teleosts measured only testosterone, until in vitro incubations showed 11-KT to be the major constituent in male fish plasma and that measurements of testosterone alone can be misleading (Kime, 1987). In trout plasma, 11-ketotestosterone predominates, but most probably there is a wide spectrum among fish from

those which only secrete 11-ketotestosterone to those which only produce 11 β -hydroxytestosterone. For example, in several species in which individuals change sex, the ambisexual fish, only 11- β -hydroxytestosterone is found in plasma and in tilapia both 11-oxygenated androgens are present in comparable amounts. Too few species have been examined to be possible to relate steroidogenic patterns to reproduction strategies (Kime, 1987). Thus, fish differ from most vertebrate groups in that the major active androgen in males is 11-ketotestosterone (Tyler et al, 1998).

Testosterone and 11-ketotestosterone may play different roles in reproduction development since in trout their peaks are temporally separated with 11-ketotestosterone coinciding with spermiation. Plasma concentrations in teleost fish show a very wide range of levels. Salmonids, in general, have extremely high values for both testosterone and 11-KT (20 – 200 ngml⁻¹), whereas most other species appear to conform more to the mammalian range (1 – 10 ngml⁻¹) (Hoar et al., 1983).

1.11.2. Liver and Steroid Catabolism

Mammalian liver has very different enzymatic activities from the interrenal and gonads since its function is to inactivate the steroids and prepare them in suitable form for excretion via the bile or urine. Too few species have been examined to give a general pattern for the very diverse class of teleost fish, but most of the mammalian enzymatic patterns appear to be present within the class. Even in mammals the liver is often neglected in endocrine studies, but it inevitably plays an essential role in steroid dynamics, which are greatly influenced by the rate of catabolism, and this may itself influence the secretion rates and morphology of the endocrine glands themselves. In nonmammalian vertebrates, and in fish in particular, where the steroid transformations of the gonads and liver are even closer it might be expected that these links may be of even greater importance. The liver may play an important role in regulating the overall biological activity of the steroids. This may be of particular importance with estrogens since in fish the liver is both the target tissue in its synthesis of vitellogenin and the site of steroid catabolism. A further possible interaction between the liver, interrenal, and gonads is suggested by the conversion of cortisol to androstenetrone by several species of teleost since this steroid may act as the precursor of the main teleost androgen, 11-KT (Kime, 1987).

1.11.3. Extragonadal metabolism and secretion of sex steroids

Circulating sex steroid levels in fish are maintained by a balance of, synthesis, degradation, solubilization, and excretion of steroids. The bioactivity of steroids in plasma can be modulated by their availability in free form, by steroid binding proteins, and/or by conjugation with glucuronic or sulfuric acid. Whereas much (but not all) steroid synthesis occurs in the gonad, the major site of catabolism of steroids and steroid conjugates is in the liver. The enzymes involved in extragonadal metabolism of steroids belong principally to the cytochrome P450 family, which is a large and diverse group of proteins involved in oxygenation of xenobiotics, steroids, and other compounds. Such enzymes have the capability to alter the hormonal milieu of a developing embryo, and thus potentially also sex differentiation (Stegeman, 1993).

1.11.4. Receptor-mediated action of sex steroids

Steroid action is mediated by binding to specific receptors. The estrogen receptor can be found in a variety of gonadal and other tissues, including liver, ovary, heart, spleen, muscle, and brain. Estrogen receptor levels are themselves influenced by estrogens, mediated by effects on estrogen receptor gene expression (MacKay et al., 1996; Pakdel et al., 1997). Such positive control of estrogen receptors by estrogen ensures that sufficient receptor is available in stimulated cell types to respond to the full degree of estrogen presentation. Estrogen receptors are DNA-binding proteins that influence gene expression (e.g. Vitellogenin), but may also function via cell-surface pathways (Kelly et al., 1999). Different genes have distinct thresholds of estrogen concentrations they will respond to: for example in cultured trout hepatocytes, the estrogen receptor gene is induced at a lower level of 17 β -estradiol than is the vitellogenin gene (Fig. 1.7). Estrogen receptor concentrations also fluctuate in different tissues, depending on the physiological state of the animal: For example, livers from previtellogenic females have very much lower levels of estrogen receptor than vitellogenic individuals. Estrogen receptor mRNA has also been detected in the pituitary and hypothalamus in trout, which may mediate steroidal control of GnRH and GtH secretion. Similarly, estrogen receptors are also found in the pineal gland, and since estrogens have been shown to affect the release of melatonin, endocrine-disrupting xenobiotics could potentially influence seasonal control of spawning by this mechanism (Pakdel et al., 1990).

Steroid hormones are lipophilic in nature and exert their known actions by either diffusing through cell membranes and binding to relatively large proteins in the nucleus called steroid hormone receptors, or by interacting via the plasma membrane, either through receptors or membrane-bound kinases. Both androgen and estrogen receptors are found in many tissues, including the brain, pituitary, gonads, and accessory sex organs. Estrogen receptors are also found in the bone (Tyler et al., 1998).

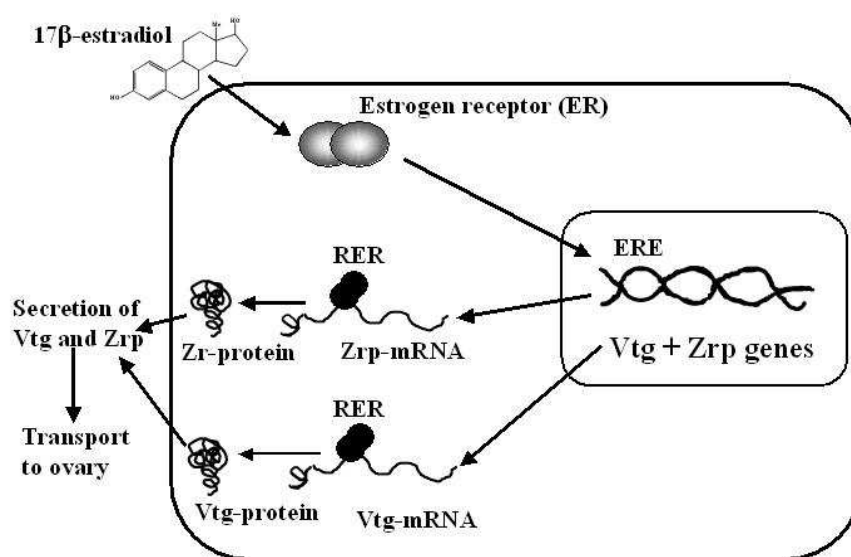


Fig.1.7: Simplified schematic representation of 17β-estradiol stimulated zonagenesis and vitellogenesis (eggshell zona radiata proteins and egg yolk protein precursor, vitellogenin, respectively, their secretion and transport in blood to the ovary and their uptake into maturing oocytes) in female teleosts (*from Arukwe, 2001d*).

1.12. The pituitary gland

The pituitary gland in teleosts consists of a central neurohypophysis interdigitating with a shell-like adenohypophysis. The adenohypophysis is subdivided into rostral pars distalis, proximal pars distalis, and pars intermedia. The gonadotropin-secreting cells are generally found in the ventral region of the proximal pars distalis. Most of the important phases in gonadal development are controlled by a hormone or hormones secreted by the pituitary gland, these being referred to as gonadotropins (GtHs). They have been purified from several species and shown to be similar in structure to the mammalian luteinizing and follicle-

stimulating hormones (LH and FSH), i.e. they are glycoproteins with 30 000 daltons molecular weight and are composed of two subunits, α and β , the biological activity being conferred by the β subunit (Scott, 1987).

Exogenous factors such as temperature, photoperiod, food availability, water quality and a variety of social factors (e.g. visual, chemical or tactile contact with conspecifics) are perceived by the brain and translated into neural impulses that stimulate the endocrine pathways of the hypothalamopituitary-gonadal axis to respond in appropriate fashion. Key endogenous hormones involved in this process include hypothalamic gonadotropin releasing hormone (GnRH), pituitary gonadotropins GTH I and GTH II (analogous to mammalian FSH and LH respectively), sex steroids, progestagens, and prostaglandins (Prat et al., 1996), (Fig. 1.8).

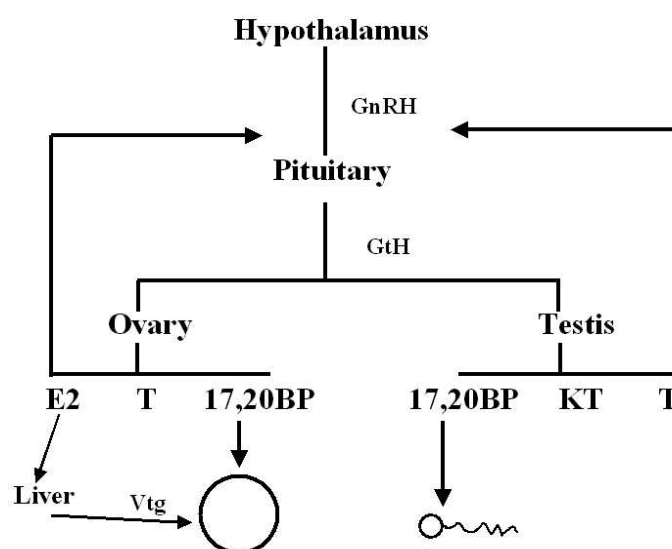


Fig. 1.8. The essentials of the reproductive system in fish. GnRH s gonadotrophin releasing hormone; GtHs - gonadotrophin; E2 - estradiol; T - testosterone; 17,20 β P - 17,20 β -dihydroxy -4 -pregnen -3 -one ; KT₁₁ – ketotestosterone (from Kime et al., 1999).

Evidence also exists to suggest that, as in mammals, a variety of growth factors and paracrine factors also play an important role in teleost ovarian development (Tyler and Sumpter, 1996). Ovulation (i.e. the release of a mature oocyte from its follicle) involves the neuroendocrine

system (Peter et al., 1978) and numerous other regulators: proteases, protease inhibitors, eicosanoids, catecholamines, vasoactive peptides and especially progestational steroids and prostaglandins (Coward et al., 2002).

1.12.1. The female-oogonial proliferation is under pituitary control

Endogenous vitellogenesis-the early stages of the secondary growth phase, involving yolk vesicle formation, are considered to be gonadotropin-dependent. GtH is released from the pituitary in response to hypothalamic signals; it is carried to the ovary, where it stimulates production of C21 steroids by follicular cells; these steroids bind to receptors in (or on) the oocyte, which trigger oocyte maturation; ovulation then follows. Ovulation, which involves the active expulsion of oocytes from the follicles, is controlled by prostaglandins (PGs), which appear to act directly on the smooth muscle fibers which are found in the theca layers of the follicles and in the ovarian lamellae (Scott, 1987).

1.12.2. The male – control of spermatogenesis

Two stages of spermatogenesis have been pinpointed as being especially dependent on the presence of the pituitary: spermatogonial mitosis and the transformation of spermatogonia into spermatocytes. Once formed, spermatocytes can transform into spermatids and these into spermatozoa in the absence of a pituitary (Hoar, 1969).

Changes in steroid hormone levels during the spermatogenic cycle in teleosts: in male rainbow trout, testosterone and 11-KT levels in the plasma increase over the period when testicular weight is increasing and spermatogenesis is most active. The levels start to fall about the time that spermiation begins, but do not fall to basal levels until spermiation has been completed and the testes have regressed. 11-KT levels may some times peak at the same time as testosterone levels or sometimes as much as 1 or 2 months later (Scott, 1987).

1.13. Sex differentiation

The gonads are composed of somatic (structural) and germinal (germ cells) elements intermixed. It is generally believed that the somatic elements of the teleost gonads derive only from cells of the peritoneal wall and thus have a monistic origin (Hoar, 1969). This is in contrast to amphibians and most other vertebrates, where stromal cells in the developing

gonads derive from two different sources: the peritoneal wall and the mesonephric or interrenal blastema. This dual origin forms the basis for the process of sex differentiation in these taxa known as cortico-medullary antagonism, under which the predominance of either portion during development gives rise to an ovary or testis (Witschi, 1957).

Endocrine control of sex differentiation involves a complex interplay between the brain and gonad through the production of pituitary-derived gonadotropins and steroids produced in the gonad and brain. Sex steroids have local, direct effects on germ-cell development, but also act as endocrine hormones to influence other cell types and organs involved in sex differentiation. This multilevel control is very complex, and involves a multitude of biochemical, neurological, and physiological pathways to provide the necessary plasticity for gonadal development to proceed in context with intrinsic and environmental factors (Hoar, 1969).

1.13.1. Development of germ cells

Several levels of control for gonadal determination seem feasible for fish, including intrinsic cell-autonomous genetic mechanisms, or endocrine, paracrine, behavioural, or environmental signals. For example, it is possible that PGCs interpret internal genetic or external environmental cues and directly transform into spermatogonia or oogonia; in turn, the surrounding somatic cells would be induced by the germ cells to differentiate accordingly to provide an appropriate hormonal environment for further gonadal differentiation .

Alternatively, somatic cells may initially interpret genetic or other sex-determination signals, and subsequent differentiation of PGCs occurs in response to signals derived from surrounding cells. The name primordial germ cell (PGC) applies to germ cells prior to gonadal sex differentiation. Once formed, PGCs remain closely associated with endodermal tissues (near yolk sac epithelium in some species) and migrate via the dorsal gut mesentery to the region of presumptive gonad, indicating that PGC migration in fish occurs via the dorsal mesentery as in other vertebrates, although some evidence exists in carps that PGC migration can occur via different pathways (Bieniarz, 1986). In most cases, PGCs appear to migrate directly to gonadal tissues, but evidence has also been presented for sturgeon that PGCs can migrate to other organs such as liver (Devlin and Naghama, 2002).

PGCs in undetermined gonads, testis, and ovaries also possess very characteristic cytological features and share unique antigenic sites between the sexes that are absent from somatic cells,

which is consistent with the idea that PGCs are similar in the two sexes, and that they remain undifferentiated and undetermined until exposed to hormonal and other influences from the developing gonad, which transforms them into spermatogonia or oogonia. Following migration of PGCs into the germinal ridge, cell division occurs to form oogonia and spermatogonia, and differentiation and migration of somatic cells is initiated concomitant with this process. Subsequently, in the testis, the somatic cells (surrounding PGCs) differentiate into seminiferous tubules and supporting connective tissue, and into cells similar to Leydig and Sertoli cells found in mammals (Devlin and Naghama, 2002).

For ovarian development, the somatic cells and PGCs begin to differentiate to form follicles, comprised of oocytes surrounded by an inner granulosa and outer thecal layer. In most species, ovarian development in females is first detectable with the proliferation of somatic cells and oogonia and early oocyte differentiation, which is then followed by the formation of the ovarian cavity (Devlin and Naghama, 2002).

1.13.2. Gonadal differentiation

In fish, the possibility to induce partial or complete functional sex reverse in many species by administration of exogenous steroid hormones during early life has been known for a long time. Moreover, development of the gonad may be influenced by fluctuations in intrinsic factors such as growth or behaviour, or by extrinsic environmental factors such as temperature, endocrine hormones or pollution (Jalabert et al., 2000).

Both estrogen and androgen receptors have been identified in early fish gonads, providing a mechanism for the action of exogenous steroids on gonadal differentiation. If sufficient levels of sex steroids are provided to fish, and particularly at stages of development when endogenous pathways have not been fully established, altered sex differentiation may occur. However, the timing and duration of treatment is of critical importance for inducing sex inversion in fishes (Hoar, 1969).

1.13.3. Gonochorism, hermaphroditism and intersexes

Many teleosts are gonochorists, where individuals develop only as males or females, and remain the same sex throughout their life spans. In differentiated gonochoristic species, also

termed primary gonochorism early gonad development proceeds from an indifferent gonad directly to ovary or testis (e.g. *Oncorhynchus kistuch*, *Abramis brama*, *E. masquinongy*, *S. schlegel*, *Dicentrarchus labrax* and *C. carpio*). Sex differentiation has been shown to begin first in the gonads of females than in males in the majority of the gonochoristic teleosts examined to date (Strussman and Nakamura, 2002). In the teleosts *Danio rerio* (zebrafish) and *Barbus tetrazona*, all gonads initially develop as ovaries, but in approximately half of the population, ovarian tissue subsequently degenerates and the gonad is invaded by additional somatic cells. Masculinization of the gonad then proceeds to produce an initially intersexual gonad that ultimately resolves into a normal testis (Devlin and Naghama, 2002).

Hermaphrodite fish can produce mature male and female gametes at some time in their lives. Synchronous hermaphrodites (also termed simultaneous hermaphrodites) produce both male and female gametes at the same time, of which some species are capable of alternating between sperm or egg delivery (e.g. Serranidae or Gobiidae), and, remarkably, some (*Rivulus marmoratus*) that are even capable of internal self fertilization. In sequential hermaphrodites, individual fish first produce one gamete type, then reverse sex and produce the other type in a subsequent spawning cycle (Devlin and Naghama, 2002).

In gonochoristic species, examples of hermaphrodites or spontaneous sex reversal are very rare. Such exceptional intersex fish, termed abnormal hermaphrodites or intersexes, are often observed by chance in field or laboratory surveys where gonad developmental stage or sex ratio is being examined (Hoar, 1969).

1.14. Sex-determination

In most of the teleosts that have been examined sex is genetically determined. A number of fish (e.g. *Sparus aurata*) are hemaphrodites and change sex naturally at some time during their lives. In virtually all species examined it has been found possible to override the genetic sex-determining mechanisms by treating the alevins or fry with sex steroids (in the water or the diet). Androgens generally cause genetic females to become functional males, and estrogens cause genetic males to be functional females. To effect functional sex inversion, the androgenic or estrogenic compounds must be administered during the indifferent stage of gonadal development and maintained up to the time that gonads are fully differentiated. Dosage is also important, with undertreatment likely to yield hermaphrodite fish and overtreatment sterile fish (Pudney, 1987).

1.14.1 Effects on sex-determination

In gonochoristic species, many influences on gonadal function occurring after sex determination are associated with impairment of reproduction. The complexity of physiological processes involved in gonadal differentiation and maturation provides many opportunities for chemical interference both in males and females. Disruptions include general effects on gonad morphology, rates of spermatogenesis, and oogenesis (including vitellogenesis), ovulation, spermiation, and/or impaired numbers or quality of gametes, as well as effects on reproductive behaviour. In many cases, the biochemical mechanisms by which reproductive impairment is mediated are not known, and impacts are noted by features such as reduced gonadosomatic index or failure to undergo sexual maturity due to arrest of gametogenesis. (McMaster et al., 1995; Van Der Kraak et al., 1992; Kime, 1995).

It is known that sex reversal of gonochoristic fishes by exposure to sex steroids highlights both the plasticity of their gonads and their vulnerability. Studies developed with eels (*Anguilla anguilla*) and flounder (*Verasper moseri*) treated with high doses of estradiol showed that, male feminization can occur even after testicular development had been initiated (Devlin and Naghama, 2002). Furthermore, Gimeno et al. (1998) demonstrated that exposure of mature male carp to the endocrine disruptors can result in degeneration of the testis and intersex.

The exposure to high estrogenic environments, such as STP effluents, show reproductive adverse effects of the endocrine disruption action including sex reversal, appearance of intersexual gonads, expression of sex-specific proteins, feminization of male gonadal ducts, or the retardation or advancement of reproductive maturity (Harries et al., 1996; Lye et al., 1997; Sumpter, 1997; Jobling et al., 1998; Rodgers-Gray et al., 2001). Some classical experiments in *Betta splendens* indicate that redetermination and redevelopment of the gonad from residual tissue (gonadal duct) can proceed in a direction opposite to the original genetic sex. This concept is also supported by the observation that PGCs (Primordial Germ cells) can be found in differentiated ovaries, testes, and ovotestes of *Liza aurata*, *Serranus hepatus*, *Coris julis* and *Xiphophorus sp.* The presence of PGCs at many stages of gonadogenesis also suggests that potential for sex reversal and redifferentiation may be maintained throughout the reproductive lifespan of many fish. Consequently, intersexes have been discovered at stages prior to sexual maturity, or in some cases, when functional gametes of both sexes are being

produced. Moreover, is of considerable interest the source of cells involved in the redifferentiation of gonads. It is not clear whether individual differentiated cells (spermatogonia or oogonia) are able to switch developmental fates during sex reversal, or whether undetermined germ cells within the gonad are recruited to initiate a new course of differentiation (Devlin and Naghama, 2002).

1.15. Hormonal control of sexual maturation

In fish, sexual maturation is central to sex differentiation since it signals the end of the growth phase of gonad development. In some species, individuals die following sexual maturation and spawning, whereas in other fish a subsequent wave of germ cell differentiation and growth (recrudescence) occurs for future spawning periods. During sexual maturation, dramatic shifts in steroid biosynthetic activity occur in both sexes, having effects on both somatic and gonadal tissues. In females, the production of testosterone and estradiol by the ovary are usually reduced, and production of 17, 20 β , dihydroxy-4-pregnen-3-one (17, 20 β -DP) is dramatically enhanced (Hoar, 1969).

1.16. GENERAL OBJECTIVES AND STRUCTURE OF THE THESIS

1.16.1. General Objectives

The major goal of this study was to assess the estrogenic potency of the effluent of a particular STP and evaluate the negative effects on fish by applying a combination of chemical analysis and various bioanalytical tools in a multi-level integrated approach

An ecotoxicological approach dealing with the interactions between environmental chemicals and fish was used in the pursuit of the main objectives. Consequently, ecotoxicological research into the selected EDCs required an interdisciplinary effort that considered physicochemical, biomolecular, toxicological, physiological and ecological processes.

To carry out laboratory and semi-field experiments different species of cyprinids (*Cyprinus carpio*, *Carassius carassius* and *Carassius auratus*) were chosen. Fish are usually used as a model in the laboratory and in semi-field studies to determine the effects of environmental estrogens on fish physiology and reproduction.

To assess estrogenic effects in fish several endpoints were used (Table 1.3), including morphological changes (e.g. the gonadosomatic index and presence of the intersex condition), histopathology (used to evaluate the effects of exposure to EDCs at tissue level), circulating steroid hormones (E2 and 11-KT) and plasmatic vitellogenin levels. The added value of histopathology in this field is its power to analyse the mechanistic effects of endocrine disruptors and characterise effects more specifically. The diagnostic power of histopathology allowed us to enhance the employment of additional histological techniques such as immunohistochemistry for Vtg detection in tissues.

Table 1.3: Major endpoints assessed in fish exposed to estrogenic chemicals.

Endpoints	Laboratory assay	Semi-field assay
Weight and Length	+	+
Somatic Indices(GSI, HSI)	+	+
Gross gonadal examination	+	+
Histopathology of gonad	+	+
Immunohistochemistry	+	+
Biomarkers		
Vtg, E2, 11-KT	+	+
Exposure period	4 weeks	4 to 8 weeks

The adverse effects can be assessed at the different levels of biological organization, from the molecular, cellular, tissue, organ and organism levels up to populations and the ecosystem. The organization levels investigated in this study are highlighted in Fig. 1.9. For instance, the condition factor was calculated to determine effects at the organism level. Gonad to body weight and liver to body weight ratios were used to determine effects at the organ level. Circulating testosterone levels and the induction of vitellogenin in males were used to assess potential impairment of reproductive endocrine homeostasis and exposure to estrogenic chemicals, respectively.

Thus, the global results have made a major contribution to our knowledge of the effects of EDCs on fish at various biological organization levels and have given a comprehensive assessment of the mechanisms of endocrine disruption in fish.

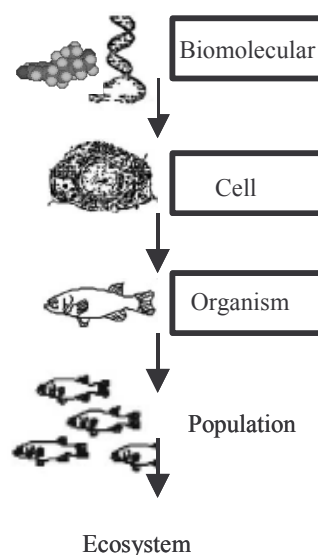


Figure 1.9: Levels of organization to be assessed (highlighted) (Adapted from Fent et al., 2001).

1.16.1.1. Specific endpoints:

1.16.1.1.1. Screening EDCs in STP water (Part III):

Since numerous EDCs such as steroid hormones are present in the STP at low environmental concentrations, sophisticated analytical techniques are required for their measurement.

Accordingly, several analytical techniques were adopted to measure EDCs in a selected STP located in Lisbon.

Analytical tools based on conventional techniques such as gas or liquid chromatography (GC and LC), coupled with sensitive detection systems such as mass spectrometry (MS) or MS-MS, were applied to detect and quantify EDCs present in the various STP treatment processes. Alternatively, immunochemical techniques (ELISA) were also applied, since they offer a selective and sensitive method that is simple to perform and can be used in small sample volumes. Figure 1.10 shows a schematic diagram summarizing the screening of the selected EDCs.

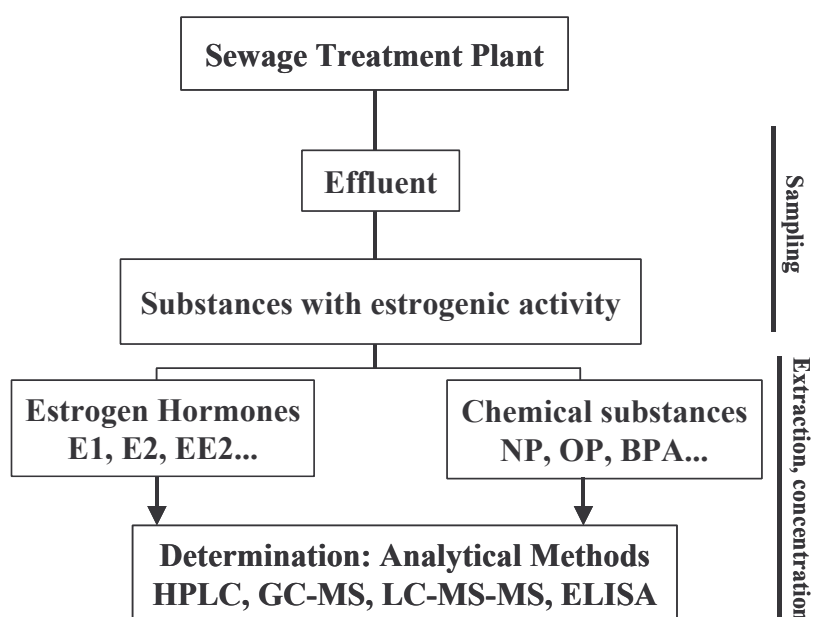


Figure 1.10: Diagram representing the methods required to analyse EDCs.

1.16.1.1.2. Laboratory Bioassays (Part IV)

The aim of these bioassays was to examine the estrogenic effects of exposure to NP, OP, BPA, E2 and EE2 at concentrations that are typical for STP effluents and above and are able to disrupt the endocrine system of vertebrates. Thus, the purpose of these studies was to investigate the estrogenic effects of five EDCs, using concentrations ranging from low to high and exposing fish to them in glass aquarium.

Different species of sexually mature carp (*C. carpio*; *C. carassius*; *C. auratus*) were exposed to different concentrations of previously selected EDCs, singly and in mixture and their potency evaluated using several endpoints. In these controlled experiments, biomarkers such as Vtg induction, steroids (E2 and 11-KT), gonad and liver weights, histopathology and the occurrence of ovo-testis were assessed. Furthermore, the selected EDCs were studied in relation to their relative estrogenic potency. In addition, the induction of vitellogenin in the liver and gonads of fish exposed to EDCs (male and female) was measured by ELISA and immunohistochemical techniques.

The endpoints used reflected disturbances to normal physiology and provided indications of pathological changes in the animal and also sensitive indications of early changes in the organism. The laboratory experiments provided evidence of toxicological consequences in the biota. Moreover, this laboratory approach was used for extrapolation to the field or semi-field experiments and provided a better understanding of the effects of these chemicals on the reproductive organs and reproductive status of fish and, also, of the mechanisms that they use.

1.16.1.1.3. Semi-field experiments (Part V)

The aim of the semi-field experiments was to assess the estrogenic potential of the treated sewage effluent from an STP (Lisbon-Chelas) by evaluating the estrogenic effects in exposed fish (*C. carassius*, *C. auratus*, *C. carpio*). To this end, carp exposure tests were performed using water tanks receiving different dilutions of the treated sewage effluent. A diagram summarizing the semi-field experiments procedure is presented in Fig. 1.11.

Thus, semi-field assays measured the effects, which are integrated responses to all estrogenic chemicals present, including those that might be missed by chemical analysis. The main objective of these experiments was to assess the negative estrogenic effects caused by potential EDCs present in the STP effluent. The main endpoints used to accomplish this task were the measurement of Vtg induction and plasma steroids concentrations, plus the histopathology and histochemistry results. Additionally, condition indices (GSI, HSI, K and KI) were calculated as complementary information on estrogenic effects. The occurrence and the nature of abnormalities in testes and ovary histology were registered as well.

The semi-field experiments were carried out several times and in different seasons to assess the persistence of EDCs in the treated sewage effluent. Furthermore, possible effects related

to different sizes were also examined in order to investigate a possible size-dependent susceptibility.

Additionally, these studies provided valuable information on the way EDCs act and affect fish. They could represent a starting point for further studies on the estrogenic potency of other STPs or to the aquatic environment.

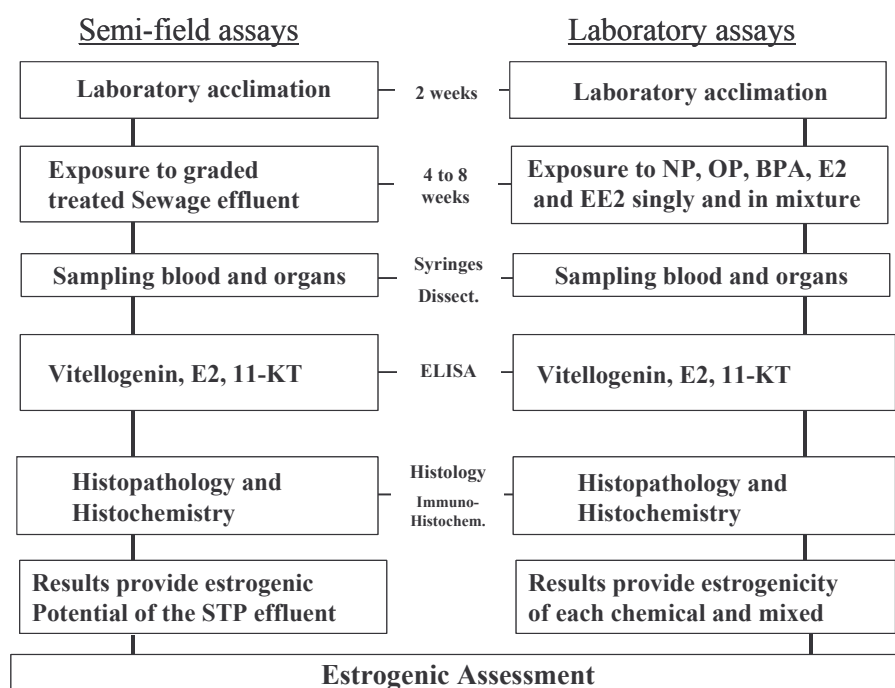


Figure 1.11: Schematic diagram of laboratory and semi-field assays.

The semi-field experiments were also complemented with the measurement of physicochemical parameters such as temperature, pH, dissolved oxygen, total suspended solids (TSS), volatile suspended solids (VSS), total nitrogen and chemical oxygen demand (COD). Heavy metals such as Ni, Cr and Cu were also determined in the STP.

1.16.2. Structure of the Thesis

The thesis is divided into seven distinct and complementary sections, composed of seven chapters and nine annexes corresponding to, or based on, articles that have been or are being published or are in preparation. In the first part, the state of the art of the research fields of this work was evaluated (and also integrated in the different chapters). The second part describes

the criteria to select the sewage treatment plant and also the fish for the experiment; the third part is directed at identifying and quantifying the presence of some EDCs at various stages of the treatment process in the selected STP in Lisbon; the fourth part describes experiments carried out in laboratory facilities under controlled conditions by exposing fish to well known EDCs which were also targeted in the first part; the fifth part describes experiments that were carried out in semi-field conditions by exposing fish to graded dilutions of a treated sewage effluent; the sixth part reviews all the EU legislative activity on the endocrine disruption issue; in the seventh part a general discussion and the overall conclusions are presented; and, finally, the annexes provide the necessary support for the information presented in the various chapters.

Accordingly, this thesis was divided into the following parts and annexes:

PART I – CHAPTER 1 -GENERAL INTRODUCTION

PART II - CHAPTER 2- SELECTION OF THE SEWAGE TREATMENT PLANT AND OF THE BIOLOGICAL MATERIAL

Parts III, IV and V includes various chapters corresponding to, or based on, the following articles that are published or under publication:

PART III - CHAPTER 3 - CHARACTERIZATION OF SELECTED ENDOCRINE DISRUPTOR COMPOUNDS IN A PORTUGUESE WASTE WATER TREATMENT PLANT

3.1. Maurício, R., Diniz, M., Petrovic, M., Amaral, L., Peres, I., Barceló, D., Santana, F. (2006). A Characterization of selected Endocrine Disruptor Compounds in a Portuguese Waste Water Treatment Plant. *Environmental Monitoring and Assessment* (in Press).

3.2. Maurício, R., Diniz, M., Petrovic, M., Amaral, L. Peres, I., Santana, F., Barceló, D. (2004). Evaluation of the estrogenic potential and physico-chemical characterization of a sewage treatment plant. Published in Proceedings of the 8th National Conference on Environment, 27-29 de October, Lisbon.

3.3. Diniz M.; Maurício R.; Mateus E.; Amaral L.; Peres I.; Castro L.; Santana F. (2001). Assessing the removal of nonylphenol in the effluent of a municipal sewage treatment plant. Exposure effects in carp (*Cyprinus carpio*). Published in Proceedings 2º Encontro Nacional de Entidades Gestoras de Água e Saneamento (ENEG), Lisboa 9 – 11 October 2001.

PART IV – Chapter 4 - LABORATORY ASSAYS

4.1. Comparative estrogenic potencies of single xenoestrogens (nonylphenol, octylphenol, bisphenol-A, 17 β -estradiol and 17 α -ethynylestradiol). In vivo assessment by exposing carp (*Carassius carassius*). (In preparation).

4.2. M.S. Diniz, I. Peres, and J.C. Pihan (2005). Biomarker and histological assessment of crucian carp (*Carassius carassius*) exposed to a mixture of xenoestrogens. (Submitted to Chemosphere).

4.3. M.S. Diniz & I. Peres (2002). Bioaccumulation and effects of 4 n-nonylphenol in *Cyprinus carpio*. Preliminary Results. Ecotoxicologia e Remoção de Poluentes. Ed. Duarte, A., Santos, A. R. Panteleitchouck, Prego, R. Instituto Piaget (2002). Pp. 55-60.

PART V – SEMI-FIELD ASSAYS

5.1. M.S. Diniz, I. Peres, I. Magalhães-Antoine, J. Falla and J.C. Pihan (2005). Estrogenic effects in crucian carp (*Carassius carassius*) exposed to treated sewage effluent. Ecotoxicology and Environmental Safety 62, 427-435.

5.2. M.S. Diniz, I. Peres and J.C. Pihan (2005). Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons. The Science of the Total Environment 349, 129-139.

5.3. M.S. Diniz, I. Peres and J.C. Pihan (2005). Crucian carp (*Carassius carassius*) exposure to treated waste water effluent: evidence of size influence on endocrine disruption? Revista de Biologia (in press).

PART VI – CHAPTER VI - EUROPEAN LEGISLATION AND POLICY ACTIONS ON ENDOCRINE DISRUPTORS

PART VII – CHAPTER 7 - GENERAL DISCUSSION AND CONCLUSIONS

ANNEXES

Annex I

Supports information to the chapter 1

Annex II

M.S. Diniz, I. Peres and J.C. Pihan (2003). Effects of nonylphenol exposure – gonadal histology and vitellogenin induction in crucian carp (*Carassius carassius*). Panel Communication Presented at the 6th National Conference on Endocrine Disruptors, 12nd SESA Technical Jornada, November (26 – 28) Elche (Spain). Supports information to the chapter 4.

Annex III

M.S. Diniz, I. Peres and J.C. Pihan (2003). Vitellogenin induction and histological effects of *Carassius carassius* exposed to 17 β -estradiol under laboratory conditions. Presented as Poster at 5th Iberian and 2nd Ibero - American Congress of Environmental Contamination and Toxicology (CICTA 2003), Porto, 22-24 September. Supports information to the chapter 4.

Annex IV

M.S. Diniz, I. Peres and J.C. Pihan (2003). Estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated sewage effluent. Presented as poster at 5th Iberian and 2nd Iberoamerican Congress of Environmental Contamination and Toxicology (CICTA 2003) Porto, 22-24 September. Supports information to the chapter 5.

Annex V

R. Maurício, M. Diniz, E. Mateus, L. Amaral, F. Santana (2001). Study of the Endocrine Disruptor Compounds Biodegradation. *Presented in Iberian Conference on Environmental*

Protection, Instituto Piaget – Viseu, 12 a 14 de Julho (Portugal). Supports information to the chapter 3.

Annex VI

Maurício, R., Diniz, M., Amaral, L., Peres, I., Santana, F. (2003). Screening Endocrine Disruptors Compounds in a Portuguese Wastewater Treatment Plant using Enzyme Linked Immunoassay (ELISA). Panel Communication Presented at the 6th National Conference on Endocrine Disruptors, 12nd SESA Technical Jornada, November (26 – 28) Elche (Spain). Supports information to the chapter 3.

Annex VII

M.S. Diniz, I. Peres, Castro, L. and J.C. Pihan (2001). Nonylphenol distribution in *Cyprinus carpio* organs. Histology as a marker of visible effects. Preliminary Results. Presented as Poster at Biomarkers of Environmental Contamination, 24 – 26 September 2001, Póvoa do Varzim, Portugal. Supports information to the chapter 4.

Annex VIII

M.S. Diniz and I. Peres (2001). Bioaccumulation and effects of 4-n-Nonylphenol in *Cyprinus carpio*. Preliminary Results. Presented as Poster at Iberian Conference on Environmental Protection. Instituto Piaget – Viseu, 12 a 14th July. Supports information to the chapter 4.

Annex IX

Supports information to the chapter 6

1.16.3. General Framework

A diagram describing the general framework of the thesis, including the main aims of each section, is presented in Figure 1.12. This diagram summarizes all the work proposed to evaluate the endocrine disruption in various species of cyprinids. The experimental design combines analytical chemistry (to screen target EDCs), bioassays (to be carried out under laboratory conditions) and semi-field assays (exposing fish to different concentrations of a treated sewage effluent in an municipal STP). The estrogenic effects on fish are evaluated using several endpoints, on the basis of a multilevel approach. The results are integrated to give an overall assessment of the estrogenic effects in fish.

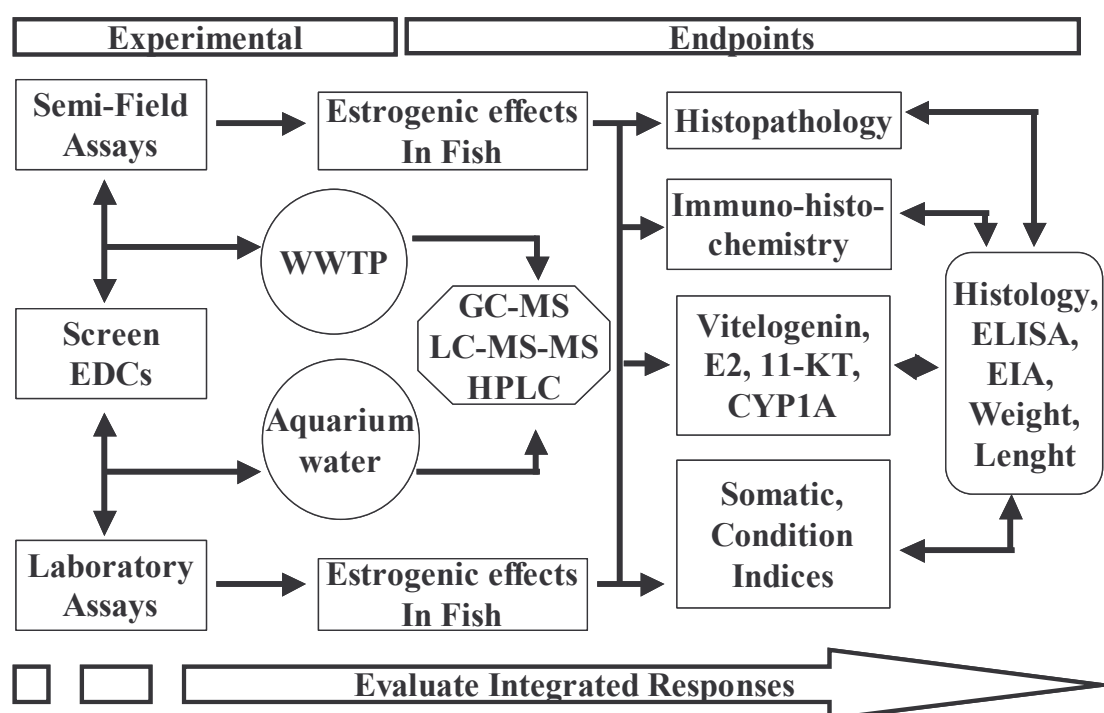


Figure 1.12: Schematic diagram representing the phases of the present study.

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PART II

CHAPTER 2- SELECTION OF THE SEWAGE TREATMENT PLANT AND OF THE BIOLOGICAL MATERIAL

2. Selection of the Sewage Treatment Plant and the fish for the experiment

2.1. Description of the Sewage Treatment Plant in Chelas (Lisbon)

The Chelas STP (Fig. 2.1) is located in the eastern zone of the city of Lisbon (Portugal) and discharges the treated effluent into the Tagus River estuary (Fig. 2.1). This sub-system is constituted by the Nereida STP (the name of one of Camões' "Tágides" or nymphs of the Tagus River) and an interceptor sewer, which includes five pumping stations (EE7 and EE1I) to collect and convey downtown waste water between the Calçada do Grilo (Xabregas) and Alfama/Santa Apolónia.



Figure 2.1: Full view of Chelas Sewage Treatment Plant.

2.2. Technical data of sewage treatment processes

According to information provided by the STP management body (SIMTEJO), the working station has secondary and tertiary treatment of the effluent and final disinfection (U.V.). The STP was designed to collect and treat 52,500m³ of urban waste water per day, corresponding to approximately 211,000 equivalent inhabitants. Table 2.1. summarizes the technical data from the Chelas sewage plant.

Table 2.1: Technical data from Chelas sewage working station.

Technical data		
Physical-chemical parameters	Concentrations (mean/maximum)	Depuration to attain
Mean daily flow ($\text{m}^3\text{day}^{-1}$)	49	
BOD5 ($\text{mgO}_2\text{l}^{-1}$)	290/270	25
COD ($\text{mgO}_2\text{l}^{-1}$)	440/595	125
TSS (mg l^{-1})	180/435	35
Ntotal (mg l^{-1})	45/55	7.5
Ptotal (mg l^{-1})	10/15	10.0

Briefly, the untreated waste water is collected by municipal pumping stations and a system of conduits conveys the effluent to the STPs where it undergoes several treatment processes to remove the grit, fat and and solids, which are dumped in a landfill after washing and conditioning. Primary sedimentation follows and the sludge produced at this treatment stage by a bottom-scraper system is pumped to a thickener. After this step the waste water is equalized in a basin by a process of dampening hydraulic or organic flow variations so that nearly constant conditions can be achieved. The removal of carbonaceous and nitrogen pollution are achieved by biological treatment (the biological reactor), with zones of anaerobic and aerobic conditioning, followed by secondary sedimentation. At the end of the biological treatment, the clarified water is submitted to an adjustment treatment in the open-air using sand and anthracite filters. The next treatment phase is the disinfection of the effluent with ultra-violet (U.V.) rays before it is discharged into the River Tagus estuary (Fig. 2.2). The sludge produced during the treatment processes can be used in agriculture as a fertilizer.

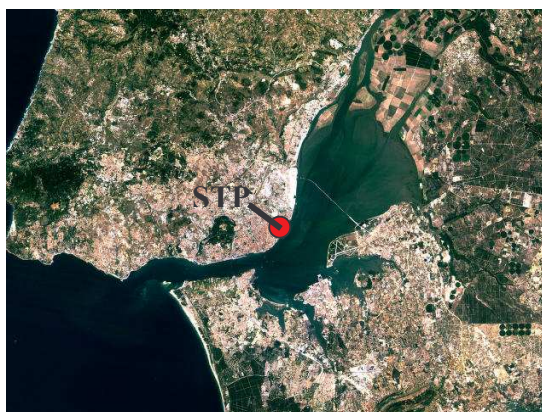


Figure 2.2: Satellite image of the River Tagus estuary (Nasa, 2003). STP (Chelas Sewage Treatment Plant).

The Chelas STP (Figs. 2.3 A and B) was selected as model to study since it receives large volumes of urban waste water and variable amounts of industrial effluent from a densely populated and highly industrialized area. Furthermore, since the treatment plant has tertiary treatment of the effluent, this allows biological assays to be carried out in relation to environmentally relevant concentrations of the chemicals discharged into the aquatic environment.

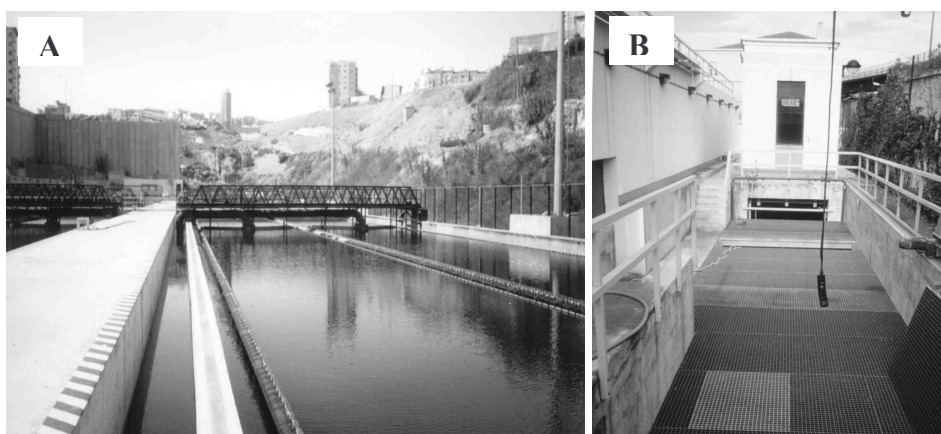


Figure 2.3: General view of the treatment steps in Chelas STP. A (secondary sedimentation); B (ultra-violet disinfection).

2.3. Selection of the fish for the experiment

2.3.1. Cyprinids

The Cyprinidae include approximately 2,070 species distributed over 194 genera worldwide and represent about 20% of the total of European freshwater species, thus constituting the most representative family of freshwater fish in Europe (Fig. 2.4). Though they originated in Europe and Asia, the fish have been introduced into environments worldwide and are presently widespread in the U.S.A, Europe and Africa (Wikipedia, 2005).



Figure 2.4: Distribution - cyprinds are the most representative freshwater fish in Europe (*Photo from earthimaging.com*).

The Cyprinidae are the largest and most important family among freshwater teleosts and have a major importance in aquaculture, principally in Asia (Horvath and Orban, 1995; Bartfai et al., 2003). The eggs are usually small (1 to 2mm in diameter) and fecundity ranges from 50,000 to 300,000 eggs per kg body weight. The incubation and eclosion occur at a temperature higher than 18 to 20 °C, within a few days. The vitelline resorption occurs in a few days and fish larvae start to feed within a few weeks after eclosion. Hybridization is frequent at the spawning period between cyprinid species as the result of changes in the environment, particularly in the habitat conditions, by natural or anthropic action (Linhart et al., 1995; Billard et al., 1995).

In general they are tolerant of wide variations in their physical conditions. For instance the common carp tolerates a temperature range of 1 to 35°C. In addition, they also tolerate variations in dissolved oxygen, which is frequent in standing and eutrophized bodies of water in the summer season. They even can survive in an almost anoxic environment ($< 1\text{ mg O}_2/\text{l}$) for a few hours and during winter they burrow into the bottom sediment and reduce their metabolic rate. They also can tolerate a pH range of 5 to 9 (Fish Base, 2004a; ITIS, 2004a).

Cypriniformes are distinguished by a single dorsal fin and by having teeth in the throat rather than the mouth, called pharyngeal teeth. Some have sensitive and gustative organs called barbels, around the mouth, which are used to find food. The diet is diverse and fish can feed on all trophic levels such as, aquatic plants, algae, aquatic insects, crustaceans, mollusks or annelids (Fish Base, 2004a; ITIS, 2004a).

Amongst the cyprinid species there are major differences concerning size and some species can reach 10 to 11 mm (e.g. *Danionella translucida*) while others can grow to a maximum length of 2 to 3 meters (e.g. *Caltocarpio siamensis*) (Hoar, 1969).

The array of reproductive strategies evident amongst cyprinids is extraordinarily diverse (see Hoar, 1969). In the vast majority of fish species eggs and spermatozoa are produced in separate individuals and gametes expelled into the external aquatic environment, where fertilization and egg activation take place. Internal reproduction may also occur (Coward et al., 2002).

Cyprinids are of great commercial value as a food fish, mainly in Asia, where they are produced by aquaculture. The world aquaculture production of cyprinid species is over 9 million tons per year. Besides the nutritional value some species of cyprinids have major value to sport fisheries and as aquarium fish (FAO, 2001; Fish Base, 2004a).

2.3.1.1. Species selected

Three different species of cyprinids (*Cyprinus carpio*, *Carassius carassius* and *Carassius auratus*) were chosen for this study. The selected species are representative of European and national rivers and lakes and are resistant to polluted environments and a variety of conditions, which makes them ideal bio-indicators and valuable as an experimental model.

Additionally, they are produced by aquaculture and therefore easy to obtain. Numerous studies have demonstrated the utility of using cyprinids as the experimental model in ecotoxicology, in particular in the evaluation of endocrine disruption in aquatic biota (Folmar et al., 1996; Goodbred et al., 1997). However, for this study *Carassius carassius* was the species most used, since it is the easiest to obtain, the fish smaller in size at maturity and it is easy to maintain and manipulate them in large numbers.

2.3.1.1.1. Crucian carp (*Carassius carassius*, Linnaeus, 1758)

Description: The body is oval and hump-backed (Fig. 2.5). The mouth is moderately small and terminal, the head about a quarter of the body length. No barbels at mouth. The dorsal fin is long, with a strong, lightly serrated third spine; the anal fin short with six to eight (exceptionally) branched rays and a strong, lightly serrated spine. Twenty-six to thirty-one gill rakers on first gill arch. Presents lateral line scales (31 to 35) and have pharyngeal teeth four each side. In colour, olive green, or reddish brown on the back, paler with a brassy tint on the sides, with a rounded dark spot, indistinct in old specimens, near the base of the tail. The pectoral, pelvic and anal fins are a bright reddish bronze (FishBase, 2004b).

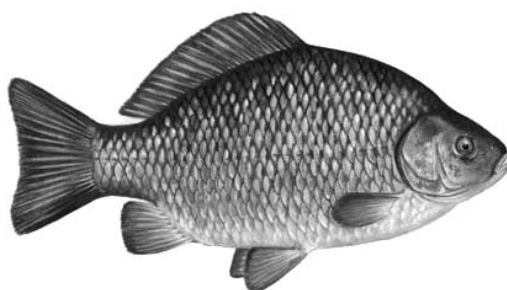


Figure 2.5: *Carassius carassius* (FishBase, 2004b).

Diet: they are omnivorous, feeding mainly on aquatic plants, plancton, benthos and insect larvae (FishBase, 2004b).

Size: the fish grows to a maximum size of 45 cm and two kilograms of total weight, however, rarely oversizes 15 cm and 0.2 Kg of weight (FishBase, 2004b).

Reproduction: The Crucian carp breeds from April/May to June, occasionally to July, in several isolated spawnings. There is no apparent segregation by size in the spawning shoals,

and fish of all ages spawn together. Reproduction occurs at a minimum temperature of 14°C and at an ideal temperature ranging from 18 to 20°C. During spawning the male develops nuptial tuberculae on the head and at the first pectoral fin rays. It is common to hybridize with common carp and the eggs are of viscous. The eggs are golden, from 1.4 to 1.7 mm in diameter, and adhere to the aquatic vegetation. They hatch in five to ten days depending on the temperature, and the alevins measure about 4 mm in length. This fish's rate of growth varies greatly depending on the habitat; many in small pools are several years old and only 2-3 in (5-7.5 cm) in length. In large waters, however, they can reach 10 to 14 cm in length at 3 or 4 years of age. After, the second year, female grow faster than males, however the males reach maturity earlier (FishBase, 2004b).

Habitat: This fish often inhabit shallow ponds, lakes rich in vegetation and slow moving rivers in Europe and Asia which becomes ice covered in the winter. Very resistant species, tolerates cold, organic pollutants, and low oxygen levels in the water. Burrows in mud in the dry season or during winter, diminishing metabolic activity (Maitland & Campbell, 1992). Its extraordinary anoxia tolerance is clearly an adaptation to this extreme environment. The Crucian carp is of considerable economic importance in eastern Europe, and is raised in fish farms, and released in poor-quality artificial waters to be cropped later. Related to common carp, have great value as an aquarium fish and to sportfishing (FishBase, 2004b).

Systematics:

Phylum	Chordata
Subphylum	Vertebrata
Class	Osteichthyes
Subclass	Actinopterygii
Order	Cypriniformes
Suborder	Cyprinoidei
Family	CYPRINIDAE
Genus	<i>Carassius</i>
Species	<i>Carassius carassius</i> (Linnaeus, 1758)

Distribution: East to western Europe, including Portugal, where it has been successfully introduced. Commonly used as an experimental model in Science, though also of major value as an ornamental fish (Fish Base 2004b).

2.3.1.1.2. Goldfish (*Carassius auratus*, Linnaeus, 1758)

Description: body morphology similar to *Carassius carassius*, they show lateral line scales (27 to 31). In color they are yellowish to gold; yellowish white or white below (Fig. 2.6).

Diet: similar to that of *Carassius carassius*, including plants, small crustaceans, insects and detritus.



Figure 2.6: *Carassius auratus* (FishBase, 2004c).

Size: grows to an average of 15-20 cm in length, at the age of 5 or 6, however in large waters can grow to 45 cm in length and 2 Kg in weight (Fish Base, 2004c; ITIS, 2004b).

Reproduction: maturity is attained at the age of 3 – 4 years, however, can show a very singular reproductive biology. In some regions of Asia and Western Siberia males and females are found at equal proportions, while in some European regions all the population was constituted by females which breed with male from other cyprinid species in a reproductive process termed as gynogenesis. They can spawn consecutively (three times), laying 160,000 to 380,000 eggs adherent to aquatic vegetation (Yue and Orban, 2002).

Habitat: they inhabit rivers, small lakes, ponds and ditches with stagnant or slow-flowing waters. They live better in cold water and show a tolerance to low oxygen conditions similar to *Carassius carassius*. Hybridize with common carp (*C. Carpio*) and crucian carp (*Carassius carassius*) (Fish Base, 2004c; ITIS, 2004b).

Systematics:

Phylum	Chordata
Subphylum	Vertebrata
Class	Osteichthyes
Subclass	Actinopterygii
Order	Cypriniformes
Suborder	Cyprinoidei
Family	CYPRINIDAE
Genus	<i>Carassius</i>
Species	<i>Carassius auratus</i> (Linnaeus, 1758)

Distribution: From Asia, was introduced throughout the world including several lakes in Europe for ornamental and recreative purposes (Fish Base, 2004c; ITIS, 2004b).

2.3.1.1.3. Mirror carp (*Cyprinus carpio*, Linnaeus, 1758)

Description: they show a long and oval body (Fig. 2.7), covered with large scales which are restricted to few at the top of the dorsal region in mirror variety. The fish have two pairs of barbels, long dorsal fin (17 to 20 rays) with serrate bony spines. Variable in color, the common carp usually show a green-olive to brownish coloration. However, mirror carp show silvery scales. In some European countries the carp has a great value as food and to gamefishing (Fish Base, 2004d; ITIS, 2004c).



Figure 2.7: *Cyprinus carpio* (FishBase, 2004d).

Diet: they are omnivorous, feeding mainly on benthos, zooplankton, and aquatic vegetation by grubbing in sediments. Can also feed on bacteria attached to suspension material in the water

column. Inhabits slow flowing rivers and lakes with dense vegetation and bottom vasoso (Fish Base, 2004d; ITIS, 2004c).

Size: the wild carp can attain 20 to 40 cm (0.3 to 1 Kg), at 3 to 4 years of age. However, there are carp with 40 years of age with 200 cm in length and 25 to 30 Kg of weight (Fish Base, 2004d; ITIS, 2004c).

Reproduction: The carp breeds from April/May to June, at an ideal temperature higher than 18 - 20°C. They easily hibridyze with *Carassius* sp. The wild carp can reach maturity at one year of age (Kuliyev & Agayarova, 1984). According to Balon (1995) wild carp spawn about three times within 14 days. The eggs are viscous, golden, with about 1.0 mm in diameter, and adhere to the shallow aquatic vegetation. Females spawn approximately 50,000 to 100,000 which increase in volume (2.5 mm) when contacts water. However, other authors states that the carp have a relative fecundity of 100,000 to 300,00 eggs per female Kg. They hatch in 3 to 8 days depending on the temperature (Fish Base, 2004d; ITIS, 2004c).

Habitat: A fish found in large bodies of water with slow flowing or standing water and soft bottom sediments. Common carp thrive in large turbid rivers. Hardy and tolerant of a wide variety of conditions, such as low oxygen levels, salinity or polluted waters. Tolerates temperatures ranging from 3 to 35°C. In the winter (when rivers and lakes are covered with ice) or in the dry season, burrows in mud and reduces metabolic rate (Fish Base, 2004d; ITIS, 2004c).

Systematics

Phylum	Chordata
Subphylum	Vertebrata
Class	Osteichthyes
Subclass	Actinopterygii
Order	Cypriniformes
Suborder	Cyprinoidei
Family	CYPRINIDAE
Genus	<i>Cyprinus</i>
Species	<i>Cyprinus carpio</i> (Linnaeus, 1758)

Distribution: Western Europe throughout eurasia to China, and South-East Asia and from Siberia to the Mediterranean and India. One of the first species to be introduced into other countries and now attains global distribution (Fish Base, 2004d; ITIS, 2004c).

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PART III

**CHAPTER 3 – A CHARACTERIZATION OF SELECTED ENDOCRINE
DISRUPTOR COMPOUNDS IN A PORTUGUESE WASTE WATER TREATMENT
PLANT**

A Characterization of selected Endocrine Disruptor Compounds in a Portuguese Waste Water Treatment Plant

3.1. A Characterization of selected Endocrine Disruptor Compounds in a Portuguese Waste Water Treatment Plant

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Abstract

Anthropogenic compounds that are able to disrupt the endocrine system of wildlife species are a major cause for concern and have led to a demand for new screening methods. The identification and quantification of endocrine disruptor compounds at wastewater treatment plant is of major interest to assess the endocrine activity of wastewater treatment plant discharges into the environment. This study consists of a preliminary survey of concentrations of previously selected endocrine disruptor compounds, undertaken to establish environmental concentrations and to support a biological program assay exposing freshwater fish to them. Selected endocrine disrupting chemicals (APEs, Bisphenol A and 17 β -estradiol) were measured in samples from a wastewater treatment plant located in Lisbon (Portugal), using recent commercial enzyme-linked immunosorbent assay kits and also LC-MS/MS. The results show that the wastewater treatment plant treatment process is efficient on the removal of target endocrine disruptor compounds. However, environmentally significant concentrations are still present in the treated effluent. The results also show that enzyme-linked immunosorbent assay kit is suitable for routine analysis of the selected compounds. The results are also useful since the wastewater treatment plant is located in a region under Mediterranean influence, which results in an effluent with its own characteristics.

Key words: Endocrine Disruptor Compounds, Wastewater; ELISA; LC-MS-MS, Environmental

3.1.1. Introduction

In the last decade there has been growing concern about a group of chemical compounds with endocrine activity which are present in the aquatic environment and can have adverse effects on reproduction in humans and wildlife species (Korner et al., 1998). Many reported effects

have been related to several classes of chemical compounds that are present in wastewater effluent, such as pesticides, aromatic compounds, alkylphenols, phytoestrogens and natural estrogens.

The non-ionic surfactants alkylphenol ethoxylates (APEOs) have been widely used in many domestic and commercial applications (Tsuda et al., 2000) and also as emulsifiers in domestic and industrial cleaning agents (Nichols et al., 2001). During wastewater treatment, APEOs undergo successive biodegradation into persistent metabolic products more toxic than the parent compound, such as alkylphenols, and are ultimately discharged to the aquatic environment. However, the estrogenicity of these compounds is less potent than that of endogenous 17β -estradiol and thus only high concentrations are liable to make a significant contribution to effluent estrogenicity (Sumpter, 1995).

Natural estrogens such as 17β -estradiol are known to be present in certain wastewater treatment plants as a result of human waste, registering levels considered sufficient to induce estrogenic responses (López de Alda and Barceló, 2001). Synthetic estrogens (e.g. ethynylestradiol) used in contraceptive formulations and for the treatment of hormonal conditions are excreted in the urine of mammals and enter the aquatic environment via wastewater treatment plant effluents, reaching sufficient concentrations (normally in the ng l^{-1} range) to trigger estrogenic responses (Petrovic et al., 2001).

Bisphenol A is widely used in households and the plastics industry, as an intermediate in the production of epoxy resins and polycarbonate plastics. It is also used in small quantities in the manufacture of thermal paper and in food and beverage can linings), dental resin composites and sealants. BPA is expected to be present in raw sewage and WWTPs and concentrated in sewage sludge. It is also considered a chemical with weak estrogenic activity, causing adverse effects on the reproductive health and fecundity of humans and wildlife (Furhacker, 2000).

Therefore the identification and quantification of these EDCs is of major importance. Additionally, it must be noted that urban wastewater effluents are usually a very complex mixture of compounds and only a small part of them have been identified (Matsui et al., 2000). However, the traditional analytical methods (e.g. GC-MS, LC-MS-MS, HPLC) are difficult, expensive and time-consuming and demand the development of specific procedures to measure these compounds in complex environmental samples. Furthermore, large sample volumes are required, besides extensive clean-up procedures to achieve sample purification.

Thus, there is a strong need for fast, simple and cost-effective methods for quantitative analysis of EDCs, such as ELISA (Goda et al., 2000).

The main objectives of this study were to screen certain EDCs (APEs, BPA and 17 β -estradiol) in a representative Portuguese WWTP, with the aid of two different analytical methods (ELISA and LC-MS-MS), and evaluate the estrogenic potential of the treated effluent discharged into the River Tagus estuary. The EDCs were selected on the basis of their known estrogenic potential, which has already been demonstrated in several studies (Routledge et al., 1998; Arukwe, 2001d; Jobling et al., 2003).

3.1.2. Materials and Methods

3.1.2.1. WWTP selection – Chelas (Lisbon)

The selection of the WWTP (Figure 3.1) as a case study was based essentially on the representative nature of its effluent, as it receives both domestic and industrial wastewater, and also on the existence of a complete treatment process (tertiary treatment) with partial reuse of treated effluent. The WWTP is also located in a region under Mediterranean influence, which results in an effluent with its own characteristics.

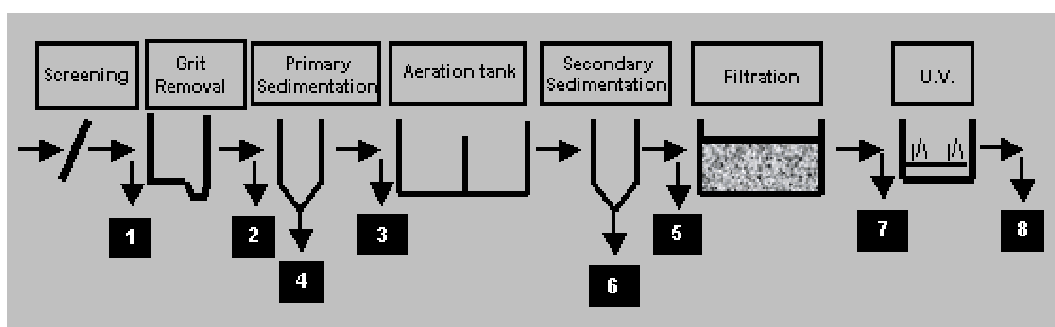


Figure 3.1: Diagram of the WWTP treatment steps and sampling points.

The WWTP was designed to treat a mean flow of 53,000 m³ per day, which corresponds to a population of approximately 211,000 equivalent inhabitants. The WWTP removes nutrients (nitrogen and phosphorus) and disinfects the final effluent before it is discharged into the River Tagus estuary. Table 3.1 shows the technical data from the WWTP for the period of the experiment.

Table 3.1: Technical data from Chelas WWTP.

Physico - chemical parameters	Mean Concentrations	Treated effluent
Mean Flow ($\text{m}^3\text{day}^{-1}$)	49,000	
BOD ₅ ($\text{mgO}_2\text{l}^{-1}$)	280.0	25.0
CQO ($\text{mgO}_2\text{l}^{-1}$)	516.0	125.0
TSS (mg l^{-1})	308.0	35.0
Total N (mg l^{-1})	50.0	7.5
Total P (mg l^{-1})	13.0	10.0

3.1.2.2. Selection of chemical compounds

To perform the screening a target-group of chemical compounds was chosen (Fig. 3.2): alkylphenol ethoxylates (APEOs), bisphenol A (BPA) and 17 β - estradiol. They were selected on the basis of their known estrogenic potential, which has already been demonstrated in several studies.

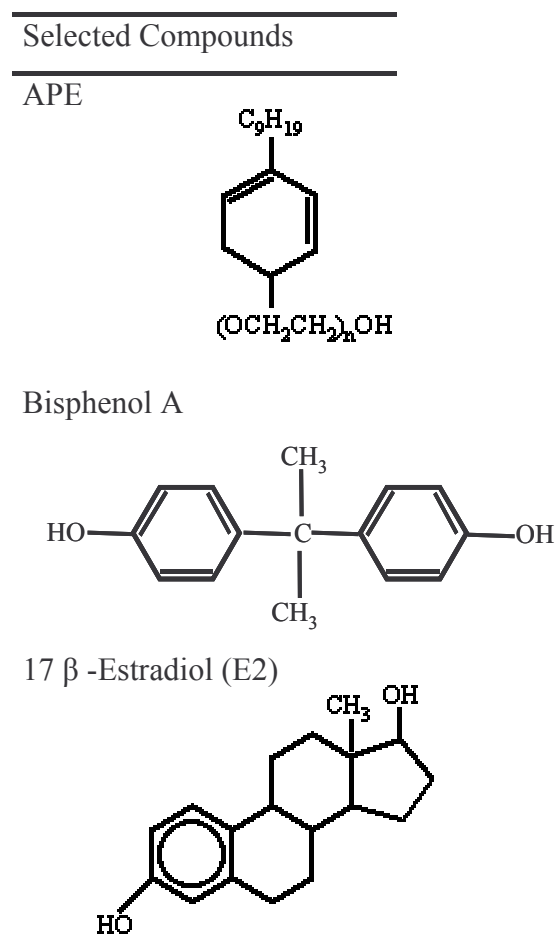


Figure 3.2: Chemical structure of selected compounds.

3.1.2.3. Sample collection

Composite samples were collected in amber glass bottles, at 4 hourly intervals over a 24-hour period, at different sampling points of the WWTP. Each point is representative of a treatment step of the working station. The aim was to evaluate the efficiency of the unit's operations in removing previously selected compounds. Other physical and chemical parameters were also measured (pH, temperature, dissolved oxygen, TSS, VSS, CDO and CBO₅) to gain a better understanding of the effluent quality in the sampling period and, thus, the plant's performance.

3.1.2.4. Wastewater Sample Pre-treatment and Extraction

The procedures for sample pre-treatment and extraction for the ELISA and LC-MS/MS analyses followed Takeda Kits instructions and also Petrovic et al. (2002).

Wastewater samples (300ml) were filtered in a glass-fibre filter with a one-micrometer pore size (Macherey – Nagel, Germany) and acidified with 1M acetic acid buffer (pH 5). The EDCs were extracted using OASIS HLB 3cc solid phase extraction (SPE) columns (Waters). Prior to extraction, the columns were conditioned with methanol and water. The EDCs were eluted with 2 x 5ml of dichloromethane (BPA and 17 β -estradiol) or methanol (APEs), depending on the EDCs for analysis, and then evaporated to dryness under a gentle stream of nitrogen and reconstituted with 10% methanol to a final volume of 2 ml.

3.1.2.5. Sludge Sample Pre-treatment and Extraction

Collection of primary and secondary sludge was performed using pre-cleaned amber glass bottles. The suspension was centrifuged at 4,500 rpm for 10 min.

The solid phase was sonicated for 20 min with 20 ml of dichloromethane-methanol (7:3, v/v). The extract was separated by centrifugation at 4000 rpm for 5 min. The extraction was repeated twice with fresh solvent mixture. Extracts were pooled and concentrated to an approximate volume of 1 ml under a gentle stream of nitrogen.

3.1.2.6. Enzyme Linked Immuno Assay (ELISA).

The ELISA technique was performed according to the protocols developed by Takeda Chemical Industries (Japan). Standards for EDCs were provided by Takeda Kits.

The ELISA test is based on a competitive reaction between a specific monoclonal antibody and the analysed compound. Briefly, the EDCs derived from samples and an antigen-enzyme conjugate, i.e. EDCs labelled with a colouring enzyme, are premixed and added into each microplate well for a competitive assay, vying for a limited number of antibody binding sites. When the EDC concentration is higher than that of the enzyme conjugate, the EDCs will predominantly bind the antibody and vice versa. Unbound APE and excess antigen-enzyme conjugates are washed out. Then, the chromogenic substrate is added to develop colour in conjunction with the conjugate's enzyme. The amount of antigen-enzyme conjugate remaining with the antibody will determine colour intensity. Higher APE concentration in the sample, for example, leads to less antigen-enzyme conjugate in a microplate well, generating a lighter colour, i.e. lower absorbance.

The standard curve, a dose-response curve from known concentrations of standard EDCs (Takeda Kits Standards), is determined from the absorbance at 450nm. The upper and lower quantification limits are shown in Table 3.2. The EDC concentration in each sample is accurately calculated from the response intensity of the absorbance. Standard solutions were prepared according to Takeda protocol instructions just before the test was performed. The results of the EDCs determination in sample extracts were corrected to the total sample volume.

Table 3.2: ELISA detection limits

Compound	Lower Limit ($\mu\text{g l}^{-1}$)	Upper Limit ($\mu\text{g l}^{-1}$)
APE	20.0	1,000
Bisphenol A	5.0	500
17 β -estradiol (E2)	0.05	1.0

3.1.2.7. Liquid Chromatography - Mass Spectrometry - Mass Spectrometry

Alkylphenols (nonylphenol and octylphenol), alkylphenol etoxylates (APnEO, nEO=1-15), bisphenol A (BPA) and 17 β -estradiol were analysed using the LC-MS-MS method (Petrovic, 2003). LC-MS-MS analyses were performed on a Waters 2690 series Alliance HPLC

(Waters, Milford, MA, USA) with a quaternary pump equipped with a 120-vial capacity sample management system. The analytes were separated on a 5 μm , 125 x 2 mm i.d. C18 reversed phase column Purospher® STAR RP-18 endcapped (Merck, Darmstadt, Germany). The sample injection volume was set at 10 μl . A binary mobile phase gradient with methanol (A) and water (B) was used for analyte separation at a flow rate of 200 μlmin^{-1} . The elution gradient was linearly increased from 30% A to 85% A in 10 min, then increased to 95% A in 10 min and kept isocratic for 5 min.

A bench-top triple quadrupole mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with a pneumatically assisted electrospray probe and a Z-spray interface was used for this study. The capillary voltage was set at 2.8 kV, extractor lens 7V and RF lens 0.6V. The source and desolvation temperatures were 150°C and 350°C, respectively. The nitrogen (99.999% purity) flows were optimized at 50 lh^{-1} for the cone gas and 540 lh^{-1} for the desolvation gas. For MS-MS experiments the argon collision gas was maintained at a pressure of 5.8×10^{-3} mbar.

Quantitative LC-MS-MS analysis of compounds detected under negative ionization (NI) conditions (NP, OP, BPA and 17 β -estradiol) was carried out in multiple reactions monitoring (MRM) mode using oxybenzoic acid as the internal standard. Compounds detected under positive ionization (PI) conditions (NPEOs and OPEOs) gave only $[\text{M}^+\text{Na}]^+$ adduct ions and produced no fragmentation. As a result these compounds were analyzed using a single stage MS in selected ion monitoring (SIM) mode using 4-nonylphenol mono ethoxylate-d2 as the internal standard. Table 3 shows the MRM and SIM ions, respectively, and the limits of detection (LODs) obtained.

Table 3.3: LC-MS-MS conditions and limits of detection

A) Negative ionisation mode

Compound	MRM 1	Cone (V)	Collision (eV)	MRM 2	Cone (V)	Collision (eV)	LOD (ngl^{-1})
NP	219 $\rightarrow$ 133	30	30	219 $\rightarrow$ 147	30	30	2
OP	205 $\rightarrow$ 133	30	30	205 $\rightarrow$ 147	30	30	2
BPA	227 $\rightarrow$ 133	30	20	227 $\rightarrow$ 211	30	25	2
E2	271 $\rightarrow$ 183	30	35	271 $\rightarrow$ 145	20	30	1

B) Positive ionisation mode

Compound	m/z	LOD (ngl ⁻¹)
NPEO ($n_{EO}=1-15$)	287 + 44n	50 (NPEO ₁)
		20 (NPEO ₂)
		10 (NPEO, $n_{EO}=3-15$)
OPEO ($n_{EO}=1-15$)	273 + 44n	50 (OPEO ₁)
		20 (OPEO ₂)
		10 (OPEO, $n_{EO}=3-15$)

3.1.3. Results and Discussion

The results for three selected estrogenic compounds are shown in Table 3.4, reporting the concentrations after the operations listed, as well as for the sludge lines. The results are presented in relation to the total sample volume.

Table 3.4: EDC concentrations measured by ELISA and LC-MS-MS.

Sample points	APEs (μgl ⁻¹)		BPA (μgl ⁻¹)		17β-estradiol (ngl ⁻¹)	
	ELISA	LC-MS-MS	ELISA	LC-MS-MS	ELISA	LC-MS-MS
1	>ld	78.15	1.02	1.55	1.12	<ld
2	Int.	55.02	0.21	0.15	1.12	<ld
3	5.77		1.32		1.73	
4	0.724		>ld		1.38	
6	>ld	4.6	1.32	0.25	0.57	
5	9.73		>ld		1.38	
7	5.77	15.53	0.55	0.31	0.91	<ld
8	5.32	5.20	0.08	<ld	0.91	<ld

Int. – interference; >ld – above limit of detection; <ld – below the limit of detection; N= 3 (mean values; sd < 7%)

The usual methods used in EDC determination are GC-MS and, more recently, LC-MS-MS (Petrovic et al., 2004). However, the analytical instruments used are very expensive and complex, involving the need for qualified personnel to operate them. Sample treatment is also complex and time-consuming and large sample volumes are needed. Nevertheless, these traditional methods are accurate and give better results.

An inter-laboratory comparison exercise conducted by Castillo et al. (2000) to determine surfactants in wastewater used different analytical strategies based on liquid chromatography (LC) coupled with mass spectrometric (MS) or fluorescent (FL) detection and a test-tube enzyme-linked immunosorbent assay kit. The study reported that LC-MS techniques are

reliable for surfactant determination and that ELISA (only used for nonylphenolpolyglycol ether determination) also gave acceptable results. However, ELISA values were higher, which was attributed to the interference of cross-reacting substances.

Fránek et al. (2001) also developed an immunoassay for NP, and nonylphenol decaethoxylate (NPEO10), for sensitive analysis of water samples. However, assay sensitivity for technical NP standard ranged within the order of mg l^{-1} . The same authors concluded that this sensitivity can be used for screening of industrial wastewater effluents rather than for ground or river water analysis where the concentrations of NP are lower, usually not exceeding mg l^{-1} concentrations.

Hence, in this study LC-MS-MS results (Table 3.4) were used in addition to ELISA methods. As it was not necessary to measure all treatment steps, two incoming and two outgoing points were chosen to carry out the LC-MS-MS analytical procedure.

Although different analytical techniques were used in order to evaluate the presence of selected endocrine compounds, results both from ELISA and LC-MS-MS showed their presence throughout the WWTP. Even if LC-MS-MS did not detect E2, this does not mean that it is not present in the wastewater since the detection limits for LC-MS-MS are lower than ELISA. Furthermore, the sample volume needed for an LC-MS-MS analysis for steroids was much higher than that the amount that was used, which was suitable for ELISA testing.

The treated effluent is discharged into the River Tagus estuary and it can be suggested that the effluent is sufficiently diluted within the estuarine waters and no effects are expected to occur in wildlife. However, it should be considered that from its source the River Tagus receives many treated and non-treated effluents. Nevertheless, further research should be carried out with estuarine organisms in order to assess possible harmful effects at the ecosystem level.

The highest APE levels were found in incoming effluent ($78 \mu\text{g l}^{-1}$ by LC-MS-MS and above detection limits with ELISA) and the lowest were recorded in outgoing effluent ($5.20 \mu\text{g l}^{-1}$ by LC-MS-MS). With respect to the final effluent, the APE ELISA kit results showed that data were overestimated in comparison with LC-MS-MS. However LC-MS-MS results after filtration were higher than those with ELISA, a fact that cannot be easily explained. Analytical interference due to the limited selectivity of antibodies is referred to as cross-reactivity and is a common phenomenon to all immunoassays (Oubina et al., 1997). Therefore, the CR of a particular compound becomes a problem when that compound is

present in the samples being analyzed at a concentration that is sufficiently high to increase the apparent concentration of the analyte of interest (Oubina et al., 1997). The experiments developed by Goda et al. (2000) also suggest that ELISA may be subject to interference that gives an overestimated value because of the broader cross-reactivity. Thus, different APEO concentrations recorded by the analytical techniques used to screen the WWTP effluent can be explained to some extent by the cross-reactivity phenomenon.

However, recently, a new monoclonal antibody-based competitive immunoassays for quantitative and semi quantitative detection of 4-*n*-nonylphenol in water was developed by Samsonova et al. (2003). This monoclonal antibody was very sensitive to 4-*n*-nonylphenol and cross-reactivity was negligible to technical 4-nonylphenol (a mixture of branch isomers).

The highest E2 level was detected after the first sedimentation (1.73 ng l^{-1}) and slight variations in E2 values were detected along the WWTP stages. They are not significantly different and could be related in part to the wastewater microbiological activity and also the cross-reactivity with other steroids, as shown in Table 3.5. Although, LC-MS-MS did not detect E2 (above the detection limit) that does not mean that the compound is not present.

Table 3.5: Cross-reactions of ELISA with APE, Bisphenol A, and 17β -Estradiol (E2). Adopted from Takeda Chemical Industries (Japan).

Compounds	% CR	Compounds	%CR	Compounds	%CR
Nonionic surfactants		Bisphenol A	100	Estrogens	
NPE(Nonylphenol ethoxylate)		Alkylphenol	<0.1	Estrone (E1)	1.3
		t-Octylphenol	0.12	2-methoxy E1	<0.4
NP10EO, NP7.5EO	100	t-Nonylphenol	<0.1	E1-3-sulfate	1.0
NP5EO	80	Phthalate Ester	<0.1	17 β -Estradiol (E2)	100.0
NP2EO	40	Diethylhexylphthalate(DEHP)	<0.1	16-keto E2	16.0
NP1EO	20	Butylbenzylphthalate(BBP)	<0.1	2-methoxy E2	2.0
OPE(Octylphenol ethoxylate)	230	Dibutylphthalate(DBP)		E2-17-glucronide	<0.4
OP10EO	<0.2			E2-3-glucronide	16.0
AE (Alkyl ethoxylate)				E2-3-sulfate-17	<0.4
				Estriol (E3)	0.6
				16-epi-E3	0.5
				E3-16-glucronide	<0.4
Anionic surfactants				Other Hormones	<0.03–0.46
LAS,SOAP,SDS, AES, Phenol, PEG	<0.2				

The highest BPA levels were found in the primary sludge (above detection limits) and concentrations underwent a significant reduction in the course of the treatment stages,

indicating that BPA is efficiently removed from wastewater. Although the methods are not comparable, ELISA and LC-MS-MS results show similar levels of the compound, suggesting that the BPA kit is reliable and can be used safely.

Table 3.6 shows that it is unequivocal that this treatment process is efficient for the removal of most target compounds (removal between point 1 and 8). However, there are some steps of the treatment process where the removal is negative, nominated between point 2 and 3 and point 3 and 5. A possible explanation can be related to biological treatment processes and cross-reactivity phenomenon.

Table 3.6: EDC's removal (%) in each step of WWTP

	Sampling points	Removal (%)					
		APEs		BPA		17 β -estradiol (E2)	
		ELISA	LC-MS-MS	ELISA	LC-MS-MS	ELISA	LC-MS-MS
Preliminary treatment (Screening and grit and grease removal)	1-2	-	30	79	90	0	-
1st sedimentation	2-3	-	-	< 0	-	< 0	-
Biological Treatment (aeration tank and final sedimentation)	3-5	69	-	-	-	20	-
Filtration	5-7	41	-	-	-	34	-
Disinfection (U.V.)	7-8	8	67	85	> 99	0	-
Influent /Effluent	1-8	> 99	93	92	>99	19	-

This study also allowed us to test recently developed ELISA kits from the point of view of their potential use in routine monitoring programs covering the presence and quantification of EDCs in WWTP effluents. The results showed that the ELISA Kits tested can be used to monitor the presence of EDCs in WWTP effluent, though precautions should be taken when interpreting results due to certain sources of interference that can alter the identification of chemicals.

Even though the kits tested are suitable for detecting the presence of the selected EDCs, the compound concentrations achieved by ELISA should be treated carefully as they may not reflect real values, due to the interference mentioned above.

3.1.4. Conclusions

The compounds screened are present in the effluent in varying concentrations throughout the treatment process and are discharged into estuarine waters at levels that may cause physiological effects in wildlife, as shown by both the ELISA and LC-MS-MS data. However, the estuarine waters may also have a diluting effect, reducing EDC concentrations and consequently the availability to their absorption by aquatic organisms.

The ELISA assays used in this study are suitable for routine screening of the selected chemicals. However, care should be taken if the results are intended to provide absolute values or are to be used to assess the kinetics of EDC bio-removal.

Considering that there are various river and estuarine organisms of commercial value that may bio-accumulate EDCs and be consumed by humans, and that river water is also used in the production of drinking water, public health problems may arise from the exposure to estrogenic chemicals. Accordingly, further research should be focused on the detection and identification of EDCs in the estuarine environment.

Acknowledgments

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Evaluation of the estrogenic potential and physicochemical characterization of a sewage treatment plant

3.2. Evaluation of the estrogenic potential and physico-chemical characterization of a sewage treatment plant

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Abstract

The aim of this study was to evaluate the estrogenic potential of a treated sewage effluent (Chelas-Lisbon) by determining certain previously selected target compounds (alkylphenols, 17 β -estradiol and bisphenol A) that are known for their ability to deregulate the endocrine system of living organisms. Samples were collected during the different treatment stages at the sewage treatment plant and analyzed by LC-MS-MS and ELISA after appropriate processing. The results show the presence of these compounds in the different phases of sewage treatment and in some cases show concentrations that have the potential to deregulate the endocrine system of living organisms. The heavy metal content of the effluent was also analyzed. Finally, certain physicochemical parameters (temperature, pH, dissolved oxygen (DO), total suspended solids (SST), volatile suspended solids (SSV) and oxygen chemical demand (OCD) were registered to complement the analytical information, as they can influence the rate of degradation and consequently the availability of the EDCs.

Key Words: Endocrine disruptors, STP, ELISA, LC-MS-MS

3.2.1. Introduction

Since the early 1990s there has been growing awareness among the scientific community and the general public of a group of compounds in the environment, which have the capacity to interact with the endocrine system and cause adverse effects in living organisms. As a result, numerous monitoring programs have identified the presence of these compounds in the effluents of sewage treatment plants (STPs), as well as in the aquatic environment (Gutendorf and Westendorf, 2001; Hemming et al., 2004). In addition, new analysis methods have been developed or proposed for the identification of these chemicals.

Thus, it is known that certain chemical compounds are present in STP effluent and their degradation products are persistent and have estrogenic activity, even after treatment. There is, therefore, growing concern about the influence of these chemicals on the environment (Desbrow et al., 1998). Generally referred as endocrine disruptor compounds (EDCs), they are capable of affecting human health and wildlife (Sumpter, 1998).

The compounds of anthropic origin usually described as mimicking endogenous steroids include the non-ionic surfactants and alkylphenol polyethoxylates (APEs), which are broadly used in a vast diversity of commercial and domestic applications (Tsuda et al., 2000) and also used as emulsifiers in domestic and industrial cleaning products (Nichols et al., 2001). During the treatment phases of urban and industrial waste waters, the APEs are degraded successively into less biodegradable forms, e.g. NP (nonylphenol) and OP (octylphenol), and end up being discharged into the aquatic environment (Arukwe, 2000).

In the aquatic environment there are natural compounds with estrogenic activity, for instance phytoestrogens and estrogens originating from human excretion, such as 17β -estradiol, estrone and estriol (Tyler et al., 1998). Moreover, in addition to the EDCs already known, many other new chemicals are synthesized every year and discharged into the environment with unknown consequences. Many of these may have estrogenic activity (Petrovic et al., 2004). Indeed, more than 70 chemicals have been referred to as potential EDCs, not to mention the fact that more than 80 000 man-made chemicals are currently in daily use and thus found in STP effluent, along with their degradation products (Sumpter, 1997).

There is little information available on the negative effects of major discharges into the aquatic environment and their toxicity for wildlife. Nor is there much information available about EDC removal at STPs. This circumstance that makes it difficult to calculate the mass balances. Thus, various bioassays and diverse analytical techniques have been employed to identify and ascertain the activity of potentially estrogenic compounds (Céspedes et al., 2004).

The present work concentrates fundamentally on the treated effluent from Chelas STP (Lisbon), which on a daily basis treats large quantities of urban wastewaters and considerable but variable volumes of industrial wastewaters. The STP serves the most highly populated city in the country and treats more than 50,000m³ per day of municipal wastewater effluents.

To study and evaluate the estrogenic potential of the STP treated effluent, certain previously selected EDCs were analyzed (alkylphenols, 17 β -estradiol and bisphenol A), taking into consideration their capacity to disrupt the endocrine system of living organisms. Certain heavy metals (Ni, Cr, Cu) were also measured. Samples were collected throughout the STP treatment stages and processed for later analysis by LC-MS-MS and ELISA. The results show the presence of these compounds in these different treatment phases and in some cases at concentrations that have the potential to disrupt the endocrine system of wildlife.

As complementary information, some physicochemical parameters (temperature, pH, dissolved oxygen, total suspended solids (SST), volatile suspended solids (SSV), oxygen chemical deficiency (OCD) and total nitrogen) were determined, since it is known that these may influence the rate of degradation and consequently the availability of EDCs. The results are discussed in relation to existing knowledge of EDC levels and a physical and chemical characterization of the STP in question.

3.2.2. Materials and Methods

The wastewater and sludge samples were collected at various stages of the STP treatment processes (Figures 3.2.1 and 3.2.2). Collection took place in June 2003, covering a daily period of 16 hours (from 8 a.m. to 12 midnight) for the duration of a week. The samples were transported to the laboratory in cooled thermal cases and then immediately filtered (Macherey-Nagel, MN GF –3, porous of 0.60 mm).

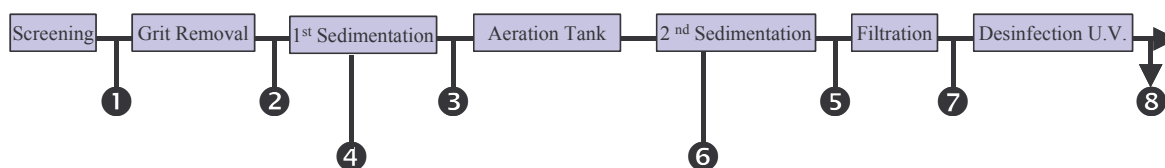


Figure 3.2.1: Sewage treatment plant – sampling points at the treatment phases. (1 – after screening; 2 - after grit removal; 3 – after 1st sedimentation; 4 – primary sludge; 5 – after 2nd sedimentation; 6 – secondary sludge; 7 – after filtration; 8 – after disinfection).

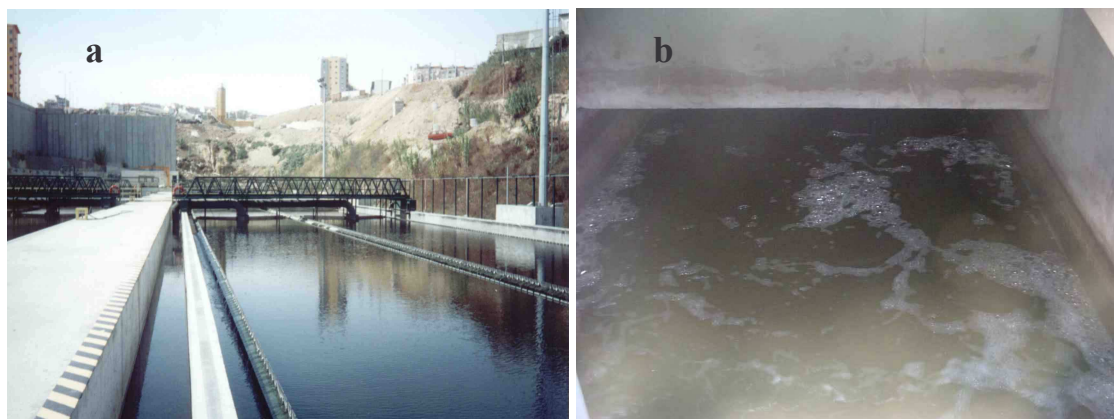


Figure 3.2.2: Chelas Sewage Treatment Plant, secondary sedimentation (a) and treated effluent after disinfection by U.V. radiation(b).

The sample pre-treatment and extraction procedures were carried out as described in the Takeda Chemical Industries user guide (ELISA) and by Petrovic et al. (2002) (LC-MS-MS).

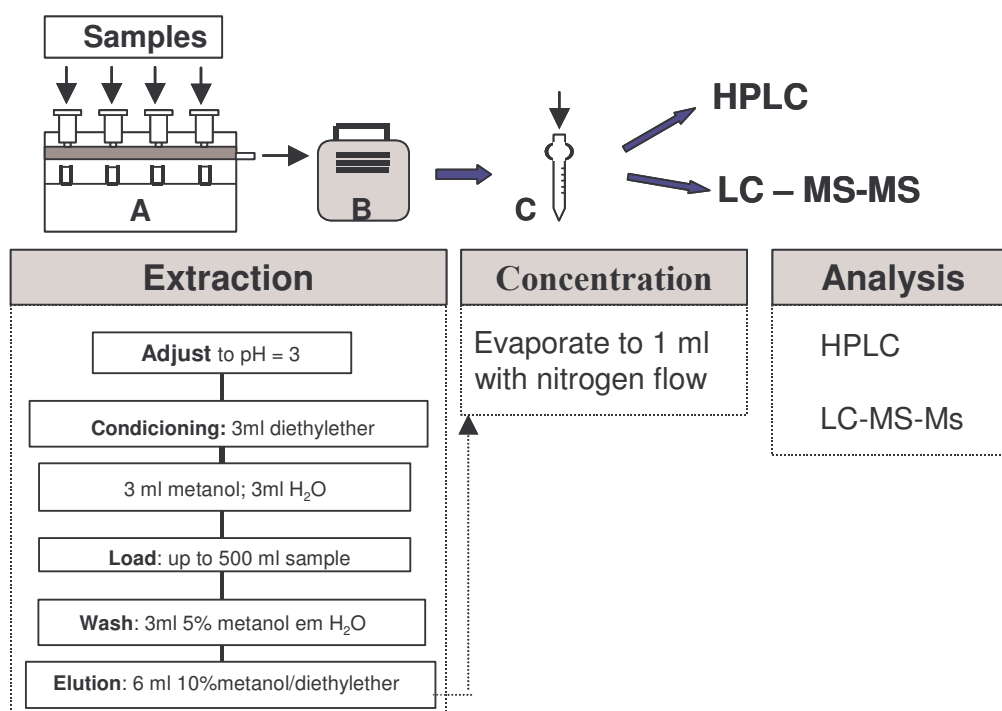


Figure 3.2.3: Schematic diagram of samples treatment and analysis.

In the ELISA analysis, the extraction of BPA, 17 β - Estradiol and APE was carried out using Waters OASIS HLB 3cc columns. They were eluted with 2 x 5 ml methanol (10%) for the BPA and APE, and with 2 x 5 ml dichloromethane (100%) for 17 β -Estradiol. Subsequently,

the samples were concentrated to two ml with liquid nitrogen at room temperature (Figure 3.2.3).

The ELISA assay is based on a competitive reaction between a specific monoclonal antibody and the compound to analyze. In this reaction, the well surface is covered with a protein (monoclonal antibody), which in turn binds exclusively to the EDC (antigen) under analysis. The EDC to be analyzed in the samples and the conjugate (antigen – enzyme) – an EDC marked with an enzyme that changes colour on reacting – were previously mixed and added to the wells of the microplate. A competitive reaction then occurred due to the limited number of connections to the antibody.

The excess conjugate and the EDC that did not react were then removed from the wells by washing the microplate well with PBS-Tween solution. Subsequently, a chromogen substrate that colors in the presence of the enzyme conjugate was added to each well. As the quantity of the antibody determines the color intensity, this was then quantified by absorbance using an ELISA reader (Biorad – Benchmark, USA).

The determination of the EDC's was carried out by constructing calibrations curves (for each EDC), also named dose-response curves, by determining known concentrations of EDCs standards at the absorbance of 450 nm. The concentration of EDCs in each sample was determined by measuring the absorbances intensity.

For the analysis by LC-MS-MS, the extraction of BPA, 17 β -estradiol and APE, was performed using OASIS HLB 3cc columns (Waters). The elution was carried out with 2 x 5.0 ml of methanol.

The LC-MS-MS analytical method LC-MS-MS consists of an automatic sampler (HP 1100; LC binary pump - HP 1090) from Hewlett Packard (Palo Alto, CA, USA), with a reverse phase column - C18 (5 μ m, 250 x 4 mm - LiChrospher 100 RP-18) and a pre-column (4 x 4 mm, 5- μ m - Merck - Darmstadt, Germany). The volume of injection was adjusted to 25 μ l at flow of 1 mlmin⁻¹.

The detection was carried out using a HP 1040 M diode array UV-Vis detector coupled in series with a mass selective detector of Waters - LC-MSD HP 1100, equipped with an

atmospheric pressure ionization source, that can be used for chemical ionization with atmospheric pressure (APCI) or by interface electrospray (ESI).

The quantitative analysis was carried out with “selected ion monitoring (SIM), using external calibration. The calibration curves were built by linear regression. The corroboration of the identity of the compounds present in the effluent was achieved by “scan mode”, comparing the time of retention and the mass spectrum of the compound analyzed with the respective standards.

The determination of some physicochemical parameters, (temperature, pH, dissolved oxygen, total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Chemical Oxygen Demand (COD), total nitrogen), were carried out using techniques described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1992).

3.2.3. Results and Discussion

Table 3.2.1 presents the concentrations of the selected EDCs at the various sample points in the sewage treatment phases. The analyses, carried out by LC-MS-MS, revealed appreciable APE levels at the beginning of the treatment line. However, a substantial reduction was found in the course of the STP processes, which indicates a high rate of efficiency in the removal systems that constitute the station.

Table 3.2.1: Measured concentrations of selected EDCs by LC-MS-MS.

Sampling Points	APEs ($\mu\text{g l}^{-1}$)	BPA ($\mu\text{g l}^{-1}$)	17 β -estradiol (ng l^{-1})
After Screening	78.2	1.6	<ld
After Grit Removal	55.0	0.2	<ld
After Filtration	15.5	0.3	<ld
After disinfection– UV	5.2	<ld	<ld

According to the scientific literature, these APE concentrations determined in the treated effluent are still sufficient to cause endocrine disruption in living organisms (Sumpter, 1998). It should, however, be noted that the dilution effect of the estuarine waters is able to neutralize the estrogenic potential of the effluent.

ELISA also analyzed the extracts of the samples collected at each sampling point of the STP process (Table 3.2.2).

Table 3.2.2: Concentrations determined by ELISA

Sampling Points	APEs ($\mu\text{g l}^{-1}$)	BPA ($\mu\text{g l}^{-1}$)	17 β -estradiol (ng l^{-1})
After Screening	>ld	1.0	1.1
After Grit Removal	Int.	0.2	1.1
After 1st Sedimentation	5.8	1.3	1.7
Primary Sludge	0.7	>ld	1.3
Secondary Sludge	>ld	1.3	0.6
After 2nd Sedimentation	9.7	>ld	1.4
After Filtration	5.8	0.6	0.9
After disinfection– UV	5.3	0.1	0.9

Int. interference; > ld :above detection limit

It is not possible to compare the values obtained by LC-MS-MS with the ELISA results, since they are different analytical techniques. ELISA is based on a method that is still being developed. It also shows a degree of interference with others chemicals through which it tends to over-estimate the results (Goda et al., 2000). Indeed, the analytic interference is due to the limited selectivity of the antibodies used, usually referred as “cross-reaction”, a phenomenon that is common to all immuno-assays (Oubina et al., 1997).

The reason for obtaining the 17 β -estradiol results using ELISA is associated with the fact that this technique is more sensitive to this compound and consequently presents lower detection limits. In addition, the sample volume necessary to analyze 17 β -estradiol was ideal for the ELISA method while it was limited for LC-MS/MS. Thus, the fact that these compounds have not been detected by LC-MS/MS does not signify that are not present in the STP effluent.

However, despite the different analytic techniques that were used and the difficulty in comparing the absolute values obtained with both techniques, the data show that EDCs are present in all STP phases.

Even if the methods cannot be directly compared, the BPA concentrations obtained by both techniques show that this compound is removed efficiently and is not expected to cause adverse effects in the biota.

Table 3.2.3: Physicochemical parameters determined at the STP sampling points.

Sampling points	pH	OD (mg l ⁻¹)	TSS (mg l ⁻¹)	VSS (mg l ⁻¹)	COD sol. (mg l ⁻¹)	COD gr. (mg l ⁻¹)	Temperature (°C)
1	7.68	0.22			148.19	375.73	23.07
2	7.68	0.25			146.37	381.31	22.99
3	7.53	0.26			137.17	258.71	22.79
4	6.62		46.12	36.52	378.78	1,8097.38	22.92
5	7.09	3.64			20.81	44.29	23.48
				1504.			
6	6.94		2239.00	00	29.97	5514.93	24.15
7	7.10	4.60	20.86	15.76	17.31	24.36	23.38
8	7.15	5.01			18.06	26.68	23.37

Table 3.2.2. show the mean values of the physical-chemical parameters. It is known that time variations for these parameters can influence the biodegradation and consequently the concentration of EDCs in the course of the STP treatment processes (Gomes and Lester, 2003).

3.2.4. Conclusions

The previously selected EDCs were detected in the different STP treatment phases. In the effluent analyzed, which is normally discharged into the River Tagus estuary, some of these compounds were found in sufficient concentrations to cause adverse physiological effects on the biota, as was shown by the results. However, it is also necessary to consider the dilution effect of the estuarine waters, which can reduce or neutralize the action of these compounds and consequently their availability for the biota. On the other hand, it is still necessary to consider that there may be other EDCs that, though not analyzed, are also discharged into the estuary.

The results also show that ELISA Kits can be used in monitoring programs to detect EDCs though care is needed with quantification when interpreting the results, as some interference and “cross-reactions” may occur and lead to over-estimation of the results.

Aknowledgments

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Assessing the removal of nonylphenol in the effluent of a municipal sewage treatment plant. Exposure effects in carp (*Cyprinus carpio*)

3.3. Assessing the removal of nonylphenol in the effluent of a municipal sewage treatment plant. Exposure effects in carp (*Cyprinus carpio*)

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L. Castro; F. Santana

Abstract

Xenobiotics can have negative effects on the aquatic environment and may cause serious changes in ecosystems, such as the disequilibrium of the male/female ratios of populations as a result of the action of endocrine disruptor compounds. The consequences for the aquatic biota are not completely understood. The object of this study was to evaluate the removal of nonylphenol in a municipal sewage treatment plant (STP) and to assess the effects of endocrine disruption in carp. A significant reduction (>95%) in the concentration of nonylphenol was observed throughout the treatment phases of the station, diminishing the potential negative effects, which in turn could affect the aquatic environment. The experimental tests were carried out using carp (*C. carpio*) exposed to different concentrations of nonylphenol under laboratory conditions. The results show that the highest levels of nonylphenol were accumulated in the liver. The histological observation showed that nonylphenol causes severe histopathological changes in exposed fish.

Key words: Endocrine Disruptor Compounds, nonylphenol, wastewater treatment, Bioaccumulation, Carp

3.3.1. Introduction

In the last 50 years, synthetic compounds like the alkylphenol polyethoxylates (APEOs) have been used in a variety of industrial, commercial and domestic applications (Tsuda et al., 2000). These compounds have an annual production of about 350 000 tonnes, in the U.S.A., Europe and Japan, which certainly explains the presence in wastewaters and the aquatic environment (Nichols et al., 2001).

Some of these xenobiotic compounds, which are present in sewage effluents and industrial sludge, possess estrogenicity (such as nonylphenol (NP)), other products of alkylphenol degradation, PCBs and diethyl-ethoxylates. They can induce alterations in the endocrine functions of fish and other organisms and cause an imbalance in the male/female ratio of populations. Studies carried out with different fish species have shown such anomalies as gross morphological and microscopical changes at the tissue level or the induction of vitellogenin in male fish (Arukwe et al., 2000; Schwaiger et al., 2000).

For that reason, there is increasing concern about the indiscriminate use of these compounds, mainly due to the relative stability of certain degradation metabolite generated from products containing APEOs during the wastewater treatment processes (Blackburn and Waldock, 1995).

The toxicity of nonylphenol has been demonstrated in freshwater and marine species. It has the ability to induce estrogenic responses in male rainbow-trout and shows bioaccumulation effects in freshwater organisms (Jobling and Sumpter, 1993; Purdom et al., 1994). Thus, there is strong evidence that these synthetic compounds are endocrine disruptor compounds (EDCs).

The information available on the effects of high wastewater discharges into the environment and the estrogenic potential of effluent is still very limited, principally in aquatic systems.

On the other hand, information on the removal of EDCs from sewage treatment plants is very scarce, a circumstance that hinders the accomplishment of mass balance estimates, which is essential to any forecast of the environmental impact on the aquatic systems.

To guarantee the correct interpretation of the environmental risk associated with these compounds, an integrated approach must be adopted, involving the mass flow from its source to the STP, to soils and to ground water reserves. It must also involve the estrogenic responses of the organisms exposed.

Any analysis of water pollution caused by organic compounds must include an evaluation of its biodegradability in the receiving system or STPs. To analyse the biodegradation rate of these compounds, taking the amounts detected in wastewaters (significant or neglected) into

account, a global evaluation of their behaviour and uses is necessary so that it is easier to foresee their effects on the aquatic systems.

Biodegradation is an excellent process for the removal of organic pollutants and, consequently, can contribute to minimizing the negative effects of the EDCs in the aquatic environment. The mechanisms of NP removal are still little known and involve volatilisation phenomena, adsorption and biochemical processes. Thus, with regard to the removal of EDCs in STPs, it is obviously necessary to improve our knowledge of removal processes in bioreactor assays and to study the kinetic mechanisms involved in the reaction.

The present study is part of a wider project that intends to study various EDCs and focus on NP removal in the soluble phase, in the course of the sewage treatment phases in Chelas (Lisbon). The project also intends to evaluate the conditions and mechanisms that are essential to the removal process and to examine aspects related with solid/liquid separation processes (Fig. 3.3.1).

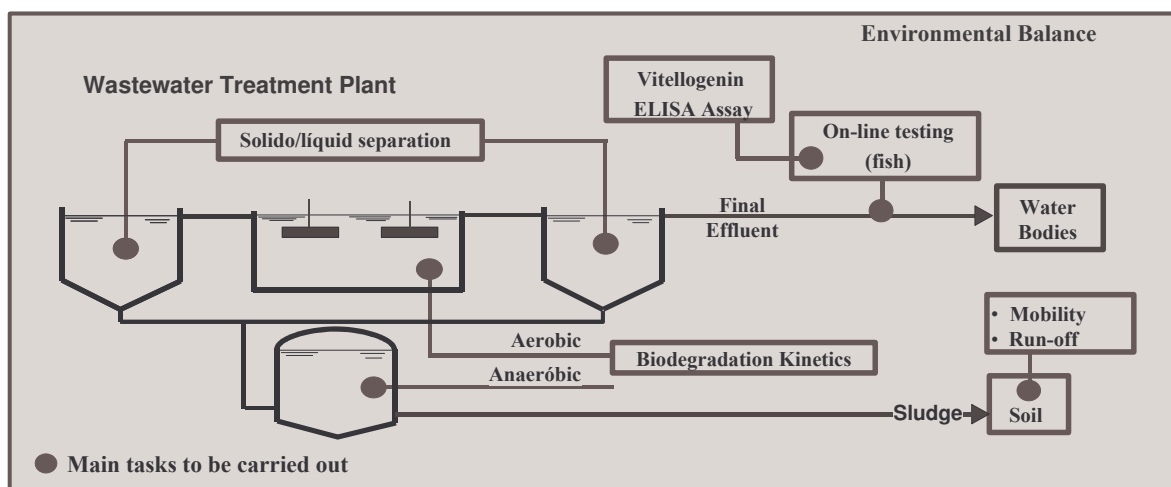


Figure 3.3.1: Evaluation of the mass balance of nonylphenol in a STP and biological assays

Laboratory bioassays were also carried out, using carp as the test fish, to assess the estrogenic effects of NP on biota.

3.3.2. Materials and Methods

3.3.2.1. Determination of nonylphenol in wastewaters

The present study was performed at the Chelas STP in Lisbon. It treats a mean flow of 49,000 m³ of wastewater per day, mainly processing urban wastewaters from about 211,000 inhabitants (Emarliz). The sampling points are identified in Figure 3.3.1.

3.3.2.2 Sample collection

The wastewater samples were collected at the STP in June 2001. The daily collection period covered 16 hours (from 8 a.m. to 12 midnight) during the course of a week. The samples were carried in coolboxes to the laboratory, where they were filtered (Macherey-Nagel, MN GF -3, porosity of 0.60 mm).

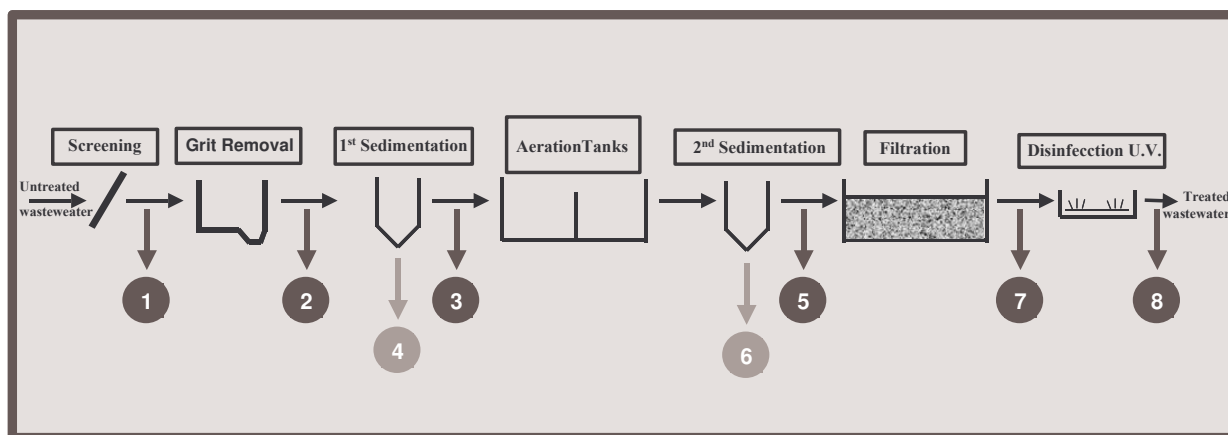


Figure 3.3.2.1: Sampling points along the treatment phases of the Chelas STP.

3.3.2.3. Sample processing

3.3.2.3.1. Compounds extraction procedure

The extraction of compounds from samples, was performed using columns with two phases (liChrolut RP - 18 (40-63mm) and liChrolut EN (40-120mm), and eluted with 2 x 5.0 ml of acetone (100%), according to a method based on Stumpf et al. (1996) and described by Weltin et al. (1998).

Later, the samples were concentrated up to one ml, with liquid nitrogen at room temperature.

Collection of primary and secondary sludge was performed using pre-cleaned amber glass bottles. The suspension was centrifuged at 4,500 rpm for 10 min.

The solid phase was sonicated for 20 min with 20 ml of dichloromethane-methanol (7:3, v/v). The extract was separated by centrifugation at 4000 rpm for 5 min. The extraction was repeated twice with fresh solvent mixture. Extracts were pooled and concentrated to an approximate volume of 1 ml under a gentle stream of nitrogen.

3.3.2.3.2. Determination of nonylphenol

To determine the NP concentration in the samples, it was used a HPLC system (Merck-Hitachi, L-6200A), with a column (Merck) of 4.0 x 125 mm, Licosphere RP18 (5mm), equipped with a fluorescence detector (Merck-Hitachi, F-1050). The operating conditions of the fluorescence detector were the following ones: Ex 275 nm, Em 300 nm; column temperature, 40°C; flow-rate 1.0 mlmin⁻¹; mobile phase, A= water (ultrapure), B = acetonitrile: linear gradient.

3.3.2.4. Fish exposure tests

3.3.2.4.1. Experimental conditions

Twelve sexually mature carp (*Cyprinus carpio*) from the Vasco da Gama Aquarium in Lisbon (average weight: 14.59 ±2.36g) were randomly distributed in three groups and placed in 50 litre aquariums, containing chlorine-free tap water at a constant temperature (15±1°C) under a photoperiod regime of 16hrs light: 8hrs dark. The fish were fed daily with commercial food (Alpis).

The water in the aquarium was contaminated with nominal concentrations of 0, 25 and 100 mg l⁻¹ of 4-n-Nonilfenol (Fluka, Chemical 74430). During the period of the experiment (28 days) the aquarium water was renewed every 48 hours.

At the end of the experiment, the fish were weighed and measured and the organs removed (liver, gonads, kidney and muscle). The gonad size was expressed as a percentage of the total body mass (Gonadosomatic Index, GSI). Sub-samples of the organs were taken and placed in a fixative solution of Bouin-Hollande. The fixed organs were processed for histology evaluation, using the usual histological methods. Other sub-samples of the dissected organs were stored to -80°C for later analysis of the NP concentration in the tissues.

3.3.2.4.2. Determination of NP in tissues

The organ samples (300 mg) were homogenised in 3ml of acetonitrile. The homogenate was centrifuged at 2000xg and the supernatant removed and filtered (Macherey Nagel with a porosity of 0.45mm) for analysis by HPLC. The recovery in this procedure was >80%. The determination of NP by HPLC was carried out according to the method previously described.

3.3.3. Results and Discussion

The concentrations of NP at the different sampling points are shown in Fig. 3.3.3.1. The analyses revealed appreciable levels of NP at the influent. However, a reduction in the concentration of nonylphenol was found during the course of the STP treatment phases, suggesting a certain efficiency by the STP treatment processes in removing NP.

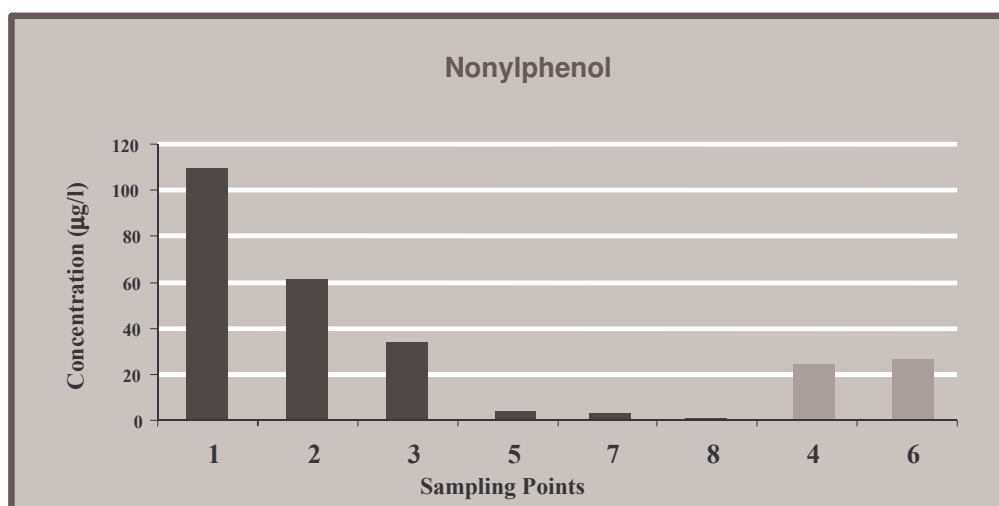


Figure 3.3.3.1: Nonylphenol concentrations at the different sampling points.

The Table 3.3.3.1 show the removal rates calculated at different types of treatment.

Table 3.3.3.1. Removal percentages of nonylphenol at the different treatment stages.

Type of Treatment	Removal Rate (%)				
	20	40	60	80	100
Preliminary treatment		◆			
Primary treatment		◆			
Secondary treatment				◆	
Reuse treatment	◆				

Analysis of the results shows that, though relative removal rates occur in the secondary treatment, in quantitative terms the largest part is removed in the preliminary treatment (Fig. 3.3.3.1). Thus, it can be said that this behaviour is a consequence of the nature of NP, which is not very soluble in water and can, therefore, be removed along with the fats.

Figure 3.3.3.2 shows NP levels in the tissues of fish exposed under laboratory conditions. The liver shows the highest concentrations of NP, suggesting that this organ plays an important role in the bioaccumulation processes of the chemical in *Cyprinus carpio*.

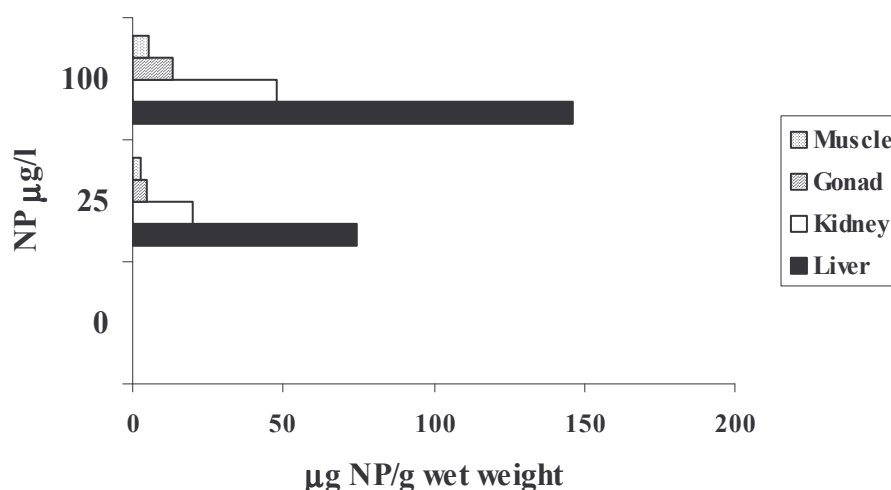


Figure 3.3.3.2: NP levels ($\mu\text{g g}^{-1}$ wet weight) in the carp muscle, kidney, gonad and liver, exposed to different concentrations of NP in aquarium water (0, 25 and 100 $\mu\text{g l}^{-1}$).

Figure 3.3.3.3 shows the gonadosomatic index (GSI), registering a decrease in the exposed fish.

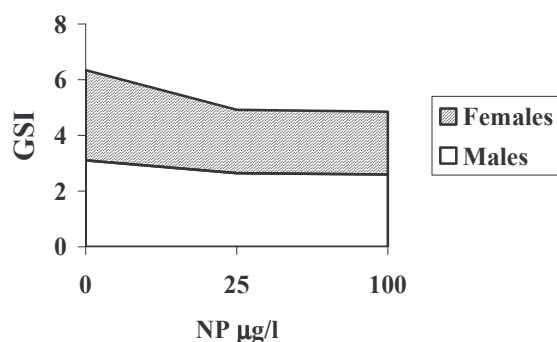


Figure 3.3.3.3: Gonadosomatic Index in carp exposed to 0, 25 and 100 $\mu\text{g/l}^{-1}$ of NP.

The testes analyses show negative effects in testis morphology in fish exposed to NP. More pronounced histopathological changes were observed in fish exposed to 100 NP $\mu\text{g/l}^{-1}$, such as a reduction in the diameter of the seminiferous lobules (Fig. 3.3.3.7). The reduction in the GSI for exposed organisms seems to indicate the effects of exposure to the NP on the gonad weight, supporting the histopathological findings. These effects may have consequences for the reproductive capacity of the organisms.

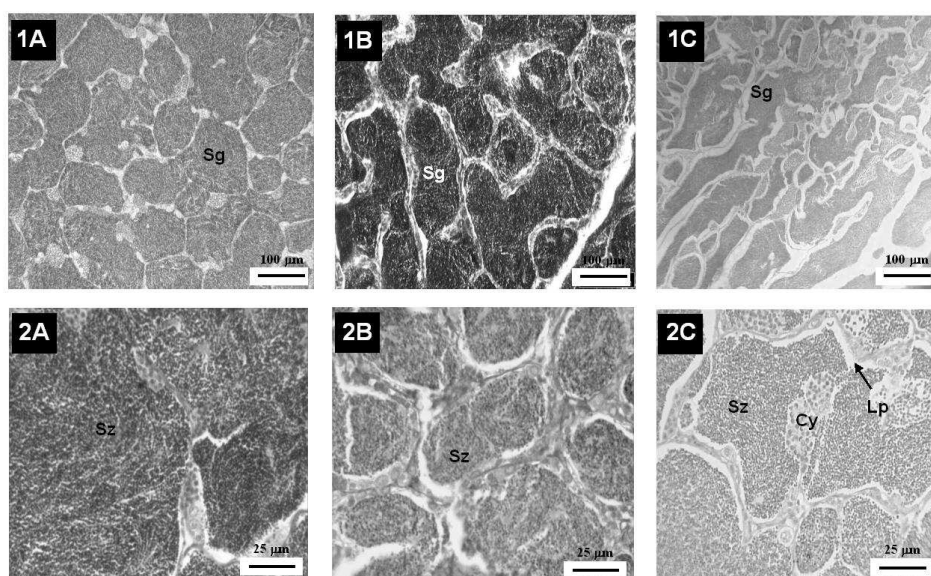


Figure 3.3.3.4. Gonad of *Cyprinus carpio* (male), showing the seminiferous lobules in controls (1A-2A) and in animals exposed to 25 and 100 $\mu\text{g/l}^{-1}$ (1B-2B and 1C-2C, respectively). Sg (seminiferous lobules); Cy (spermatogenic cysts) Sz (spermatozoa). Bar: 100 μm (1A, 1B, 1C); Bar: 25 μm (2A, 2B, 2C). Coloration HE, (cuts 5 μm).

The results obtained with the NP exposure test show that, for concentrations usually found in STP effluent, significant effects are observed in fish, a result that accentuates the importance of the treatment systems. On the other hand, it is important to assess the effect for substantially lower concentrations of the compound and to simulate the conditions of the aquatic environment that receives the discharges of treated effluents. This was taken into consideration in the current R&D project, which will include a study of the biological materials in assays using different dilutions of the treated effluent.

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PART IV

CHAPTER 4 - LABORATORY ASSAYS

Comparative estrogenic potencies of single xenoestrogens (nonylphenol, octylphenol, bisphenol-A, 17 β -estradiol and 17 α -ethynylestradiol). In vivo assessment by exposing carp (*Carassius carassius*)

4.1. Comparative estrogenic potencies of single xenoestrogens (nonylphenol, octylphenol, bisphenol-A, 17 β -estradiol and 17 α -ethynylestradiol). In vivo assessment by exposing carp (*Carassius carassius*)

M.S. Diniz, I. Peres, and J.C. Pihan

Abstract

The estrogenic potencies of single chemicals that are known to be estrogenic substances (nonylphenol, octylphenol, bisphenol A, 17 β -estradiol and 17 α -ethynylestradiol) were evaluated. Sexually mature carp of both sexes were exposed for four weeks to different doses (0, 1, 25 and 100 $\mu\text{g l}^{-1}$) of each of the single chemicals under controlled laboratory conditions. Vitellogenin (Vtg), the gonadosomatic index (GSI), the hepatosomatic index (HSI) and histological abnormalities were used to assess the estrogenic potency of each chemical. Results show a significant ($P < 0.05$) increase in Vtg induction in exposed male fish for each of the single chemical treatments. The results for females only show significant ($P < 0.05$) Vtg induction in fish exposed to NP and BPA (100 $\mu\text{g l}^{-1}$). A significant GSI reduction was also found in males exposed to all chemicals. Histopathological effects observed in testes (e.g. ovo-testis, restricted spermatogenesis) seemed to be dose- and chemical-related. The results suggest that the strongest estrogenic responses for the endpoints assessed are caused by the steroids that were tested.

Key words: vitellogenin, xenoestrogens, *Carassius carassius*, estrogenic response

4.1.1. Introduction

Although it has been known since the 1930s that exogenous substances are capable of interfering with the endocrine system. Endocrine Disruption has only been recognized as an environmental issue since about 1992, when it started to receive considerable scientific, government and public attention (Lye et al., 1997; Mathiessen, 2000).

A variety of natural products and synthetic chemicals, known collectively as endocrine-disrupting compounds (EDCs), mimic or interfere with the mechanisms that govern vertebrate

reproductive development and functions (Crews, 2000). A number of reports have documented the fact that these chemicals, which are found in many STP effluents and in receiving waters such as rivers and estuaries, are sufficiently concentrated to induce estrogenic responses in fish species (Tyler et al., 1998; Vos et al., 2000).

Alkylphenol ethoxylates (APEs) are non-ionic surfactants that have been used in a wide range of industrial, domestic and commercial applications such as detergents, plastics emulsifiers, pesticides and consumer cleaning agents (Nichols et al., 2001). Since APEs are primarily used in aqueous solutions, they enter the environment mainly through sewage and industrial wastewater treatment plants where they may undergo biodegradation processes that result in less degradable small-chain ethoxylates, primarily mono- and diethoxylates, carboxylates and alkylphenols, which are more toxic and persistent than the parent compound (Maguire, 1999; Arukwe, 2000). Nonylphenols (NPs) and octylphenols (OPs) are widely employed in industry and constitute the predominant components of alkylphenols found in the aquatic environment, possessing estrogenic effects that have been reported in fish, amphibians, birds and mammals (Thibaut et al., 2002).

Bisphenol A (BPA) is an intermediate in the production of epoxy resins and polycarbonate plastics in the plastics industry and it is also used in the paper industry. It is used in many food and drink packaging applications to line metal food cans, bottle tops and water supply pipes. Some polymers used in dental treatment also contain Bisphenol A. (Furhacker et al., 2000).

Natural and synthetic estrogens such as 17β -estradiol (E2) and 17α -ethynylestradiol (EE2) have been identified in certain sewage treatment plants (STPs) as resulting from human and animal waste, in sufficient concentrations to induce estrogenic responses (López de Alda and Barceló, 2001; Folmar et al., 2002; Lee et al., 2002). As these estrogens are excreted primarily in conjugated forms, the presence of unconjugated forms in the aquatic environment is probably the result of their deconjugation by the STP biological processes (Johnson and Sumpter, 2001).

The estrogenic compounds, such as the natural hormone E2 and the synthetic pill component EE2, have been under special attention in pollution monitoring, due to their possible involvement in endocrine disruption in wild life (Rodgers-Gray et al., 2000). Natural steroid estrogens are very potent hormones and have biological activity at very low concentrations.

However, the significance of the natural estrogens in the environment is presently unknown; once these estrogens reach the river it is still unclear how available they are to aquatic organisms (Tyler et al., 1998).

To be successfully measured, the very low environmental concentrations expected for natural and synthetic steroid hormones normally need sophisticated and expensive analytical techniques. For this reason, biological tools are needed to measure effects, i.e. the integrated responses to all estrogenic chemicals present, including those that might be missed by chemical analysis (Aerni et al., 2004).

Even though several endpoints can be used to assess the effects of EDCs, it is vitellogenin (Vtg), zona radiata proteins (Zrp), histopathology and steroid concentrations that are the most commonly used. Vtg is a phospholipo-glycoprotein that is used as a useful and sensitive biomarker of exposure to xenobiotic effects in fish and other animals. It is usually synthesized in the female's liver during oogenesis and is a major precursor of egg-yolk proteins in oviparous vertebrates (Wallace, 1985). The synthesis of this protein is normally restricted to females but it can be induced in males and juveniles by exposure to estrogens. Therefore, vitellogenin is commonly used as a biomarker of exposure to estrogenic compounds (Sumpter and Jobling, 1995; Denslow et al., 1999).

The main objective of the present study was to assess the estrogenic effects of exposure to a variety of known chemicals that are able to disrupt the endocrine system of fish. The fish were exposed to single chemicals and their potency was evaluated using Vtg induction and histological effects in both female and male adult crucian carp. They were exposed to different concentrations of NP, OP, BPA E2 and EE2 under laboratory conditions so that we could gain a better understanding of the effects and mechanisms of these chemicals on the reproductive organs and reproductive status of fish. The study also provides useful information for comparison with field studies.

4.1.2. Materials and Methods

4.1.2.1. Test chemicals

Test chemicals E2 (97% purity), EE2 (98% purity); OP (97% purity) and BPA (99% purity) were purchased from Sigma-Aldrich Chemical Company (Germany); 4-n-nonylphenol (96.4% purity) was purchased from Fluka Chemika (Germany).

Chemical stock solutions were prepared individually at the appropriate concentrations (1.0 g l^{-1}) using methanol (pró-analysis, Merck) as the solvent. Further, spiking solutions were premixed by diluting stock solution in 1000 ml of distilled water and stored at 4°C in the dark room prior to addition to the 30 liters aquarium. The concentration of solvent in experimental media was less than 0.1%. Solvent control conditions were also tested. Nominal concentrations were used throughout the experiments.

4.1.2.2. Fish selected and the design of the experiment

Sexually mature crucian carp (*Carassius carassius*), aged 24 months or more, were used as a test organism in this laboratory experiment. They were obtained from local producers (Estação Aquícola da Azambuja and Aquário Vasco da Gama), weighing an average of $15 \pm 2 \text{ g}$. For the 2 weeks before the exposure experiments they were acclimated in 300L tanks with a continuous flow of recirculated, filtered and aerated tap water at $16 \pm 2^{\circ}\text{C}$.

The experiments were conducted in four glass aquaria each supplied with 30 L of tap water previously dechlorinated. The aquaria were aerated, the water temperature was maintained at $15 \pm 2^{\circ}\text{C}$ and the photoperiod at 12h light: 12h dark, throughout the experiments. The pH values ranged between 7.2 ± 0.3 . During the experimental period, fish were fed daily with commercial dry flake food (Tetra brand). The fish were acclimatized to the laboratory conditions 2 weeks prior to the experiments. Mortality was observed daily.

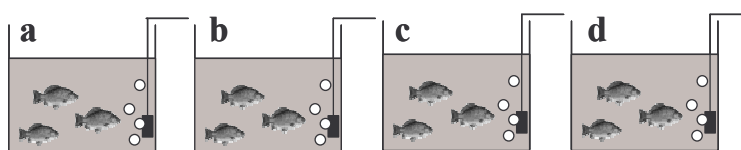


Figure 4.1.1: Experiment design. (a) controls; (b) $1 \mu\text{g l}^{-1}$ chemical; (c) $25 \mu\text{g l}^{-1}$ chemical; (d) $100 \mu\text{g l}^{-1}$ chemical.

The fish were then transferred from the acclimation aquarium and randomly distributed into the glass aquaria (Fig. 4.1.1), in four groups of 12 fish for each treatment group. Each treatment was duplicated (24 fish for each treatment group), totaling 120 animals. All experiments were conducted during the reproductive resting period (November – January).

Five previously chosen chemicals (E2, EE2, NP, OP and BPA) were tested, by adding serial concentrations of each single compound to aquaria water, in five separate experiments to test each compound singly. Treatments were duplicated, repeating each experiment. The nominal concentrations of the selected chemicals were: 1, 25 and 100 $\mu\text{g l}^{-1}$. A solvent-free control and a solvent control (0.1% methanol) were also included. During the experimental period (28 days) the aquarium test conditions were renewed every 48 h.

4.1.2.3. Analytical chemistry

The HPLC system used was a L-6200A (Merck-Hitachi, Germany), connected to a fluorescence detector F-1050 (Merck-Hitachi, Germany). Chemicals were detected using excitation wavelength of 275 nm and emission wavelength of 300 nm. The chromatographic column applied, a 4.0 x 125 mm, Licosphere RP18 (5 μm), was purchased from Merck (Germany). HPLC determinations were performed in the following operating conditions: column temperature, 40°C; flow-rate 1.0 ml min^{-1} ; mobile phase, A= water (ultrapure), B= acetonitrile: linear gradient.

4.1.2.4. Sampling Fish

After 28 days' exposure, fish were measured and blood was taken by caudal vein puncture using a heparinized syringe (2cc) for vitellogenin analysis. The blood was centrifugated (15 min at 4,000xg at 4°C) to obtain the plasma, which was transferred to plastic Eppendorf tubes treated with aprotinin (10 TIU/ml). The tubes were immediately stored at -80°C until further use.

Fish were killed by decapitation, organs (liver and gonads) were quickly excised and total body weight and the wet weights of the isolated liver and gonads were obtained. The gonadosomatic index (GSI) and the hepatosomatic index (HSI) from males and females were determined as gonad weight/total body weight x 100 and liver weight/total body weight x 100,

respectively. The total fish weight was used to determine the total condition index ($K = 10^5 \times \text{total weight} \times \text{length}^{-3}$).

4.1.2.5. Vitellogenin analysis

To quantify the vitellogenin, an enzyme-linked immunoassay (ELISA) method was used following a protocol described by Denslow et al. (1999).

Briefly, plasma samples were appropriately diluted (1:200 for males and 1:1000 for females) in coating buffer (phosphate buffered saline solution, PBS). Then samples were coated (50 μl /well) on Certified Maxisorp F96 polycarbonate immunoassay plates (Nunc-Roskilde, Denmark) and incubated overnight at 4°C. After washing microplate wells with PBS-Tween (pH 7.3), the residual binding sites were saturated by adding 250 μl blocking buffer (1% BSA in PBS) to each well and incubated overnight at 4°C. After further washing, the microplates were incubated overnight at 4°C with an anti-carp Vtg monoclonal antibody (G10/5/1), kindly furnished by the Laboratory of Immunology and Microbiology (LIM/ESE), University of Metz, France. Subsequently, plates were washed and the labeled secondary antibody (goat-antimouse immunoglobulin-IgG conjugated to alkaline phosphatase (SIGMA-Aldrich) diluted to the appropriate concentration (1:1000) in blocking buffer was added to wells and incubated for 2 hours at room temperature. Following this washing, the substrate (p-nitro-phenylphosphate – PNPP, SIGMA-Aldrich) was added (100 μl) to each microplate well and incubated at room temperature (10-30 min) in the dark. After adding the stop solution (50 μl , 3N NaOH), the plates were read in a 96-well microplate reader (BioRad-Benchmark, USA) at A405 nm.

The amount of VTG in samples was quantified using a calibration curve created by preparing serial dilutions of standard purified carp VTG (LIM/ESE, University of Metz) to provide a range from 10 to 1000 ngml^{-1} . A standard female plasma sample diluted to the required concentration to fall within the standard curve was chosen for interassay control and to ensure that every assay was operating correctly.

4.1.2.6. Histological examination

Samples of the removed organs (liver and gonads) were fixed in Bouin-Hollande's fixative for 48 h followed by dehydration through a series of graded ethanol solutions (30–70%) and toluene. The organs were embedded in paraffin and 5 µm sections cut and stained with hematoxylin and eosin for further histological analysis, using a Leica microscope (Leica-ATC 2000, Germany). The maturation stages were classified according to the scale proposed by Gupta (1975).

4.1.2.7. Statistical Analysis

Statistical analysis was performed with Statistica software (Statistica version 5.0; Statsoft Inc., USA). Data was checked for normality and variance homogeneity using Levene's test and, if necessary, was appropriately transformed (e.g. Log10) prior to analysis. One-way analysis of variance (ANOVA), followed by a Tukey test, was used on the results to compare the effect of different treatments on vitellogenin. The differences described in the results section were statistically significant at $P < 0.05$. To construct the standard curve and quantify vitellogenin measurements Microplate Manager 4.0 (BioRad Software, USA) was used, extrapolating absorbances to the standard curve.

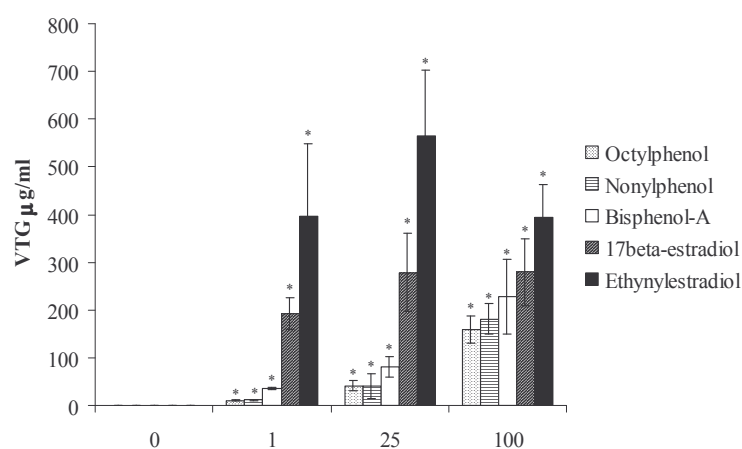
4.1.3. Results

The general water quality parameters monitored in the glass aquaria remained constant during the experiment. The cumulative mortality was less than 10% in each treatment group.

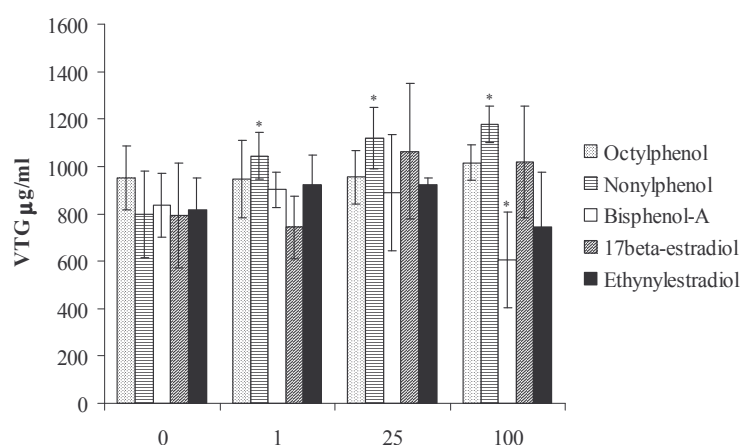
The analysis showed significant ($P < 0.05$) induction in Vtg concentration in all males exposed to all the different treatment groups (Fig. 4.1.3.1.a). Treatment with 25 and 100 µg l⁻¹ EE2 resulted in a major increase in Vtg, 565.4±137.9 and 394.0±68.8 µg ml⁻¹ (mean±sd) respectively. With regard to females, significant ($P < 0.05$) Vtg induction was observed in fish exposed to NP (1, 25 and 100 µg l⁻¹) and BPA (100 µg l⁻¹) in relation to the respective control (Fig.4.1.3.1.b).

Table 4.1.3.1. shows the K, GSI and HSI results according to the treatment groups for each single chemical. The significant differences ($P < 0.05$) observed between treatments and

controls are marked with an asterisk (*). The control fish results from the different experiment groups were also analyzed but no significant differences were detected between them.



(a)



(b)

Figure 4.1.3.1: Vitellogenin levels in males (a) and females (b) exposed to different single chemical treatment under laboratory conditions. Significant differences if $*(P<0.05)$.

No effects on the condition factor (K) were observed in fish, male or female, exposed to 1, 25 and 100 $\mu\text{g l}^{-1}$ of each chemical for 28 days. Similarly, no significant differences were observed in controls for each treatment (chemicals).

Treatment with OP, BPA, E2 and EE2 (1, 25 and 100 $\mu\text{g l}^{-1}$) or NP (25 and 100 $\mu\text{g l}^{-1}$) resulted in significant ($P<0.05$) reductions in the male gonadosomatic index (GSI) compared with the respective control value (Table I). With regard to females, treatment with NP and E2 (1, 25 and 100 $\mu\text{g l}^{-1}$), BPA and EE2 (25 and 100 $\mu\text{g l}^{-1}$) and OP (100 $\mu\text{g l}^{-1}$) resulted in significant differences in comparison with controls.

Table 4.1.3.1: Condition factor (K), gonadosomatic index (GSI) and hepatosomatic index (HSI) calculated for all treated groups.

Concentration	K		GSI (%)		HSI (%)	
	Male	Female	Male	Female	Male	Female
control	1.86±0.21	1.61±0.35	1.94±0.31	4.79±1.09	1.80±0.30	2.47±0.52
1 µgl ⁻¹ OP	1.89±0.43	1.90±0.31	1.62±0.23*	3.69±1.13	2.56±1.36	3.87±0.73*
25 µgl ⁻¹ OP	1.43±0.10	1.99±0.37	1.17±0.30*	3.45±0.98	1.63±0.25	3.76±1.05*
100 µgl ⁻¹ OP	1.73±0.26	1.84±0.26	0.67±0.57*	1.87±0.84*	2.91±1.25	3.90±1.00*
control	1.70±0.10	1.66±0.46	1.76±0.55	4.24±0.63	1.84±0.60	2.41±0.41
1 µgl ⁻¹ NP	2.11±0.50	1.93±0.50	0.72±0.08	2.69±0.22*	2.58±0.22*	2.80±0.41
25 µgl ⁻¹ NP	1.65±0.39	2.19±0.47	0.57±0.08*	2.59±0.54*	2.64±0.39*	2.86±0.33
100 µgl ⁻¹ NP	1.90±0.50	1.78±0.34	0.28±0.06*	2.56±0.40*	2.01±0.16	2.74±0.55
control	2.12±0.27	1.95±0.22	1.90±0.31	3.94±0.59	2.01±0.59	2.53±0.86
1 µgl ⁻¹ BPA	2.16±0.58	1.87±0.41	0.92±0.10*	3.48±0.50	2.50±0.16	2.50±0.80
25 µgl ⁻¹ BPA	2.12±0.41	2.88±1.84	0.80±0.10*	2.40±0.39*	2.54±0.39	2.51±0.33
100 µgl ⁻¹ BPA	2.88±0.66	2.40±0.46	0.78±0.30*	2.15±0.28*	2.19±0.87	2.52±0.24
control	2.17±0.28	2.55±0.42	1.98±0.22	4.70±0.76	2.46±0.56	2.55±0.50
1 µgl ⁻¹ E2	2.34±0.41	2.73±0.68	0.79±0.22*	3.12±0.55*	2.55±0.34	3.56±1.06*
25 µgl ⁻¹ E2	2.19±0.49	2.18±0.33	0.55±0.17*	2.65±0.39*	2.61±0.31	3.82±0.44*
100 µgl ⁻¹ E2	2.21±0.42	1.92±0.39	0.60±0.06*	2.68±0.40*	3.00±0.64	3.32±0.93
control	2.15±0.26	2.18±0.36	1.89±0.16	4.12±0.74	2.34±0.42	2.74±0.74
1 µgl ⁻¹ EE2	2.16±0.18	2.14±0.52	1.05±0.25*	3.16±0.69	2.60±0.82	3.05±0.33
25 µgl ⁻¹ EE2	1.72±0.27	2.06±0.20	0.89±0.44*	1.65±0.47*	2.62±0.65	2.82±0.20
100 µgl ⁻¹ EE2	2.14±0.80	1.96±0.39	0.85±0.22*	1.64±0.83*	2.39±0.44	2.77±0.46

A plot of the GSI index as a function of the plasma Vtg concentrations for all treated groups showed that these parameters ($P < 0.05$) had a significant negative relationship in all exposed males ($r = -0.59$, NP; $r = -0.56$, BPA; $r = -0.81$, OP; $r = -0.87$, E2; $r = -0.68$, EE2). With regard to females significant ($P < 0.05$) negative correlations were found in fish exposed to NP ($r = -0.66$) and E2 ($r = -0.33$).

No significant differences in the male hepatosomatic index (HSI) were observed for any of the OP, BPA, E2 or EE2 treated groups compared with controls in any of the experiments, although we can observe a tendency to increase (Table 4.1.3.1.). However, significant differences were found in the HSI for males exposed to 1 and 25 NP µgl⁻¹. Concerning female HSI, significant differences were observed for the fish exposed to 1, 25 and 100 OP µgl⁻¹ and to 1 and 25 E2 µgl⁻¹.

4.1.3.1. Histological observations

4.1.3.1.1. Controls

Testes from controls belonging to all groups were analyzed by light microscopy and no differences were detected among them. All the testes were normal in gross appearance and most of them in advanced stages of spermatogenesis. Control testes were classified at stage III (all features of active spermatogenesis could be observed, including spermatocytes, spermatids and spermatozoa) and IV (few spermatogonial cells around the periphery of the lobules, which are filled with spermatozoa). However, in a few cases testes classified at stage II (presence of spermatogonia and primary and secondary spermatocytes) were also detected.

Ovaries had a typical sacular structure limited by a thin gonad wall. They contained all stages of oocyte development, showing mostly maturing oocytes (Stage II) and advanced maturing oocytes (Stage III) but also a few small, young oocytes.

4.1.3.1.2. Treated

Histological examination of the testes revealed a similar dose-dependent pattern of effects though they varied in intensity according to the exposure chemical. The most severe effects on testicular structure were observed in fish exposed to steroids (E2 and EE2).

4.1.3.1.3. NP and OP

Histological observations showed that gonad growth is affected by NP exposure. The majority of the fish exposed to 25 and 100 $\mu\text{g l}^{-1}$ presented seminiferous lobules with reduced diameter and in some cases it was only possible to observe remains of spermatozoa. In most cases spermatogenesis seemed to be inhibited. In a few cases it was possible to observe the absence of lumina in the lobules, composed mostly of spermatogonia. Aggregates of melanomacrophage type cells were detected in 40% of the testes. Fish exposed to 25 and 100 $\mu\text{g l}^{-1}$ showed amorphous eosinophilic tissue around or in the seminiferous lobules. We also detected *foci* of lipidic cells within the cellular structure of testes. No intersex condition was noticed in males exposed to NP or OP.

Fish exposed to OP responded similarly to the NP treatment group, though the effects seem to be less pronounced and, at a lower concentration ($1 \mu\text{g l}^{-1}$), no observable effects were found. The testes exposed to 25 and $100 \mu\text{g l}^{-1}$ showed diminished seminiferous lobules, restricted or even completely absent spermatogenesis. No ovo-testes were observed.

4.1.3.1.4. BPA

Testes from the fish exposed to BPA showed identical changes to those in fish exposed to alkylphenols (NP and OP). However, it is difficult to assess if the effects were greater in intensity. Most of the fish exposed to 25 and $100 \text{ BPA } \mu\text{g l}^{-1}$ showed inhibited spermatogenesis and a decrease in the diameter of the seminiferous lobules, which contained remains of spermatozoa. Fibrous and amorphous eosinophilic tissue around seminiferous tubules was detected as well. No ovo-testes were found. Fish exposed to $1 \mu\text{g l}^{-1}$ BPA presented slight changes or no changes.

4.1.3.1.5. Steroids (E2 and EE2)

Histological analysis revealed progressively regressed testes in fish according to the E2 and EE2 concentrations. Fish exposed to all different EE2 and E2 concentrations showed spermatogenesis inhibition, with few spermatozoa and a reduction in the diameter of the seminiferous lobules. All exposed fish also showed vacuoles and fibrous and amorphous eosinophilic tissue around or in the seminiferous lobules and melanomacrophage aggregates within testis tissue. Also noticed was the presence of intersex in 10% of fish exposed to $25 \mu\text{g l}^{-1}$ and 20% in fish exposed to $100 \mu\text{g l}^{-1}$ E2 (Fig. 4.1.3.2): male tissue was scattered with oocytes. The intersex condition was also observed in 20% of fish exposed to 25 and $100 \mu\text{g l}^{-1}$ EE2.

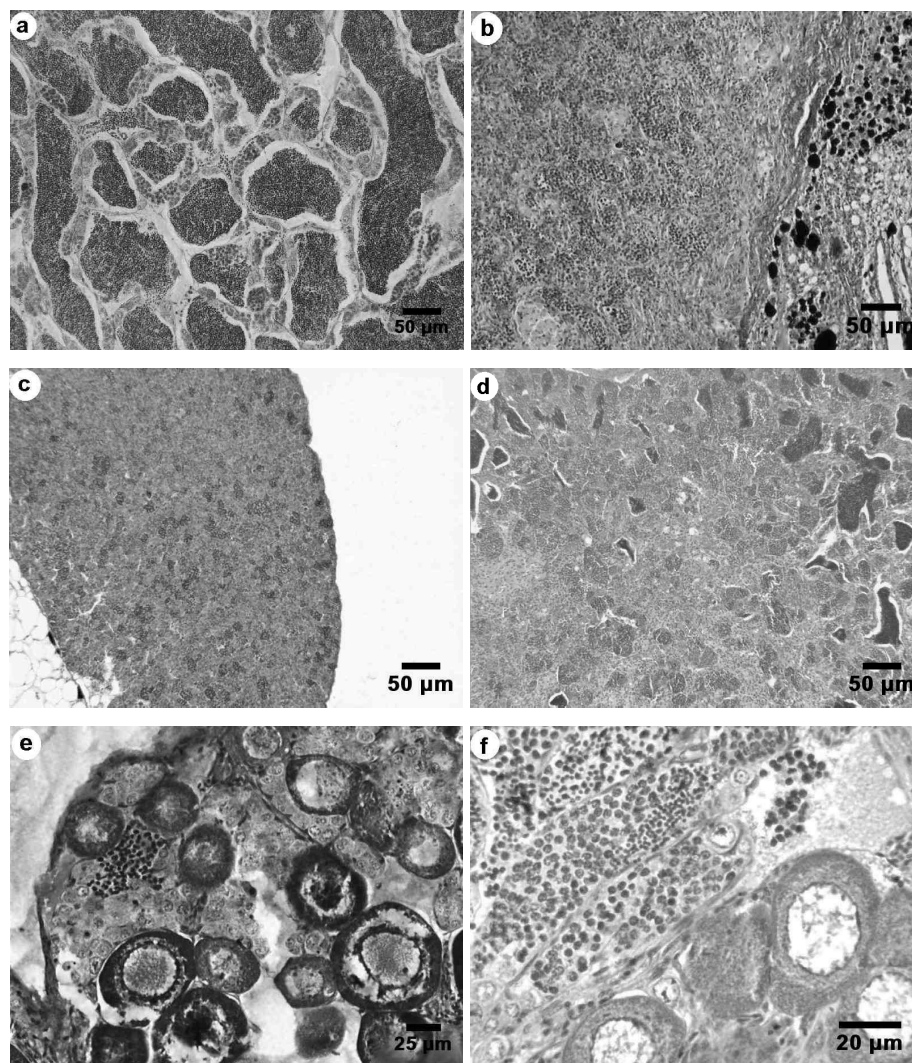


Figure 4.1.3.2: Testes histology in fish exposed to 100 µg l⁻¹ of each single chemical treatment (a: control; b: NP; c: OP; d: BPA; e: E2; f: EE2); section cuts: 5µm; colouration: H&E.

4.1.4. Discussion

Laboratory studies are used to determine the effects of exposure to a chemical and the range of concentrations over which the chemical causes effects (Sumpter, 1997). Vtg is widely used as biomarker of exposure to xenoestrogens (Nichols et al., 2001; Tilton et al., 2001) and numerous authors have demonstrated the induction of this protein in fish exposed to EDCs in laboratory and field studies (Denslow et al., 1997; Folmar et al., 1996; Heppell et al., 1995; Jobling and Sumpter, 1993; Pelissero et al., 1993; Sumpter and Jobling, 1995; Hemming et al., 2004; Ishibashi, et al., 2001; Christiansen et al., 1998; Purdom et al., 1994; Panter et al., 1998).

The chemicals tested were able to induce Vtg synthesis in males in a dose-dependent manner, indicating that all selected chemicals are estrogenic. However, the results suggest that they possess different estrogenic potencies. Steroids, such as E1, E2 or EE2, are commonly referred as the most potent endocrine disrupting chemicals (Sumpter and Jobling; 1995; Tyler and Routledge, 1998) and studies have indicated that concentrations of steroids such as E2 and EE2 in the water, as low as 0.1 ng l^{-1} , are sufficient to elicit endocrine disruption (Petrovic et al., 2001; Aerni et al., 2004). However, to date, few studies have been carried out to assess their estrogenic potency, which is still limited, and the results of various authors and different assays do not always agree (Petrovic et al., 2001). The results of the present work suggest that E2 and EE2 are the most potent EDCs, confirming other studies that have shown the high potency of E2 and EE2 in inducing Vtg (Rodgers-Gray et al., 2000; Bjerselius et al., 2001).

The presence of abnormal concentrations of Vtg in females has been advocated as a reliable and complementary biomarker of potential reproductive disturbances (Folmar et al., 2001; Solé et al., 2003). However, the present study shows that in general no significant variations were observed in female plasmatic Vtg, with the exception of females exposed to NP and $100 \mu\text{g l}^{-1}$ of the BPA. Similar results were found in female carp exposed to different dilutions of STP effluent, which did not show any significant disturbances (Rodgers-Gray et al., 2000; Solé et al., 2003).

The results suggest that alkylphenols (NP and OP) are weaker xenoestrogens. It was shown that alkylphenol ethoxylated (APE) metabolites can also act as a steroid mimic, although to a much lesser extent (Jobling et al., 1996). They show estrogenic potencies of three and more orders of magnitude lower than E2 and can produce estrogenic effects in fish in the microgram per liter range (Routledge and Sumpter, 1997; Thorpe et al., 2001; Ackerman et al., 2002).

However, despite showing less estrogenic activity than E2 or EE2, their persistence and the high concentrations found near STPs can cause estrogenicity problems in cyprinids and therefore be responsible for endocrine disturbances in fish (Purdom et al., 1994; Routledge et al., 1998; Folmar et al., 1996, 2001; Solé et al., 2003).

The laboratory evidence of the estrogenic potency of these chemicals provides valuable information with which to compare and understand the estrogenicity of municipal STP effluents that have been shown to contain natural estrogens and other estrogenic substances

that disturb reproduction in fish (Purdom et al., 1994; Sumpter and Jobling, 1995; Folmar et al., 1996, 2001; Lye et al., 1997; Harries et al., 1999; Nichols et al., 1999; Ternes et al., 1999; Korner et al., 2001). In addition, it has also been demonstrated that in many countries the human estrogens estrone (E1), estradiol (E2) and estriol (E3) and the pharmaceutical estrogen ethynylestradiol (EE2) are common constituents of sewage effluent (Desbrow et al., 1998; Larsson et al., 1999; Folmar et al., 2001).

The significant reduction in the GSI of males exposed to treatments can be the result of the estrogenic action of the chemicals which in turn affects gonad development. The GSI decrease seems to be related in a dose-dependent manner. The correlation analysis also showed a significant negative relationship between Vtg and GSI with regard to males in all treatment groups, which could indicate a direct association between Vtg induction and negative effects in male testes.

Similar results have been found by other authors. Bram (2004) showed a decrease in the GSI of male fish exposed to EE2 and Van den Belt (2001) also found a significantly reduced GSI in female zebrafish exposed to 10 ng l⁻¹ EE2 for 3 weeks. Lower GSIs were also observed in female fathead minnows exposed to 10 and 100 µg l⁻¹ µg NP for 3 weeks (Harries et al., 2000).

Laboratory studies have already demonstrated the estrogenicity of some EDCs. For instance, male flounder (*Platichthys flesus*) injected with NP (Christensen et al. 1998), zebrafish (*Danio rerio*) and juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to 4-tert-octylphenol (OP), 17α-ethynylestradiol, 4-nonylphenol, bisphenol A, and 17β-estradiol (E2) showed an increase in Vtg, though a difference in species sensitivity was found, with NP and OP being less sensitive than the other compounds (Van den Belt et al., 2003). Lindholm et al. (2001), measured high levels of Vtg in rainbow trout (*Oncorhynchus mykiss*) exposed to BPA in water. Moreover, Japanese medaka (*O. latipes*) exposed to octylphenol showed that reproduction of the exposed males was affected (Gray et al., 1999) and another study with the same species showed that exposure to OP induced a change in the sex ratio and ovotestis (Gray and Metcalfe, 1997; Knorr and Braunbeck, 2002).

The histological evaluation revealed that steroids caused the most pronounced effects in exposed males, although no severe changes were visible in the ovaries. The exposure to both steroids caused the inhibition of spermatogenesis and even complete regression of the testes.

In some cases the intersex condition was also detected. The effects caused by exposure to E2 and EE2 confirm findings from other authors, though different responses have been reported according to the doses administered and the stage of testicular development at the time of treatment (Tyler et al., 1998; Aerle et al., 2002; Van den Belt et al., 2003; Kang et al., 2002).

Although the nominal concentrations of E2 and EE2 used in the present study were higher than those normally found in the aquatic environment, they are a valuable tool that helps with the evaluation of gonadal histopathology.

The exposure to NP, OP and BPA also affected testes growth and in some cases inhibited spermatogenesis, though no ovotestes were found, which may support the evidence that they are less potent xenoestrogens. Similar studies with rainbow trout (*Oncorhynchus mykiss*) indicated that low $\mu\text{g l}^{-1}$ concentrations of NP inhibited testicular growth (Jobling et al., 1996; Arukwe et al., 2000b).

Studies carried out by Pastva et al. (2001) exposing medaka (*Oryzias latipes*) at environmentally relevant concentrations of BPA caused transient embryonic deformities. BPA is also known to bind competitively to sex steroid-binding protein in humans and rainbow trout and was found to have estrogenic activity in a yeast two-hybrid assay. High doses of BPA cause reproductive toxicity and affect cellular development in rats, while an apparent dose-response induction of Vtg and eggshell zona radiata protein levels have been observed in BPA-treated juvenile salmon (Ishibashi et al., 2001).

In vitro assays are also used to assess the estrogenic potency of suspected endocrine disruptor compounds. Recent in vitro assays, e.g. yeast estrogen screen (YES) and MCF-7 breast tumor cell proliferation (E-Screen)) tested the estrogenic potency of E2 and EE2 and compared it with studies on the in vivo production of male sheepshead minnow vitellogenin (Vtg). The in vitro tests were substantially less sensitive (at least 1000 times) than the sheepshead minnow Vtg assay for estimating the estrogenic potency of the two xenobiotic chemicals, which suggests that in vitro-based, large-scale screening programs could potentially result in many false negative evaluations. (Folmar et al., 2002).

4.1.5. Conclusions

The present study provides information on the estrogenic potencies of a group of chemicals that have estrogenic activity and can affect the physiology and reproduction of fish and other living organisms.

The results can thus contribute to a better understanding of the way EDCs function in the endocrine system of fish. This evaluation of the histological abnormalities caused by exposure to EDCs allows subsequent comparison with histological changes found in wildlife exposed to EDC-polluted environments.

Acknowledgments

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A biomarker and histological assessment of crucian carp (*Carassius carassius*) exposed to a complex mixture of xenoestrogens.

4.2. A biomarker and histological assessment of crucian carp (*Carassius carassius*) exposed to a mixture of xenoestrogens.

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Abstract

This work study the estrogenic effects of xenoestrogens in crucian carp (*Carassius carassius*). The mixture of five selected endocrine disruptor chemicals was prepared (nonylphenol, octylphenol, bisphenol A, 17 β -estradiol and 17 α - ethynylestradiol) and added to glass aquarium at different nominal concentrations (0.1, 1, 25 and 100 $\mu\text{g l}^{-1}$). Crucian carp, male and female, were then exposed to those concentrations during 28 days. The assessment of the estrogenic effects was carried out by measuring the plasma Vtg concentration and plasma steroid levels (11-Ketotestosterone and 17 β -estradiol) and carrying out a histological observation of the gonads. The results show that, even at the dose of a 0.1 $\mu\text{g l}^{-1}$ mixture of chemicals, Vtg was significantly induced in male fish. A significant decrease in the gonadosomatic index was also observed in males exposed to the mixture. Steroid concentrations revealed an inverse relationship between 11-Ketotestosterone and 17 β -estradiol in males, whereas elevated levels of 17 β -estradiol were also detected in exposed females. The most severe effects on the testes of exposed fish included spermatogenesis inhibition and the presence of an intersex condition, which were observed in all treatments.

Key words: vitellogenin, steroids, chemical mixture, intersex

4.2.1 Introduction

There are many environmental chemicals that may disrupt the endocrine system of organisms, affecting growth, sexual maturation and reproduction (Gimeno et al., 1998; Ackermann et al., 2002). They may act by modulating or mimicking the action of the endogenous steroid hormones, such as 17 β -estradiol (E2) (Routledge et al., 1998; Sumpter, 1995).

These chemicals, also known as endocrine disruptor compounds (EDCs), enter aquatic ecosystems through specific points (effluent from sewage treatment, industry and pulp mills) and non-specific points (urban and agricultural runoff) (Folmar et al., 2002).

The presence of significant concentrations of natural sex hormones, such as the endogenous E2 and estrone, and of synthetic steroids, such as 17 α -ethinylestradiol (EE2), in municipal treatment plants, are of particular concern in the risk assessment of endocrine disrupting compounds (Kramer et al.1998; Lopéz de Alda and Barceló, 2001). In addition to natural steroids, many anthropogenic chemicals have been identified as endocrine disruptor chemicals, including such chemicals as organochlorine pesticides, polychlorinated biphenyls, dioxins, alkylphenols, phthalates, bisphenol A and synthetic steroids that can be found in sewage treatment effluent and in the aquatic environment (Van den Belt et al., 2003; Kinnberg and Toft, 2003).

Steroidal estrogens (e.g. E2 and EE2) possess the highest estrogenic activity and have been shown to induce estrogenic effects in fish in the nanogram per liter range (Ackerman et al., 2002; Örn et al., 2003). Alkylphenols are persistent biodegradation products of the widely used non-ionic surfactants alkylphenolpolyethoxylates (APEs) that have properties mimicking the natural estrogen E2 (Christensen et al., 1999; Scott and Jones, 2000).

Alkylphenols (e.g nonylphenol (NP), octylphenol (OP)) are considered weak estrogens from the wildlife perspective, as demonstrated both in vivo and in vitro assays (Sumpter and Jobling, 1995; Routledge and Sumpter, 1997; Christiansen et al., 1998a,b). These compounds are commonly used in a wide variety of industrial, agricultural, and domestic products, including detergents, pesticides, paints, and cosmetics (Kinnberg and Toft, 2003). Bisphenol-A (BPA) is another compound that has estrogenic potency and is widely used in the production of epoxy resins and polycarbonate plastics (Toppari et al., 1995).

Traditional analytical instruments are used to perform chemical analyses of EDCs, singly and mixed or in complex mixtures from STPs and the environment. However, chemical analyses are not sufficient to assess the estrogenic contaminants present in the environment since they do not consider the biological aspects (Desbrow et al., 1998; Harries et al., 1997; Pawlowski et al., 2000; Petrovic et al., 2004). Therefore, it is necessary to use in vivo systems to assess the true environmental risk of potential endocrine disruptors.

In addition to documented EDCs, every year numerous new chemicals are synthesized and discharged into the environment without any knowledge of their estrogenic potential with regard to wildlife. Moreover, identification of all these new chemicals is impractical: hence, the assessment of estrogenic effects in complex environmental mixtures needs an integrative approach combining chemical analysis and biological assays. (Petrovic et al., 200, 2004).

Several studies have reported estrogenic responses in wildlife, showing vitellogenin (Vtg) induction and in some cases an increased prevalence of ovo-testis in male fish (*Rutilus rutilus*) near to or exposed to STPs effluents (Purdom et al., 1994; Sumpter, 1995). Further studies using caged rainbow-trout (*Oncorhynchus mykiss*) also showed Vtg induction and a decrease in male gonad growth in fish exposed to sewage treated effluent or downstream of a treatment plant (Harries et al., 1997; Sumpter et al., 1997). At that time the identity of the compound(s) was unknown, however, alkylphenolic chemicals were associated with the observed effects (Sumpter and Jobling, 1995; Harries et al., 1997; Christiansen et al., 1998a). Further laboratory studies have demonstrated the estrogenic effects of several suspected EDCs (Sumpter, 1997; Gimeno et al., 1998; Furhacker, et al. 2000; Arukwe et al., 2000)

To assess responses to estrogenic exposure in fish several endpoints are commonly used, such as developmental abnormalities of the gonads, the induction of Vtg and steroid concentrations in males (Folmar et al., 2002). Vtg is commonly used as a biomarker to assess estrogenic responses as it is an estrogen-dependent egg yolk phospholipo-glycoprotein that is secreted by the liver of oviparous vertebrates (Wallace, 1985). It is usually restricted to females but it can be induced in males and juveniles by exposure to estrogens (Sumpter and Jobling, 1995; Denslow et al., 1999).

The aquatic environment and obviously STP effluents contain complex mixtures of chemicals (Sumpter, 1997). However, the current scientific literature frequently reports on the effects of chemicals singly or in binary mixtures but there are no or few studies that have focused on mixtures of more than two chemicals. Thus, the purpose of this study is to investigate the effects of a mixture of five chemicals known as endocrine disruptors that are usually found in STPs receiving domestic and industrial effluents. Normal to high concentrations of chemical mixtures commonly found in STP effluent were used to assess endocrine disruption in fish.

In the study, crucian carp were exposed to $0.1 - 100 \mu\text{g l}^{-1}$ of a mixture of chemicals (NP, OP, BPA, E2, EE2). The results obtained with the present work are intended for future use – for comparison with exposure effects found in the field (near STPs). To attain this objective, Vtg induction, condition indices, histopathology and steroids were analyzed in the exposed fish.

4.2.2. Materials and Methods

4.2.2.1. Test fish

Both male and female crucian carp (*Carassius carassius*), sexually mature (aged approximately two years), were obtained from national producers (“Estação Aquícola da Azambuja” and “Aquário Vasco da Gama”). Their body weight was $8.4 \pm 1.5 \text{ g}$ and length $7.2 \pm 0.3 \text{ cm}$ (mean $\pm$ sd; N=90 x 2). Prior to the start of the experiment, the fish were acclimatized to laboratory conditions over a two-week period. The fish were maintained at $16 \pm 2^\circ\text{C}$ in a 300 L glass aquarium supplied with filtered, re-circulated, constantly aerated tap water, under a 12h light:12h dark photoperiod. They were fed daily with dry commercial flakes.

4.2.2.1.1. Test chemicals

The test chemicals 17β -estradiol (97% purity), 17α -ethynylestradiol (98% purity), 4-tert-Octylphenol (97% purity) and Bisphenol-A (99% purity) were obtained from Sigma-Aldrich (Germany) and 4-n-nonylphenol (96.4% purity) from Fluka Chemika (Germany).

A chemical stock solution was prepared by mixing all five compounds (MIX) at the appropriate concentrations (1.0 g l^{-1} MIX) using methanol (pro-analysis, Merck) as the solvent. Further spike solutions were prepared by diluting stock solutions into 1000 ml of distilled water and storing them at 4°C in the dark before adding them to the aquaria. The concentration of solvent in the experimental medium was less than 0.1%, as recommended by the Organization for Economic Cooperation and Development (OECD). Solvent control conditions were also tested. Nominal concentrations were in use throughout the experiments and were determined weekly, 24 hr after being added to each aquarium.

4.2.2.1.2. Exposure procedure

Fish were randomly distributed into six 50 liter glass aquarium (Fig.4.2.1), each supplied with 30L of de-chlorinated tap water and aerated (temperature: $15\pm 2^{\circ}\text{C}$; pH 7.4 ± 0.2 ; dissolved oxygen $>6 \text{ mg l}^{-1}$), under a constant photoperiod of 12h light:12h dark. They were fed with dry commercial flakes (Tetra) once a day. Mortality was also recorded.

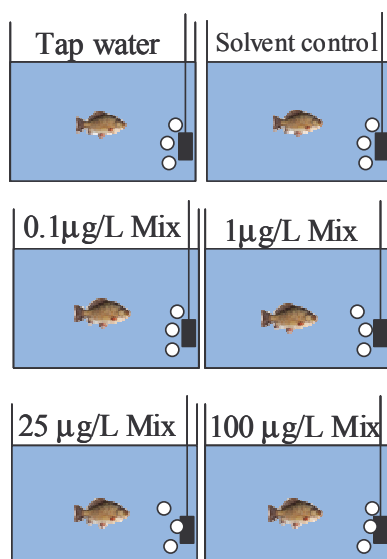


Figure 4.2.1: Experiment design.

Fish were exposed to tap water (control), solvent control (0.1% methanol) and nominal concentrations of 0.1, 1, 25 and 100 $\mu\text{g l}^{-1}$ MIX for a period of four weeks. During the experiment (4 weeks) the water and nominal concentrations of the chemicals were renewed every 48h in the aquarium.

At the end of the experiment, fish were weighed and measured. Blood was collected from the caudal vein using a heparinized syringe (2cc), transferred to Eppendorf tubes (treated with aprotinin) and maintained on ice. The plasma was obtained by centrifuging the blood for 15 min at $4,000\times g$ (4°C) and stored at -80°C until further analysis of Vtg.

Fish were killed by thermal shock on ice and dissected to remove the organs (liver and gonads) and then weighed. The male and female gonadosomatic index (GSI) and the hepatosomatic index (HSI) were calculated (to assess physiological effects of the chemical mixture) as follows: $\text{GSI} = (\text{gonad weight}/\text{total body weight}) \times 100$ and $\text{HSI} = (\text{weight}/\text{total}$

body weight) x 100. The total fish weight was used to determine the condition factor ($K = \text{total weight} \times 10^5 / \text{Length}^3$) for individual fish, to assess fish health.

4.2.2.1.3. Determination of vitellogenin

The ELISA assay was performed using a protocol based on the method described by Denslow et al. (1999). In this assay, purified Vtg developed by the Laboratory of Immunology and Microbiology (LIM/ESE) at the University of Metz, France, was used as standard after being tested. The standard Vtg was diluted to give a range of 10 to 1000 ngml⁻¹ and a calibration curve was constructed allowing the quantification of Vtg in plasma samples. After dilution, at least 1:200 for males and 1:1000 for females, the samples were pipetted (50 µl) in triplicate into a Maxisorp F96 microtiter plate (Nunc-Roskilde, Denmark) and incubated overnight at 4°C. After removing the coating reagent by inversion the plate was washed (three times) with PBS-Tween. Free binding sites in the wells were blocked by adding 280 µl blocking buffer (1% BSA in PBS-Tween) into wells and the plate was incubated overnight (4°C). After this, the plate was washed three times (PBS-Tween) and coated (50 µl) with a monoclonal antibody (G10/5/1) developed by the Laboratory of Immunology and Microbiology (LIM/ESE) at the University of Metz, France, and then incubated overnight at 4°C (or for 60 min at 38°C). After washing, a secondary antibody (goat anti-rabbit antibody) coupled with alkaline phosphatase (SIGMA-Aldrich) diluted to the appropriate concentration (1:1000) in a blocking buffer was added (100 µl) and incubated for 2h at room temperature (or 60 min at 38°C). After a final washing step, 100 µl of the substrate (p-nitro-phenylphosphate – PNPP, SIGMA-Aldrich) was added to each microplate well and incubated at room temperature (10-30 min) in the dark. Then the enzyme reaction was stopped by adding 50 µl of stop solution (3N NaOH). The plates were read in a 96-well microtiter plate reader (BioRad-Benchmark, USA) at 405 nm.

4.2.2.1.4. Plasma steroid analysis

Plasma concentrations of 11-Ketotestosterone (11-KT) and 17β-estradiol were measured using EIA Kits (Estradiol EIA Kit and 11-KT EIA Kit, Cayman Chemicals, USA), according to kit protocols provided by Cayman Chemicals (2002, 2003).

Both the assays are based on the competition between free estradiol or 11-KT and a tracer (estradiol linked to an acetylcholinesterase or 11-KT-acetylcholinesterase (AChE) molecule) for a limited number of estradiol or 11-KT-specific rabbit antiserum binding sites. The concentration of the estradiol tracer (or 11-KT tracer) is held constant while the concentration of free estradiol or 11-KT (standard or sample) varies. The amount of free estradiol (or 11-KT) tracer able to bind to the rabbit antiserum will be inversely proportional to the concentration of free estradiol (11-KT) in the well. This rabbit antiserum estradiol (or 11-KT) (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has previously been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. After developing in the dark (60 to 90 min.), the plates are read at 405 nm in a microplate reader. The assay results were calculated using a computer spreadsheet provided by Cayman Chemical for data analysis.

Plasma steroid concentrations were measured by constructing standard curves, using standard estradiol and 11-KT, by standard serial dilutions to a range between 7.8 and 1,000 pgml⁻¹, according to information furnished by Cayman Chemical (2002, 2003). To calculate the assay results and analyze data a computer spreadsheet provided by Cayman Chemical was utilized.

4.2.2.1.5. Analytical Chemistry

The chemical analyses were performed adopting a method from Weltin et al. (2000) and Mateus and Santana (2004). Water samples were extracted using SPE cartridges OASIS HLB 3cc (Waters), by passing samples at a flow rate of about 1 mLmin⁻¹ with the aid of a vacuum pump. Nonylphenol, octylphenol and bisphenol-A residues were eluted from the cartridge with an eluent consisting of 2 x 5.0ml methanol and 17β-estradiol, 17α-ethynylestradiol with 2 x 5.0 ml dichloromethane (9:1, v/v) eluent. The extracts were then concentrated by evaporating to 2 ml using a stream of nitrogen. After adding an internal standard (2,4,6 – Trimethylphenol, Fluka – Chemika), samples extracts were ready for HPLC analysis.

Analyses of chemicals were performed on an L-6200A HPLC system (Merck-Hitachi, Germany) equipped with a F-1050 fluorescence detector (Merck-Hitachi, Japan) and a Licrospher RP18 (5 μm) HPLC column), 4.0 x 125 mm (Merck). Operating conditions were: Ex 275 nm, Em 300 nm; column temperature, 40°C; flow-rate 1.0 mlmin⁻¹; mobile phase, A = water (ultra pure), B = acetonitrile: linear gradient. The detection limit in the concentrated

extract was 5 $\mu\text{g l}^{-1}$. Calibration curves were constructed with serial dilutions of standard solutions of the selected chemicals.

4.2.2.1.6. Histological examination

After removing organs (gonads and livers), samples were taken and fixed in Bouin-Holland solution for 48h. Samples were routinely processed: after dehydration in ethanol series they were embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. Sex was determined and maturity stages of the gonads of both males and females were evaluated by light microscopy on a classification scale proposed by Gupta (1975) for cyprinids.

4.2.2.1.7. Statistical Analysis

All data were tested for normality and variance homogeneity. If these assumptions were not accomplished then data were transformed to obtain normality and homogeneity of variances. One-way analysis of variance (ANOVA) followed by a Tukey post-test was performed on the results to make comparisons among treatments. Correlation analysis (Pearson) was carried out to examine the significance of the relationships among vitellogenin (Vtg) concentrations and GSI, HSI and steroid (11-KT and E2) concentrations. Statistical analysis was performed with Statistica software (Statistica version 5.0; Statsoft Inc., USA). To construct a standard curve and quantify Vtg measurements Microplate Manager 4.0 (BioRad Software, USA) was used. Absorbances were extrapolated to the standard curve.

4.2.3. Results

Cumulative mortality observed throughout the experiment showed that more than 90% of fish survived in each treatment. The nominal and determined concentrations of test chemicals added to the aquarium water are presented in Table 4.2.1.

Table 4.2.1: Measured concentrations of test chemicals in aquarium water.

Test Chemicals	Nominal/ measured concentrations ($\mu\text{g l}^{-1}$)			
	0.1	1	25	100
Nonylphenol	0.06 $\pm$ 0.01	1.10 $\pm$ 0.20	20.60 $\pm$ 2.61	89.43 $\pm$ 8.00
Octylphenol	0.07 $\pm$ 0.01	1.37 $\pm$ 0.25	23.22 $\pm$ 0.78	89.05 $\pm$ 5.10
Bisphenol A	0.08 $\pm$ 0.02	1.29 $\pm$ 0.37	27.30 $\pm$ 1.00	107.26 $\pm$ 7.12
17 β -estradiol	0.03 $\pm$ 0.01	0.58 $\pm$ 0.16	19.87 $\pm$ 1.62	83.24 $\pm$ 12.32
17 α -estradiol	0.03 $\pm$ 0.02	0.59 $\pm$ 0.17	20.15 $\pm$ 3.63	92.09 $\pm$ 6.05

The Vtg levels of plasma from male and female fish exposed to different concentrations of a mixture of selected chemicals are presented in Figure 4.2.2. A minimum exposure of nominal 0.1 $\mu\text{g l}^{-1}$ MIX was sufficient to induce Vtg synthesis in male crucian carp. The analysis showed a significant ($P < 0.05$) increase in Vtg concentration for all treated male fish (Fig. 4.2.2a) comparatively to controls. A maximum response (254.54 $\pm$ 36.89 Vtg $\mu\text{g ml}^{-1}$) was found in males exposed to 100 $\mu\text{g l}^{-1}$ MIX and a dose response was found as well. Statistical analysis showed that Vtg induction in response to MIX treatments was significantly correlated ($r = 0.76$, $P < 0.05$) with the GSI decrease in male fish. No statistical differences were found between controls and solvent controls. No significant changes were observed in plasma Vtg from females exposed to MIX treatments.

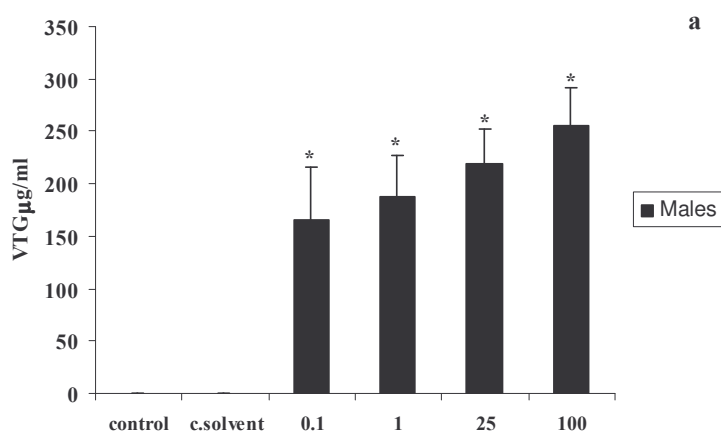


Figure. 4.2.2 (a)

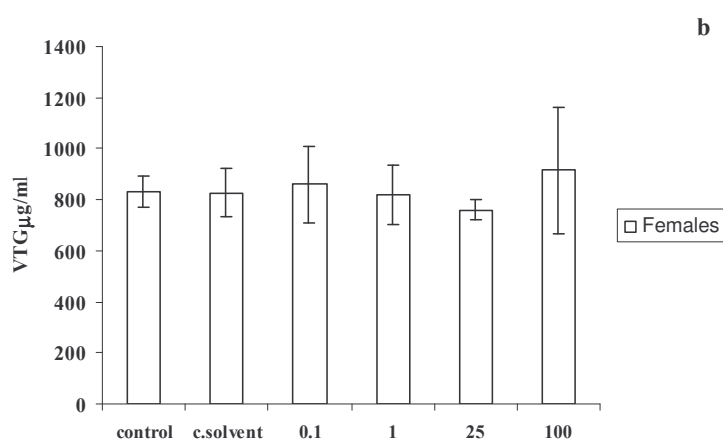


Figure. 4.2.2: Plasma vitellogenin concentrations in *C. Carassius* exposed to different concentrations of a mixture of chemicals. Significant differences if * $P < 0.05$. (a) males; (b) females.

Table 4.2.2 presents the results for the gonad, liver and condition indices in control and treated fish. Fish health was assessed by calculating the condition factor (K). The measured K values from males exposed to $100 \mu\text{g l}^{-1}$ were significantly lower than those of control fish, which may indicate an increase in toxicity as treatment concentrations increase. Regarding females, no significant differences were found to K values. The lowest GSI and the highest HSI values were found in male fish exposed to $100 \mu\text{g l}^{-1}$ MIX. HSI data analysis revealed that significant differences were found between male fish exposed to $100 \mu\text{g l}^{-1}$ MIX and controls. With relation to females, no significant differences were found among treatments and HSI.

Table 4.2.2: Indices values (GSI and HSI) calculated in fish exposed to MIX treatments. Significant differences if (* $P < 0.05$).

	K (mean $\pm$ sd)		GSI (mean $\pm$ sd)		HSI (mean $\pm$ sd)	
	Male	Female	Male	Female	Male	Female
Control	2.32 $\pm$ 0.25	2.35 $\pm$ 0.28	1.98 $\pm$ 0.26	3.89 $\pm$ 0.59	1.70 $\pm$ 0.35	2.19 $\pm$ 0.19
Control solv.	2.30 $\pm$ 0.11	2.20 $\pm$ 0.20	1.87 $\pm$ 0.24..	4.01 $\pm$ 0.67	1.67 $\pm$ 0.29	2.24 $\pm$ 0.19
0.1 $\mu\text{g l}^{-1}$	2.32 $\pm$ 0.14	2.27 $\pm$ 0.22	0.85 $\pm$ 0.08*	3.12 $\pm$ 0.96	2.08 $\pm$ 0.85	2.31 $\pm$ 0.41
1 $\mu\text{g l}^{-1}$	2.28 $\pm$ 0.42	2.38 $\pm$ 0.37	0.77 $\pm$ 0.09*	2.82 $\pm$ 0.69	2.12 $\pm$ 0.96	2.34 $\pm$ 0.11
25 $\mu\text{g l}^{-1}$	2.22 $\pm$ 0.26	2.50 $\pm$ 0.30	0.62 $\pm$ 0.10*	...3.92 $\pm$ 1.71	2.53 $\pm$ 0.96	2.37 $\pm$ 0.97
100 $\mu\text{g l}^{-1}$	2.07 $\pm$ 0.13	2.42 $\pm$ 0.32	0.40 $\pm$ 0.19*	..2.63 $\pm$ 0.26	3.84 $\pm$ 1.12*	3.35 $\pm$ 0.45

The data analysis revealed a significant decrease ($P < 0.05$) in the male GSI (Table 4.2.2) for all treatments. In addition, significant differences were found in the male GSI between fish

exposed to $100 \mu\text{g l}^{-1}$ and 0.1 and $1.0 \mu\text{g l}^{-1}$. With regard to females no significant differences were found.

A significant increase ($P < 0.05$) was observed in male E2 plasma concentrations in comparison with controls, while a significant decrease ($P < 0.05$) was observed in plasma 11-KT measured in all experiment treatments (Fig. 3 a and b). Regarding male mean E2 and 11-KT concentrations, they ranged from 10.3 to $9308.8 \text{ pg ml}^{-1}$ and from 1658.7 to 82.7 pg ml^{-1} , respectively.

With reference to females, the lowest mean concentration of plasma E2 was $2464.6 \text{ pg ml}^{-1}$ and the highest $12894.5 \text{ pg ml}^{-1}$. Significant increases in E2 concentrations were observed in all treatments with respect to controls, with the exception of females exposed to $0.1 \mu\text{g l}^{-1}$. The E2 concentrations were higher in females than in males (Fig. 4.2.3b). The statistical analyses of female 11-KT concentrations at the different MIX concentrations, revealed no significant differences among treatments.

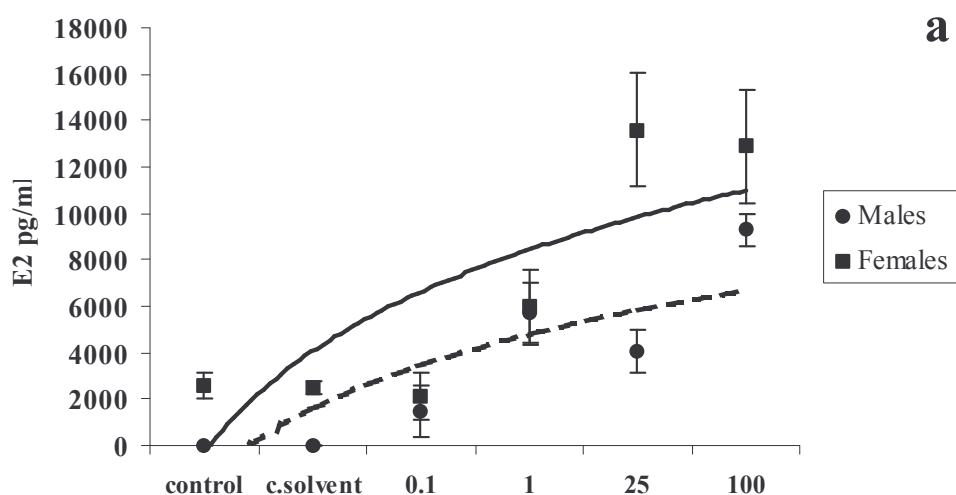


Figure 4.2.3 (a).

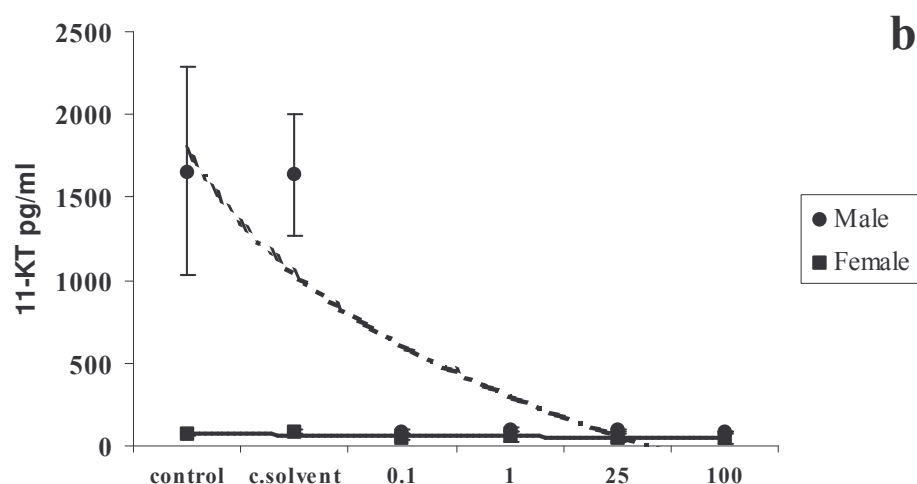


Figure 4.2.3. Trend lines of the steroids concentration E2 (a) and 11-KT (b) in fish exposed to different doses of a mixture of chemicals.

4.2.3.1. Histological observations

The histological gonad observations covered the maturation stage, oogenesis and spermatogenesis, respectively, according to a classification proposed by Gupta (1975).

The testes of control fish presented a normal appearance, showing clumps or cysts of spermatogonial cells at the lobule periphery and spermatocytes, spermatids and spermatozoa towards the interior. Some fish seemed to be at more advanced stage of spermatogenesis, with spermatids and spermatozoa visible. Fish were classified as at the maturing and advanced maturation stages.

The major effects observed in male fish exposed to different chemical mixture concentrations are shown in Table 4.2.3.

Table 4.2.3: Occurrence of main effects observed in treated male fish (N= total males found in each treatment).

Treatment($\mu\text{g l}^{-1}$)/observed effects	0.1 (N=8)	1 (N=8)	25 (N=6)	100 (N=6)
Diameter of seminiferous lobules reduced or absent	6	6	4	4
The lobules lumen contains remains of spermatozoa	2	2	2	4
Absence of lumen in the lobules, composed mostly of spermatogonia	2	2	2	4
Degeneration of the seminiferous lobules structures	4	4	4	6
fibrous and amorphous eosinophilic tissue around seminiferous lobules	4	2	2	5
Presence of melanomacrophages	4	4	4	4
Presence of ovo-testes	nd	2	2	2

Few testes of the treated male fish showed a normal appearance. The major effects observed in most treated testes included a restriction of spermatogenesis and a diminishing of the diameter of most lobules.

All male fish exposed to treatments presented similar effects, though the severity of the effects seems to increase with treatment concentrations. For instance, male fish exposed to $100 \mu\text{g l}^{-1}$ showed the most severe effects, such as an intersex condition (Fig. 4.2.4b).

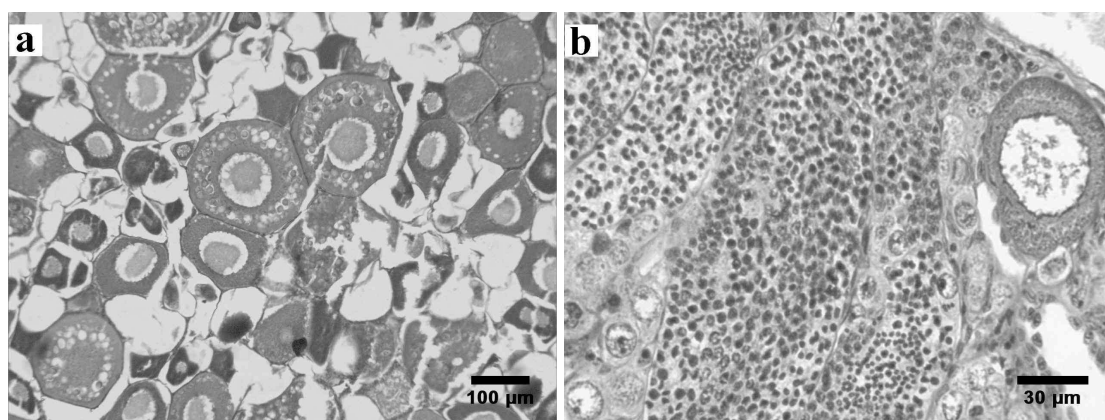


Figure 4.2.4. Ovary of female (a) and inter sex condition in testis (b), both exposed to $100 \mu\text{g l}^{-1}$ Mix.

With respect to treatments (0.1 ; 1 ; 25 ; $100 \mu\text{g l}^{-1}$ MIX), in a few cases it was observed that spermatogenesis was inhibited and the seminiferous lobules were composed mostly of spermatogonia. Degeneration of the structures of the seminiferous lobules, fibrous and amorphous eosinophilic tissue and yellowish groups of cells with melanin or not (melanomacrophages) were also detected in all treatments.

Most of the control females were classified at stages II and III, i.e. they were showing maturing oocytes and advanced maturing oocytes, respectively. A few mature oocytes were also observed. After 28 days of exposure to MIX, histological observation of ovaries revealed that they were normal in gross appearance and all stages of oocyte development, though atretic follicles were also detected in all experiment treatments. As with controls, the ovaries of exposed females were classified at stages II and III (Fig. 4.2.4b).

4.2.4. Discussion

The synthesis of measurable quantities of Vtg in male fish exposed to xenoestrogens is commonly used as a biomarker of estrogenicity (Sumpter, 1995; Folmar et al., 2001b). The results show that Vtg is significantly induced in males exposed to all treatments of the chemical mixture and that Vtg induction occurs even at the lowest mixture concentration ($0.1 \mu\text{g l}^{-1}$).

According to the current scientific literature, fish exposed to sewage effluents containing mixtures of environmental chemicals register Vtg induction and, in the case of males, severe testicular effects, such as the presence of oocytes within testis tissue (Sumpter, 1997; Tyler and Routledge, 1998).

The natural hormone E2, from human waste, and the synthetic estrogen EE2, used in oral contraceptives, are considered as two of the most potent estrogenic compounds, as demonstrated in *in vitro* assays for estrogenic activity (Soto et al., 1995; Coldham et al., 1997) and *in vivo* studies (Scholtz and Gutzeit, 2000; Van den Belt, 2002, 2003).

In this study, most probably the major estrogenic effects were caused by action of the steroids E2 and EE2, the most potent estrogens present in the chemical mixture. In addition, these chemicals have been shown to induce estrogenic responses in fish at low concentrations in water ($0.1 - 1 \text{ ng l}^{-1}$), lower than those usually found in the environment (ng l^{-1}) (López de Alda and Barceló, 2001; Kramer et al., 1998).

With regard to females, no significant differences were detected in Vtg induction, which may suggest that females are apparently less affected by exposure to the xenoestrogen mixture or the duration of the experiment was too short. Most of the studies that use Vtg as a biomarker

have been restricted to males, though it has been shown that females are also likely to be affected by xenoestrogens and, therefore, Vtg measurement can be used as a reliable and complementary biomarker of potential endocrine disturbance (Kime et al., 1999; Folmar et al., 2001a). Nevertheless, the use of Vtg as a biomarker of exposure to EDCs in females is complicated by natural seasonal variations in protein levels (Solé et al., 2003).

Studies using mixtures of EDCs either do not exist or are insufficient, which makes comparisons difficult. However, laboratory studies using single or binary mixtures are a valuable aid to understanding the effects and mechanisms of EDCs in organisms. For example, laboratory studies have showed that Vtg synthesis has been significantly induced in various fish species as a response to BPA exposure (Christiansen et al., 2000; Lindholm et al., 2000, 2001; Segner et al., 2003), in male fathead minnows (*Pimephales promelas*) exposed to NP (Pickford et al., 2003), in flounder (*Platichthys flesus*) (Madsen et al., 2003) and rainbow-trout (*Oncorhynchus mykiss*) (Pedersen et al., 2003) exposed to dietary 4-tert-octylphenol, in prespawning adult male and female carp (*Cyprinus carpio*) injected with a single dose of EE2 (Solé et al., 2000) and in male and female fathead minnows (*Pimephales promelas*) exposed to different EE2 concentrations (Pawlowski et al. 2004). Exposure of zebrafish embryos (*Danio rerio*) to 17 α -ethynylestradiol for 3 months also resulted in an increase in total body Vtg content, a reduction in total body weight and an increase in morphological abnormalities. A function of exposure time was also found (Van den Belt et al., 2003).

The present study registered a significant decrease in the male GSI. Pawlowski et al. (2004) also registered a decrease in the GSI after exposure of mature male and female fathead minnows (*Pimephales promelas*) to EE2 and Kinnberg et al. (2000) showed a reduction in the GSI in platyfish (*Xiphophorus maculatus*), dose-dependent, after 28 days' treatment with nonylphenol. In addition, testes were negatively affected, showing pronounced effects at the highest concentrations of nonylphenol. Christiansen et al. (1998a) showed a GSI reduction in eelpout (*Zoarces viviparus*) exposed to nonylphenol and E2, though the mechanisms whereby nonylphenol causes the observed effects on the testes are still unknown. A possible explanation is that direct or indirect effects on the Sertoli cells may result in testis changes.

In the present study no significant differences were found in female GSI, probably because they are less affected by xenoestrogen exposure or need longer exposure before the xenoestrogens produce negative effects.

Significant differences were found in the HSI of males exposed to $100 \mu\text{g l}^{-1}$, which shows a rising trend in all treatments. These results agree with other studies, which have demonstrated an increase in HSI in male flounder (*Platichthys flesus*) after exposure to water containing nonylphenol, and with field surveys, where flounder have showed high Vtg content and had correspondingly high HSI values (Matthiessen et al., 1998; Allen et al., 1999). However, exposure to water containing 4-tert-octylphenol in eelpouts (*Zoarces viviparus*) resulted in no changes in HSI, even though there was a significant induction of Vtg (Christiansen et al., 1998a).

This study shows that fish exposed to treatments exhibited severe testicular effects, confirming findings from other studies that used different species (Tyler and Routledge, 1998; Gimeno et al., 1998; Panter et al., 1998).

The spermatogenesis inhibition and/or the intersex condition observed in fish exposed to the chemical mixtures confirm other findings, which have found spermatogenesis inhibition in adult male rainbow trout (Jobling et al., 1996) and the development of testis-ova in Japanese medaka (*Oryzias latipes*) (Gray et al., 1999).

Steroid 11-KT concentrations in male plasma showed a significant dose-dependent decrease with regard to treatments with the MIX chemicals. At the same time a significant increase was registered in male E2 plasma concentrations. Significant differences were also found in female E2 concentrations, which increased in a dose-dependent manner. Females exposed to $100 \text{ E2 } \mu\text{g l}^{-1}$ had mean plasma concentrations about five times greater than those of the controls.

Experiments with goldfish (*Carassius auratus*) exposed to different concentrations of E2 for 28 days showed that male goldfish reproductive behavior and physiology were severely affected as a decrease in 11-KT concentrations was observed and it was suggested that the mechanisms behind the altered male behavior are probably related to the effects of E2 on the testes, resulting in decreased androgen production (Bjerselius et al., 2001). Nichols et al. (2001) suggested that a reduction in male plasmatic T is the consequence of treatment blocking.

Studies carried out by Condeça and Canario (2001) showed testis regression but no significant changes in ovary development after treatment with diet E2 in sea bream (*Sparus aurata*).

Furthermore, fish walleye (*Stizostedion vitreum*) collected from Mississippi River locations below an STP also exhibited male depressed serum T concentrations and elevated serum 17 β -estradiol (E2) whereas females had elevated serum E2 levels but no significant differences were found in T levels. These results were attributed to the estrogenic release from STP effluent (Folmar et al., 2001a). Thus, the altered serum concentrations of reproductive steroids can indicate changes in synthesis, plasma storage or hepatic degradation (Guillette et al., 1999).

However, in an extensive study with carp conducted in the USA, besides the Vtg response, no other alterations were observed (Goodbred et al., 1996). Moreover, in addition to the reported effects, estrogenic exposure can also cause alterations to the xenobiotic metabolizing capacity of fish, as experiments with the natural estrogen E2, the synthetic estrogen EE2 (Solé et al., 2000) and the xenoestrogen *p*-nonylphenol (NP) (Arukwe et al., 1997; Thibaut et al., 2002) have shown. Studies carried out by Solé et al (2003) in teleost fish, such as carp, showed that an increase in Vtg could be the result of a plasmatic E2 increase, although this was not always apparent since E2 and T exhibited high individual variations.

4.2.5. Conclusions

It is known that the aquatic biota is exposed to a mixture of chemicals rather than any single one. Therefore, this study shows the relevance of studying mixtures rather than single chemicals, which reflects a more realistic aquatic environment model. In addition, it has demonstrated that low levels of chemical mixtures are able to disrupt the endocrine system of fish. The results are also useful for future comparisons involving effects identified in field studies where chemical mixtures occur. Finally, this work provides a valuable contribution to our knowledge of the histological effects and mechanisms of endocrine disruption, a contribution that can be useful for further studies.

Acknowledgments

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Bioaccumulation and effects of 4 n-nonylphenol in *Cyprinus carpio*. Preliminary Results

4.3. Bioaccumulation and effects of 4 n-nonylphenol in *Cyprinus carpio*. Preliminary Results

M.S. Diniz & I. Peres

Abstract

The presence and identification of xenoestrogens, such as alkylphenols, in urban wastewater treatment plants and its possible relationship with endocrine disruption in fish and the study of the effects and distribution of these compounds in organisms are of major interest. Additionally there is little information concerning this issue. In this study, four groups of carp (*Cyprinus carpio*) were randomly distributed in three aquaria, under a semi-static regime and exposed to 0, 25 and 100 $\mu\text{g l}^{-1}$ of 4-n-nonylphenol concentrations during 28 days at a constant temperature ($15\pm 1^\circ\text{C}$). Levels of nonylphenol in the liver, kidney, gonads and muscle were determined using an HPLC system with a fluorescence detector. Organ samples were also collected to carry out histological analyses and evaluate nonylphenol effects at tissue level. The results show that the organisms bioaccumulated increasing levels of nonylphenol, according to the experimental treatment. In addition, the histological results show histopathological effects in *C. carpio*.

Key words: *Cyprinus carpio*, Nonylphenol, gonad, endocrine disruption

4.3.1. Introduction

The OECD (Organisation for Economic Cooperation and Development) defined an endocrine disruptor compound (EDC) as an exogenous substance that causes adverse health effects in living organisms, and their progeny, as a consequence of alterations in endocrine function (Jounay, 2000). A vast group of chemicals is already known to cause or is suspected of causing endocrine disruption, e.g. the non-ionic surfactants alkylphenol polyethoxylates (APEOs).

APEOs have been widely used in the last 50 years in a vast array of commercial and domestic applications (Tsuda et al., 2000), for example as emulsifiers in industrial and domestic cleaning products (Nichols et al., 2001). APEOs are degraded successively by STP (sewage

treatment plant) processes to form less biodegradable compounds, e.g. NP (nonylphenol), before being discharged into the aquatic environment.

Recent studies have shown that NP is one of the most potent metabolites of APEO degradation (Arukwe et al., 2000; Nichols et al., 2001), as it has a similar structure to the endogenous estrogens in fish and can, consequently, act by deregulating their endocrine system.

It has been suggested that the disruption of endocrine homeostasis due to exposure to weak agonist estrogens, such as nonylphenol or octylphenol, can compromise the reproductive success and even survival of wildlife (Nichols et al., 2001).

Thus, studies performed in various fish species exposed to NP have shown various anomalies, such as morphological and histological changes in gonads (Arukwe et al., 2000; Schwaiger et al., 2000). In addition, it has been shown that alkylphenols such as NP have the capacity to induce vitellogenin synthesis in male and juvenile bony fish, e.g. the rainbow-trout (*Oncorhynchus mykiss*) (Jobling et al., 1996) or carp (*Cyprinus carpio*) (Gimeno et al., 1998).

These alterations have often been associated with wastewater discharges, though it has not been possible to establish a correlation with a specific compound. It is more probable that several compounds are responsible for the adverse effects observed in fish and other living organisms (Jounay, 2000).

There is little information available on the effects and presence of APEOs in the aquatic environment. Thus, since NP possesses estrogenic activity, it is of the greatest importance to study its adverse effects and the degree of bioaccumulation with respect to living organisms, particularly at the reproductive level. The main objective of this study is to contribute to a better comprehension of the bioaccumulation and adverse effects of NP in fish.

4.3.2. Materials and Methods

4.3.2.1. Exposure Tests

Sexually mature carp (*Cyprinus carpio*) (N=12, mean weight: 14.59±2.36g) were randomly distributed into three groups and maintained in 50 liter aquaria containing tap water from

which the chlorine had been removed. The fish were kept under a constant temperature of $15\pm 1^{\circ}\text{C}$ and subjected to a photoperiod regime of 16 hours light: 8 hours dark. The animals were fed daily with pellet food (Alpis).

The water in the aquarium was contaminated with nominal concentrations of 0, 25 and $100\ \mu\text{g l}^{-1}$ of 4-n-Nonilfenol (Fluka, Chemika 74430). During the experiment period (28 days) the water in the aquarium was renewed and contaminated with the different concentrations of NP.

After 28 days the fish were sampled, weighed and measured and the organs removed (liver, gonads, kidney and muscle). The size of the gonads was expressed as a percentage of the total body mass (Gonadosomatic Index – GSI). Sub-samples of the organs were taken and placed in Bouin's fixative for 48 hours. The organs were then processed using the usual histological techniques for light microscopy examination. Other sub-samples were stored at -80°C until further analysis of the NP concentrations in fish tissue.

4.3.2.2. Determination of NP in tissues

Sub-samples of the organs (liver, gonad, muscle and kidney) weighing 300 mg were homogenized in 3 ml of acetonitrile (Riedel de-Haen, Germany). The homogenate was centrifuged at $3,000\times g$ during 10 minutes and supernatant removed and filtered (Machery-Nagel, with porous of $0.45\ \mu\text{m}$ for HPLC analysis).

The determination of nonylphenol concentrations in the organs was performed by a HPLC system (Merck-Hitachi, L-6200A), using a column (Merck) of $4.0\times 125\ \text{mm}$, Licosphere RP18 ($5\ \mu\text{m}$) equipped with fluorescence detector (Merck-Hitachi, F-1050).

4.3.3. Results and discussion

Figure 4.3.1 shows the nonylphenol content in some of the tissues of the exposed fish. The liver shows the highest concentrations, which suggests the possibility that the liver plays a major role in the NP bioaccumulation and metabolizing process in *Cyprinus carpio*.

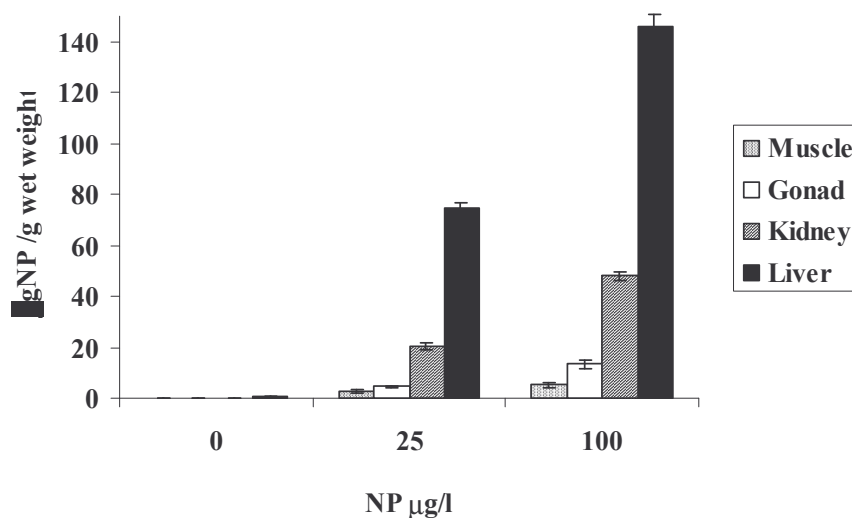


Figure 4.3.1: NP levels ($\mu\text{g g}^{-1}$ wet weight) in muscle, kidney, gonads and liver of carp, exposed to different concentrations of NP (0, 25 and $100 \mu\text{g l}^{-1}$).

Figure 4.3.2 shows a slight decrease in the gonadosomatic index (GSI) of fish exposed to the different treatments. The reduction in the gonad weight seems to be dose-dependent.

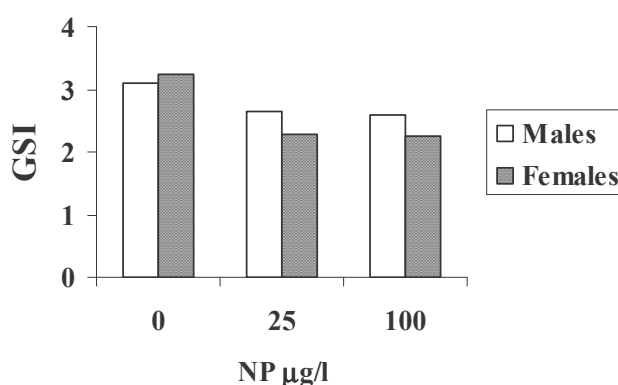


Figure 4.3.2: Gonadosomatic Index of carps exposed to 0, 25 and $100 \mu\text{g l}^{-1}$ of NP.

Although the reduction in the GSI observed in the fish was not statistically significant, it may, however, indicate the adverse effects of male gonad exposure to NP.

The light microscopy examination revealed that the testes of the fish exposed to the nominal concentrations of $100 \text{ NP } \mu\text{g l}^{-1}$ show morphological alterations in the gonads (Fig. 4.3.3).

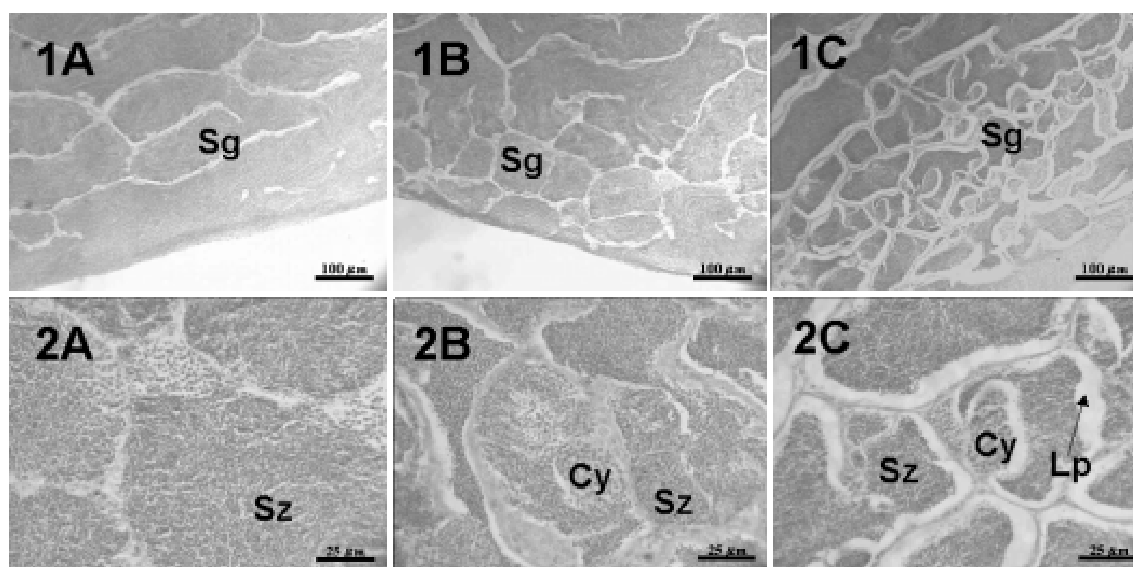


Figure 4.3.3: *Cyprinus carpio* gonad (male), showing the seminiferous lobules in controls (1A-2A) and in the animal exposed to 25 and 100 $\mu\text{g l}^{-1}$ (1B-2B and 1C-2C, respectively). Sg (seminiferous lobules); cy (spermatogenic cysts); Sz (spermatozoa); Lp (peripheral lacuna - arrow). Magnification 100x (1A, 1B, 1C); Magnification 400x (2A, 2B, 2C). Coloration H&E (cuts 5 μm).

The histological analysis of the testes shows that spermatogenesis remains active in exposed fish, though a decrease in the number of Sertoli cells seems to occur. In addition, the existence of peripheral lacunae (Lp) should be noted, possibly caused by the reduction in Sertoli cells.

The results thus show that for concentrations of 100 $\mu\text{g l}^{-1}$ morphological and histological changes seem to take place in the fish gonads, which in turn may cause reproductive dysfunction.

The results also show the importance of and the need for more studies on the detection of these compounds in the aquatic environment, as well as in STP effluents, as the latter constitute a great source of EDC into the aquatic ecosystem.

There is also a need to carry out long term exposure tests, with lower concentrations that simulate the aquatic environment, in order to assess the possible long-term effects on exposed fish.

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PART V

CHAPTER 5 – SEMI-FIELD ASSAYS AT THE SEWAGE TREATMENT PLANT OF CHELAS

Estrogenic effects in crucian carp (*Carassius carassius*) exposed to treated sewage effluent.

5.1. Estrogenic effects in crucian carp (*Carassius carassius*) exposed to treated sewage effluent.

M.S. Diniz, I. Peres, I. Magalhães-Antoine, J. Falla and J.C. Pihan

Abstract

In order to assess the estrogenicity of a municipal sewage treatment plant, sexually mature crucian carp of both sexes were exposed for 28 days to domestic treated sewage effluent running with a constant flow of water, with tap water and with different concentrations of the sewage effluent (25, 50 and 100 percent). Vitellogenin (Vtg), gonadosomatic index (GSI), hepatosomatic index (HSI) and histological abnormalities were used to assess the estrogenic potency of the effluent. Results show a significant ($P<0.05$) increase in Vtg levels in all exposed male fish. A significant ($P<0.05$) reduction in the GSI was only observed in fish exposed to 100 percent effluent. Morphological changes were detected by histological evaluation revealing severe effects on the testes. Spermatogenesis was progressively reduced to total inhibition in fish exposed to 100% effluent. One of the most pronounced effects detected was the presence of oocytes in male gonads, observed in 20 percent of males exposed to 100 percent sewage effluent.

Key words: vitellogenin, sewage effluent, *Carassius carassius*, estrogenicity, EDC

5.1.1. Introduction

Since the early 1990s there has been an increasing interest in EDCs (Endocrine Disruptor Compounds), a group of chemicals present in sewage effluents, which are considered responsible for sewage estrogenic activity. Although the sewage effluent matrix is usually very complex and many of its components unknown from the point of view of toxicity and estrogenic potency, there are several compounds, known to be estrogenic, which studies have focused on. Some of these compounds are alkylphenol polyethoxylates (APEs), which are used in a variety of industrial and household applications. The breakdown products of APE are persistent metabolites such as nonylphenol or octylphenol, which have been shown to be

estrogenic to fish although they are considered weak (Sumpter and Jobling, 1995; Cox, 1996; Coldham, 1998; Tyler et al., 1998; Harries et al., 1999; Jones et al., 2000; McArdle, 2000). Other important chemicals found in STPs (Sewage Treatment Plants) are natural (e.g. 17- β -estradiol) and synthetic (e.g. 17- α -ethynylestradiol) steroids, the most potent endocrine disruptors in sewage effluents, which are from human waste and are excreted primarily as a variety of inactive glucoronide or sulfonide conjugates that are rapidly cleaved and metabolised by the STP biological processes (Johnson and Sumpter, 2001). The concentration of “free” steroidal estrogens, such as 17- β -estradiol and 17- α -ethynylestradiol, measured in some STPs and aquatic environment, suggests that conjugated estrogens suffers deconjugation by bacteria into an active form during STP process. This is supported by the fact that there are enzymes, such as β -glucuronidase, that are capable of hydrolysing steroid glucuronides. β -glucuronidase is found in many species of bacteria, including *Escherichia coli*, which are probably the most common bacteria found in sewage (Tyler and Routledge, 1998) .

Several environmental contaminants have been identified as xenoestrogens because of their ability to induce responses similar to those caused by 17- β -estradiol. These compounds include the therapeutic natural estrogen 17- β -estradiol, phytoestrogens, mycoestrogens and also the synthetic 17- α -ethynylestradiol (Jones et al., 2000). Additionally, there are other chemicals frequently found in STP that have weak estrogenic activity (e.g. APEs, bisphenol A, phthalates, pesticides and dieldrin) (Harries et al., 1999).

The presence of these compounds in STPs has been shown by various studies (Sumpter and Jobling, 1995; Arnold, 1996; Desbrow et al., 1998; Rudel et al., 1998; Blackburn et al., 1999; Korner et al., 2000; Barber et al., 2000; Alda and Barceló, 2001; Thomas et al., 2001; Snyder et al., 2001; Lee et al., 2002) which have demonstrated not only the presence of these xenoestrogens in sewage but also the frequent existence of quantities that are able to cause endocrine disruption. Additionally, xenoestrogens have been associated with reproductive abnormalities in different wild fish species as they mimic the natural estrogen 17- β -estradiol and compete with estrogen receptor binding (Arnold, 1996; Tyler et al., 1998). These EDCs can thus interact with physiological systems and cause alterations in development, growth and reproduction in wildlife that is exposed to them. Furthermore, it has been reported that exposure to EDCs has resulted in altered fecundity in female fish, and reduction in testicular development and fertility in male fish (Jobling et al., 2003).

One of the most sensitive responses to estrogens in fish is the induction of vitellogenin (Vtg), which is used as a biomarker of exposure to estrogenic chemicals in an aquatic environment. Vtg is a phospholipoglycoprotein (synthesised in all oviparous liver vertebrates) that serves as the major precursor to the egg yolk proteins. Significant levels of Vtg are normally found only in sexually maturing females, however both male and juveniles are also capable of synthesizing Vtg when exposed to exogenous estrogens (Heppel et al., 1995; Sumpter and Jobling, 1995; Lomax et al., 1998; Denslow, 1999; Flammarion et al., 2000).

The first reports showing the effects of estrogen in sewage on fish were carried out by Purdom et al. (1994), followed by other reports in the U.K. and North America showing estrogenicity in fish linked to sewage effluent. These studies showed that caged fish exposed directly to the flow of effluent or downstream of an STP exhibited high levels of plasma vitellogenin (Sumpter and Jobling, 1995; Jobling et al., 1998). Subsequently, several studies were carried out and reported high vitellogenin levels in blood plasma and, in a few cases, developing oocytes in the testes of male rainbow trout (*Onchorynchus mykiss*), native flounder (*Platichthys flesus*), fathead minnows (*Pimephales promelas*) and roach (*Rutilus rutilus*), collected near or exposed to sewage effluents (Purdom et al., 1994; Sumpter and Jobling, 1995; Jobling et al., 1998; Allen et al., 1999; Harries et al., 1999; Hemming et al., 2001; Stentiford et al., 2003).

Furthermore, many laboratory studies have also clearly showed the estrogenic potency of some of these environmental chemicals in sewage effluents, which cause endocrine disruption at environmentally realistic exposure levels in several fish species (Vos et al., 2000). Thus the laboratory experiments have demonstrated that exposure to EDCs can cause severe alterations of the endocrine and reproductive organs (Jobling and Sumpter, 1993; Christensen, 1999; Bjerselius, 2001; Casini, 2002; Segner et al., 2003; Van den Belt et al., 2003).

The main objective of this study was to assess the estrogenic potential of a treated effluent from an STP (Lisbon-Chelas), by evaluating the estrogenic effects in exposed fish (*Carassius carassius*). The STP serves one of the most densely populated areas in Portugal and handles large amounts of domestic waste along with varied amount of industrial wastewater that, at the same time varies in composition. The STP undergoes tertiary treatment, with activated sludge treatment and the treated effluent is discharged into the Tagus estuary after ultra-violet treatment. Additionally, this study provides valuable information for further studies on the

estrogenic potency of STPs. Thus, to our knowledge, it is the first time that the estrogenic effects of sewage have been assessed using fish exposed to sewage effluent in Portugal.

5.1.2. Materials and Methods

5.1.2.1. Experimental Design

A group of four square fibreglass tanks (Fig. 5.1.1), each with a 1m³ capacity was set up in Chelas, a district of Lisbon. Each tank was supplied with a constant flow of water (8 L/min.), with tap water and with different concentrations of the sewage effluent, as follows: 0, 25, 50 and 100 percent. The experiment was conducted at ambient temperature ($14\pm4^{\circ}\text{C}$) over 28 days. The control tank (tap water) and all the dilutions used tap water that had previously been dechlorinated with an activated carbon filter. The tanks were also aerated and the chlorine, temperature, pH and conductivity of the tap water were checked weekly.

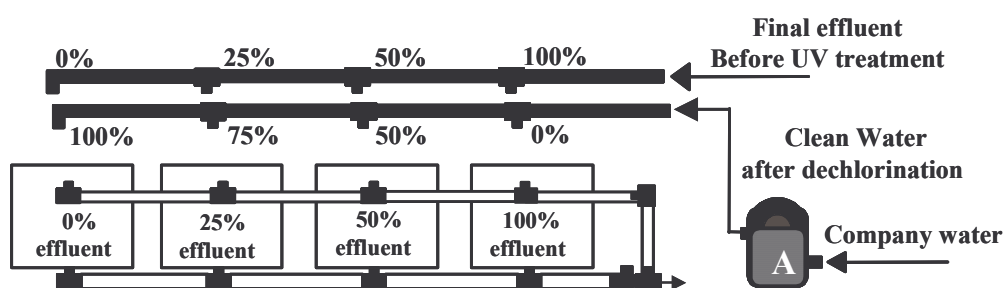


Figure 5.1.1: Design of experimental tanks. A – activated carbon filter.

Sixty carp of both sexes, aged 1 to 2 year and sexually mature were obtained from a state producer (Azambuja Aquaculture Station). The fish were acclimatized in the laboratory facilities for at least 2 weeks before the beginning of the experiments in the winter. At the sewage treatment plant, the fish were randomly divided into four groups of 15 fish and placed in the tanks. They were fed once a day with commercial pelletized food (Alpis) during the experimental period. After 28 day's the fish were sampled. The weight and fork length were recorded and blood was collected Vtg analysis. Mortality was also recorded.

5.1.2.2. Vitellogenin Analysis

After the experimental period, blood was sampled from the caudal vein using heparinized syringes treated with aprotinin (10 TIU ml^{-1}) and kept in ice prior to centrifugation ($4,000 \times g$ for 15-20 mins at 4°C). After centrifugation, the plasma was then frozen at -80°C and stored in eppendorf tubes until further Vtg analysis. The fish plasma of both sexes was analysed by a quantitative enzyme-linked immunosorbent assay (ELISA) adapted from Denslow et al. (1999). Briefly, diluted plasma samples (1:200 for males and 1: 1000 for females) were coated ($50 \mu\text{l/well}$) on Maxisorp polycarbonate immunoassay plates (Nunc-Roskilde, Denmark) and incubated with a carp Vtg monoclonal antibody G10/5/1, furnished by the Laboratory of Immunology and Microbiology (LIM/ESE, University of Metz, France) and diluted to an appropriate concentration ($0.1 - 0.5 \mu\text{g ml}^{-1}$). The plates were then incubated with an alkaline phosphatase conjugated secondary antibody (1/1000, Sigma-Aldrich), which uses p-nitrophenyl phosphate (PNPP, Sigma-Aldrich) as a substrate. After adding the stop solution (3N NaOH), the plates were read in a 96-well microplate reader (BioRad-Benchmark, USA) at 405 nm .

For quantification purposes, Vtg standards were prepared by serial dilutions of purified carp Vtg (LIM/ESE, Metz University) to give a range from 10 to 1000 ng ml^{-1} . The detection limit of Vtg was 10 ng ml^{-1} . For interassay control we chose a standard female plasma sample diluted to the required concentration to fall within the standard curve as described by Denslow et al. (1999).

The coefficient of variation was calculated for each triplicate sample and, if it exceeded 15 percent, samples were runned again. Standard curves, fitted by log-linear regression, were used to quantify Vtg concentration, with R^2 values of 0.96 to 0.99.

5.1.2.3. Histological techniques

After the blood sampling, the fish were euthanized and weighed. All fish (15 for each treatment) were then dissected and the organs removed. Gonads, liver and kidneys were excised and weighed. Small portions of the organs were fixed in Bouin's solution for 48 hrs and subsequently placed in 70% ethanol until further histological processing. In the processing procedure samples were embedded in paraffin, sectioned to $5 \mu\text{m}$ and stained with

haematoxylin & eosin for histological evaluation. The gonadosomatic and hepatosomatic indices, GSI and HSI respectively, were calculated as a percentage of total body weight, in order to provide information on the gonad and liver conditions, using the following formula:

$$\text{GSI} = (\text{total gonad weight} / \text{total body weight}) \times 100$$

$$\text{HSI} = (\text{liver weight} / \text{total body weight}) \times 100$$

Male gonads were classified as being in one of four maturation stages and female gonads were classified in one of six maturation stages according to a classification scale proposed by Gupta (1975).

For male gonads, Stage I (immature testes) shows the lobules containing spermatogonial cells. Stages II and III (maturing testes) show clumps or cysts of spermatogonial cells at the periphery of the lobules and primary and secondary spermatocytes and spermatids towards the interior. Stage IV corresponds to few spermatogonial cells around the periphery of the lobules, which are filled with spermatozoa.

For females, Stage I corresponds to immature ovaries, with youngest oocytes (diameter: 0.05 – 0.15 mm). Stage II shows maturing oocytes, characteristically containing small vacuoles in the cytoplasm (diameter: 0.15 – 0.3 mm). Stage III is characterized by advanced maturing oocytes (diameter: 0.3 – 0.7 mm). Then, as the ovaries mature, from Stage III onwards a number of ova undergo a process of resorption. At this stage the whole follicle loses its shape, and is described as atretic follicle. Stage IV represents mature oocytes (diameter: 0.7 – 0.8 mm), filled with chromophilic yolk. At Stage V, the mature ova increase in size with accumulation of yolk, and at this stage they are ripe (this stage is observed only after hypophysation). At Stage VI, the ovary does not differ greatly from the previous stage, with the remaining ova not as closely packed together since some have been extruded.

5.1.2.4. Statistical analysis

The data was analysed using a one-way ANOVA with a Tukey post-test (Statistica version 5.0; Statsoft Inc., USA) for significant differences. In order to calculate Vtg in unknown samples Microplate Manager 4.0 (BioRad Software) was used, by linear-log regression extrapolation of absorbance values to the standard curve.

5.1.3. Results

The cumulative mortality rate after 28 days was 5% for control fish and fish exposed to 25 percent sewage effluent, 6.7% for fish exposed to 50 percent sewage effluent and 8.3% for fish exposed to 100 percent sewage effluent.

At the end of the experimental period, high levels of vitellogenin were detected in the blood plasma of both males and females. The highest Vtg concentration levels were detected in fish exposed to 100 percent sewage effluent, 249.57 μgml^{-1} for males and 1467.26 μgml^{-1} for females (mean values).

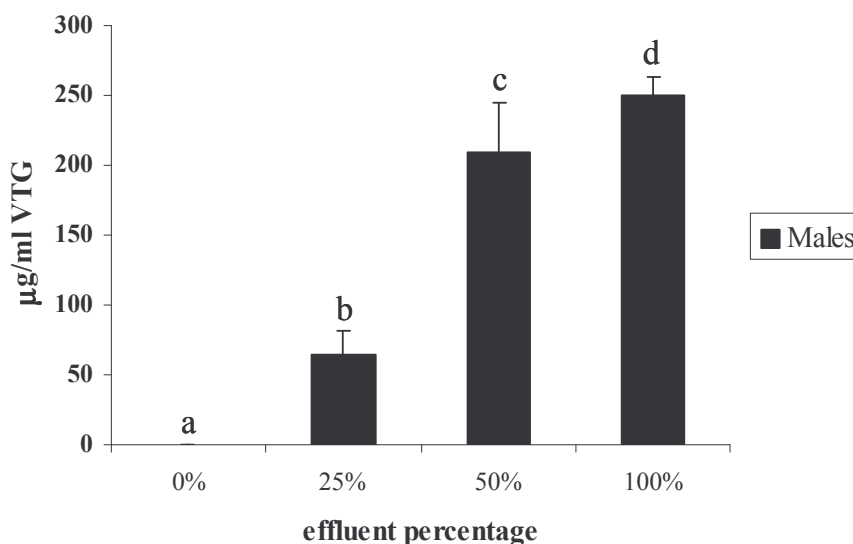


Figure 5.1.2: Vitellogenin induction in fish males exposed for 28 day to sewage effluent. Significant differences if letters are different ($P < 0.05$).

The vitellogenin analysis revealed a significant increase ($P < 0.05$) in all exposed male fish in comparison with control ones. Significant differences were also observed between each treatment (Fig. 5.1.2).

Regarding exposed females, the results show significant differences ($P < 0.05$) between controls and females exposed to 50 and 100 percent of the sewage effluent (Fig. 5.1.3).

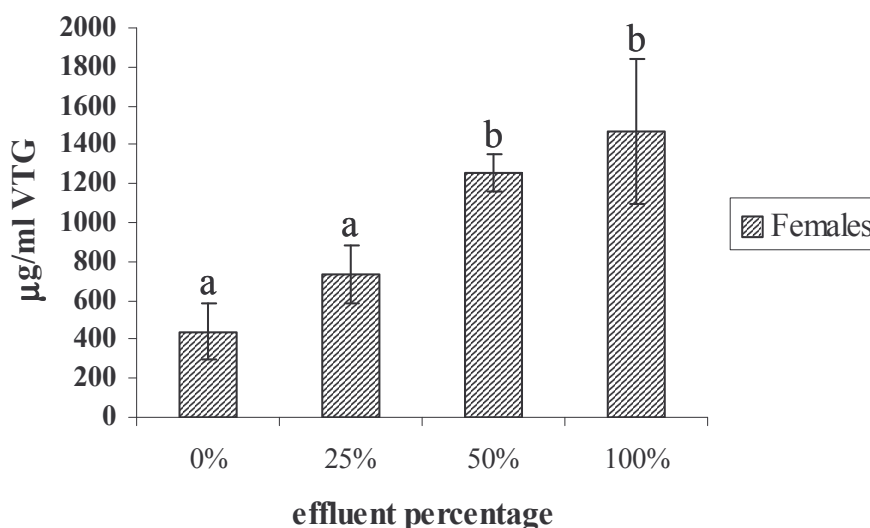


Figure 5.1.3: Vitellogenin induction in fish females exposed for 28 day to sewage effluent. Significant differences if letters are different ($P < 0.05$).

Analysis of the gonadosomatic index revealed a decrease in both males and females, with a more pronounced effect in fish exposed to 100 percent of effluent (Fig. 5.1.4). Statistical analysis showed significant differences only between males exposed to 100 percent effluent and the other treatments, including controls ($P < 0.01$). With regard to females, significant differences were also observed between females exposed to 100 percent effluent and the other treatments including controls ($P < 0.01$).

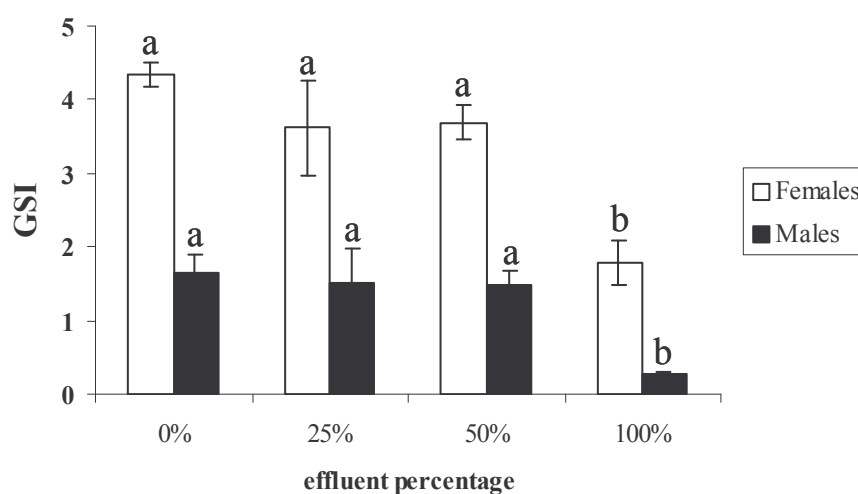


Figure 5.1.4: Gonadosomatic index (GSI) in fish exposed to sewage effluent. Significant differences if letters are different ($P < 0.05$).

Statistical analysis of the hepatosomatic index (HSI) reveals that there are no significant differences (Fig.5.1.5) between controls and both exposed male and female fish.

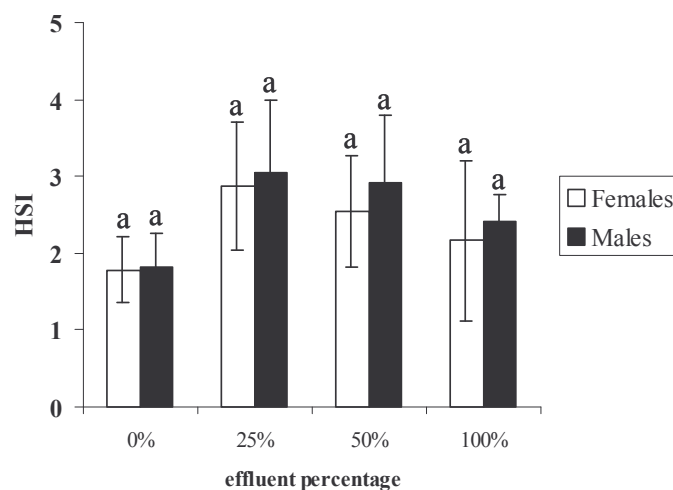


Figure 5.1.5: Hepatosomatic index (HSI) in fish exposed to sewage effluent. Legend: Significant differences if letters are different ($P < 0.05$).

Histological analysis of the testes in exposed male fish revealed estrogenic effects from sewage effluent (Fig.5.1.6 and 5.1.7).

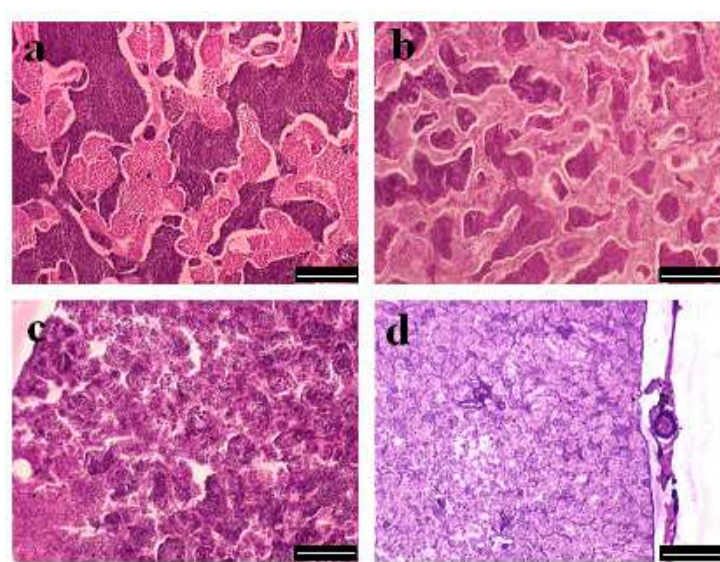


Figure 5.1.6: Testis histology: a – control males; b – males exposed to 25 percent effluent dilution; c - males exposed to 50 percent effluent dilution; d - males exposed to 100 percent effluent. Bar: 100 μ m. Section cuts: 5 μ m; coloration: H&E.

After 28 days the testes of control fish had a normal appearance, containing cells at all spermatogenic stages (Fig. 5.1.6 - a), and were classified as maturing testes (Stage II). The lumina were filled with spermatozoa and the lobules contained numerous spermatogenic cysts. The spermatogenesis was active although in two control fish the spermatogenesis seemed to be at a less advanced stage. No ovo-testis condition was observed in male control fish.

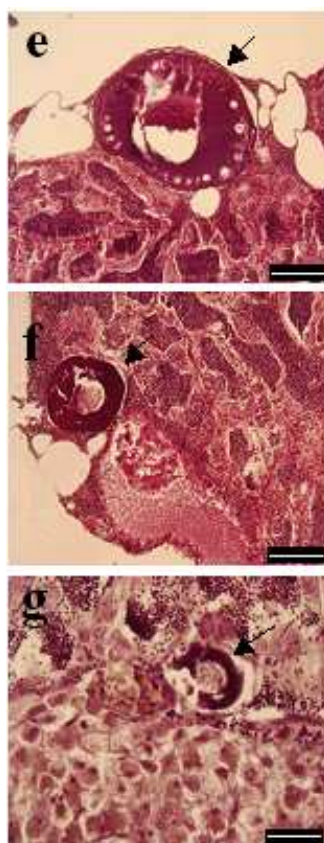


Figure 5.1.7: Testis histology: showing growing oocytes (arrowhead) within testis of fish exposed to 100 percent effluent. Bar (e and f): 100 μ m; Bar (g): 30 μ m Section cuts: 5 μ m; coloration: H&E.

The general aspect of the testes of the individuals exposed to 25 percent of sewage effluent was similar to that of the controls, except for the lesser abundance of spermatogenic cysts and the fact that sperm cells seem to be hypertrophied (Fig. 5.1.6 - b). Fish exposed to 50 percent sewage effluent also exhibit spermatozoa in the seminiferous lobules but the spermatogenic cysts were less abundant than in previous treatments. The tissue seems to be degenerated and hypertrophied cells were observed around seminiferous lobules (Fig. 5.1.6 - c). Most fish

exposed to 100 percent of the effluent presented regressed testes and an absence of spermatogenesis and spermatogenic cysts. Moreover, recrudescence tissue, the presence of several vacuoles within testis tissue and numerous dilated blood capillaries were also observed (Fig. 5.1.6 - d). In two exposed males exposed to 100 percent effluent the spermatogenesis was still active, with spermatozoa in seminiferous lobules lumina, but we also detected the presence of oocytes at the periphery of the gonad (Fig. 5.1.7 – e and f).

The maturation stages of female fish exposed to different sewage dilutions (Table 5.1.1) were assessed by histological observation of tissue slides using the Gupta (1975) classification.

Table 5.1.1: Number of female *Carassius carassius* at different maturation stages, exposed to different sewage dilutions.

Sewage percentage	Maturing Stages					
	I	II	III	IV	V	VI
0%	2	5	n.o.	n.o.	n.o.	n.o.
25%	1	5	1	n.o.	n.o.	n.o.
50%	1	4	1	n.o.	n.o.	n.o.
100%	1	4	1	n.o.	n.o.	n.o.

n.o. (not observed).

The majority of control females were classified as being at Stage II, characteristically showing maturing oocytes, though also the presence of young oocytes at different stages of development. Females exposed to 25, 50 and 100 percent of sewage effluent, were also classified as being at Stage II, but there were also females at Stage I and III, corresponding to immature and advanced maturing oocytes respectively. Atretic oocytes and oocyte degeneration with oogenesis inhibition were also observed in some females exposed to the different exposure treatments, with the exception of control fish.

5.1.4. Discussion

Prior to the present study, treated sewage effluent was screened to assess the presence of certain chemical compounds that are well known to be estrogenic (APEs, bisphenol A, 17 β -estradiol and ethynilestradiol), using an ELISA KIT developed by Takeda Chemical Industries, LDA (Japan). The data showed that the selected chemicals were in the range of ng l⁻¹ to μ g l⁻¹, with APE presenting the highest concentrations and 17 β -estradiol the lowest, with values in the ng l⁻¹ order of magnitude (unpublished data). Thus, although we do not know all the compounds present in sewage effluent that can also have estrogenic activity, the

compounds screened are effectively present in amounts that may give rise to an estrogenic response in fish.

Jobling et al. (1998) suggested that the high incidence of intersexuality found in wild populations of river fish (*Rutilus rutilus*) were consistent with exposure to hormonally active substances and are associated with discharges from sewage treatment plants that are known to contain estrogenic chemicals. The present study indicates that treated sewage effluent apparently shows some estrogenic activity, as suggested by the elevated Vtg levels and morphological abnormalities in exposed crucian carp.

Elevated serum Vtg concentrations have been reported in male wild carp, *Cyprinus carpio* (Folmar et al., 1996; Solé et al., 2002), and walleye (*Stizostedion vitreum*) (Folmar et al., 2001) exposed to or living near Sewage Treatment Plants and in laboratory studies with chemicals known to be present in sewage. Ishibashi et al. (2001) showed that goldfish (*Carassius auratus*) exposed to EDCs (under laboratory conditions) suffered Vtg elevation. Our data shows a progressive increase in Vtg levels, in comparison with controls, which is related to the effluent concentrations of the experiment, with the highest values being determined in fish exposed to 100 percent effluent. The elevated levels in females were also closely related to sewage effluent concentrations, which may suggest a stimulus to VTG production caused by EDCs present in the sewage, a situation that can have direct repercussions in the reproductive capacity of fish (Kime, 1999). Furthermore, studies with rainbow trout exposed to cyanide during late vitellogenesis showed increasing plasma vitellogenin, while gonadal levels fell and hepatic concentrations were unchanged (Kime, 1995). It is known that the Vtg is a highly sensitive biomarker of exposure, as has been demonstrated by many different studies (Tyler et al., 1996; Sherry et al., 1999; Flammarion et al., 2000; Solé, 2000). Thus, the results observed in the present work clearly indicate that sewage effluent possesses an estrogenic effect by stimulating Vtg production.

Kime (1995) pointed out in a review paper that one of the most common measures of the effects of pollutants in males and females is a decrease in GSI. Moreover, studies carried out by Ashfield et al. (1998) reported a reduction in the GSI in trout exposed to alkylphenolic compounds, considering it as a crucial factor to successful reproduction. Another study performed by Hemming et al. (2001) also reports a decrease in GSI in exposed fathead minnows. If the sewage compounds have no effect on gonads, no significant differences should be found between controls and other treatments. In the present study the gonad growth

seems to be affected by exposure to the effluent, as is shown by the GSI reduction both in males and females, particularly in fish exposed to 100 percent effluent, which was significantly different from other treatments and controls ($P < 0.05$), suggesting that sewage compounds can have a negative effect in fish reproduction. Thus, the sewage effluent studied seems to have a specific effect on gonad growth.

Mukherjee et al. (1991) pointed out that an increase in HSI caused by exposure to pollutants may cause a liver dysfunction capable of interfering with normal development and other reproductive processes. However, no statistical differences ($P > 0.05$) were detected in the HSI. Therefore, sewage effluent estrogenicity may not in itself explain GSI and HSI data and we should consider effluent toxicity and other environmental parameters that are not measured but which could affect fish physiology.

Histopathological assessment of carp testes have shown that control males evaluated have normal spermatogenic activity with cells at all stages of differentiation. There were some males with apparently low spermatogenic activity, which could be related to the seasonal period of the experiment.

Some field and laboratory studies have shown the occurrence of female features in some male fish species exposed to EDCs, with histological abnormalities like the presence of oocytes scattered within apparently normal testicular tissue (Hashimoto et al., 2000) or the development of an oviduct (Gimeno et al., 1996).

Most of the fish exposed to 100 percent effluent showed an inhibited spermatogenesis and in two cases the development of oocytes, suggesting a strong effect, resulting from the sewage chemicals, that disrupt the reproductive functions of male fish. Enlargement of testis blood capillaries and several vacuoles were also observed within male gonads. Fish exposed to 25 and 50 percent effluent dilutions present a progressive reduction in spermatogenesis and fewer spermatozoa in lobules lumina in comparison with controls. The estrogenic response seems to be less potent than in fish exposed to 100 percent effluent but may be enough to compromise reproductive success.

The oocytes found in the testes, seem to develop mostly at the border of the peritoneum membrane although we found, in one case, oocytes in a seminiferous lobule. We can not easily explain this location but we can hypothesize that it is related to the migration of

undifferentiated cells along the peritoneum that encloses the testis. These cells develop into oocytes as a consequence of exposure to EDCs. We also must note that it was very difficult to hold on to these peripheral oocytes because histological procedures in the sample processing displace them from their location and they are easily lost.

Females exposed to different sewage effluent dilutions were classified as being at the initial maturation stages (I and II), which is related to the season (winter). However, we found few mature oocytes in the ovaries of females exposed to the different concentrations of sewage effluent, which could be indicative of a disruption of the endocrine system due to an external stimulus such as the EDCs present in an STP. Additionally, females show atretic oocytes and in some cases oocyte degeneration that could be the result of exposure to EDCs or other unknown chemical compounds, with toxicological effects on gonads affecting the normal development of oogenesis. Kime (1995) also noted that several studies concerning the effects of pollution in the teleost ovary show that exposure to pollutants leads to less-developed oocytes and fewer large, mature oocytes, and an increase in the number of atretic follicles.

Large amounts of industrial and domestic sewage are discharged into the River Tagus, reaching estuarine waters. The river and its estuarine waters contain environmental estrogens, such as APEs and NP and synthetic estrogens, as shown by Azevedo et al. (1999). In the course of those studies Portuguese rivers were screened and the presence of these compounds was detected, in some cases in amounts that can cause endocrine disruption. The present study has also recorded significant amounts of EDCs in a sewage treatment plant's effluent discharged into the Tagus Estuary (Lisbon), which is indicative of the main sources of aquatic contamination.

5.1.5. Conclusions

This study shows that treated domestic effluent from an STP located in Lisbon (Portugal) possess estrogenic activity. This is clearly shown by the gonadal reduction and the histological abnormalities (e.g. presence of oocytes) observed in the testes of male fish exposed to it and is also shown by an induction of vitellogenin, which provides evidence that sewage effluent contains chemical compounds capable of affecting the endocrine system of fish. This is the first Portuguese study that has tried to assess the estrogenic potency of sewage using fish. Further work must be done to screen suspected estrogenic chemicals in sewage, which could be responsible for the estrogenicity detected in the present experiments.

Acknowledgments

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Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons

5.2. Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons

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Abstract

To assess the estrogenic potency of the treated domestic sewage effluent from a sewage treatment plant (STP) located in Lisbon (Chelas), sixty mirror carp (*Cyprinus carpio*) were exposed to different concentrations of the sewage effluent (0%, 25%, 50% and 100%) for two periods of 28 days in two different seasons (winter/spring). Vitellogenin induction in males was used as a biomarker of exposure to xenoestrogens. At the end of the experiment, blood samples were taken for vitellogenin analysis and the fish were sacrificed and dissected. Gonad samples were taken for histological evaluation of the sewage effects. The results showed an increase in vitellogenin induction in exposed fish, both males and females, depending on the different dilutions of the sewage effluent. In comparison with controls, the gonadosomatic index decreased significantly ($P < 0.05$) in fish exposed to 100% treated effluent. Although statistically not significant, the hepatosomatic index (HSI) was high in all exposed fish. Histological abnormalities in fish gonads were evaluated and related to the different percentages of sewage effluent. Seasonal variations found in estrogenic responses were attributed to weather influences on sewage dilution.

Key words: endocrine disruption, vitellogenin, *Cyprinus carpio*, sewage effluent, seasonal variations

5.2.1. Introduction

The number of anthropogenic chemicals released into the aquatic environment is increasing year by year and is the cause of great concern, particularly with regard to xenoestrogens (Allen et al., 1999; Harris, 2000), which have bioactivity similar to endogenous steroid hormones and are known to affect the development, sexual maturation and reproduction of vertebrates (Ackermann, 2002). Some of these xenoestrogens, also called EDCs (Endocrine

Disruptor Compounds), are structurally similar to the endogenous steroid hormone 17β -estradiol, while others may be not structurally related to naturally occurring steroids. Thus, many non-steroidal compounds, such as flavonoids, lignins, sterols and fungal metabolites, and synthetic chemicals can interact with sex hormone receptors or can modulate their metabolism and biosynthesis (Combes, 2000).

In the current scientific literature, treated and non-treated sewage effluents have been identified as the main sources of EDC discharges into the aquatic environment (Sumpter, 1995; Tyler and Routledge, 1998; Solé et al., 2001, 2002). Indeed, STPs (Sewage Treatment Plants) receiving domestic and industrial sewage release a complex mixture of chemicals (natural and synthetic), some of which are difficult to biodegrade in the treatment process (Sumpter, 1997, 1998; Petrovic et al., 2002; Solé et al., 2003).

According to previously published literature, estrone, 17β -estradiol (E2), synthetic estrogen 17α -ethinylestradiol (EE2) and the degradation products of alkylphenol polyethoxylates (APEOs) are major causes of estrogenic effects from STP effluents (Harries et al., 1997; Desbrow et al., 1998; Sonnenschein and Soto, 1998; Petrovic et al., 2002). APEOs are a group of non-ionic surfactants that are microbially degraded into more lipophilic products with estrogenic potential (Hawrelak, 1999), such as nonylphenol or octylphenol, substances that may act as endocrine disruptors at the estrogen receptor level by mimicking the activity of E2. There is also evidence that alkylphenols can disrupt endocrine-mediated events by inhibiting enzymes involved in the metabolism of sex steroids (Harris, 2000).

Moreover, synthetic estrogens, such as EE2, are even more potent than natural estradiol (Allen, 1999; Van den Belt et al., 2004). These chemicals can reach the aquatic environment in biologically active amounts and bioaccumulate to significant concentrations in aquatic organisms (Sumpter, 1995; Arukwe et al., 2000).

The first reports in U.K. rivers concerning intersex fish (presence of oocytes within the testes) were related with the proximity to STPs (Purdom et al., 1994). Subsequent studies identified EDCs in lagoons and rivers close to STPs in the U.K, where intersex fish were often found (Allen et al., 1999). Other studies identified and quantified chemicals with estrogenic activity in STPs (Spengler et al., 2001). Studies carried out by Harries et al. (1999) showed estrogenic potency from STPs in rainbow trout (*Oncorhynchus mykiss*) and Solé et al. (2002) reported high levels of vitellogenin (Vtg) in male carp (*Cyprinus carpio*) collected downstream of a

sewage treatment plant in Spain. In the U.S.A, Folmar et al. (2001) demonstrated that STPs release estrogenic effluents capable of inducing vitellogenin production and altering normal serum sex steroids in a commercially valuable fish, the walleye (*Stizostedion vitreum*).

The use of biomarkers of potential endocrine disruption in fish is important for the detection and monitoring of the potentially adverse effects of environmental contaminants on aquatic organisms. Usually Vtg is used as a monitoring tool to assess estrogenic effects in studies involving EDCs (Allen, 1999). Vtg is an estrogen-inducible phosphoprotein that is normally synthesized by the liver of female oviparous vertebrates during oogenesis. It is the precursor of egg yolk proteins but both males and juveniles can synthesise Vtg if estrogens or xenoestrogens are present. Accordingly, this protein can be used as a biomarker for estrogenic exposure in male and juvenile fish (Folmar et al., 1996).

Desbrow et al. (1998) isolated and identified the major estrogenic chemicals present in sewage effluents discharged into U.K. rivers. Synthetic estrogen EE2, natural hormone E2 and estrone were present in all effluents assessed, which suggests that these chemical compounds may be responsible for the induction of Vtg synthesis in male fish near effluent discharges from STPs.

Few studies have focused on seasonal variations in sewage effluent estrogenicity or on how these changes can be influenced by variations in STPs efficiency or by the volume/dilution of the influents (Rodgers-Grey et al. 2000). Nevertheless, there is a little information available and a strong need to understand which factor(s) or mechanisms contribute to seasonal variations and how this can influence estrogenic responses in fish.

The main objective of the present work is to evaluate the estrogenic responses of mirror carp (*C. carpio*) exposed to treated sewage effluent from an STP located in Lisbon (Portugal), in two periods in different seasons (winter and spring). To achieve the proposed objectives Vtg was used as a biomarker of the response and histopathological analyses were also performed. Additionally, condition indices (GSI, HSI, K and KI) were calculated in order to complement the information and assess the influence of the season on the effects of exposure to sewage effluent EDCs.

It must also be noted that studies carried out in other countries have shown that treated sewage effluents may have certain common characteristics with regard to the presence of EDCs, although they can vary specifically according to local conditions (Tyler and Routledge,

1998; Korner et al., 2000; Fox, 2001). Furthermore, the presence and extent of EDCs in STP effluents is mainly related to industrial and/or highly populated areas which produce large quantities of domestic wastewater (Sumpter, 1997).

5.2.2. Materials and Methods

5.2.2.1. Selection of the Sewage Treatment Plant

An urban STP was selected to study the seasonal estrogenic effects of treated effluent on carp. This STP is located in a densely populated area and has the capacity to treat 52,500 m³ of wastewater per day, corresponding to more than 200,000 inhabitants. The STP was chosen because it receives large quantities of domestic wastewater and variable amounts of industrial effluents which undergo tertiary treatment with nutrient removal (nitrogen) and disinfection by ultra-violet treatment before being discharged into the River Tagus.

5.2.2.2. Endocrine Disruptor Compounds Analyses

To perform a screening evaluation of the presence of EDCs in the wastewater, a group of chemicals known to be estrogenic were selected (alkylphenol ethoxylates (APEOs), bisphenol A (BPA) and 17 β -estradiol). Samples were collected prior to the experiments from the STP influent and effluent. Sample treatment and EDC measurements were performed according to the protocols provided by Takeda Chemical Industries (Japan). The concentrations of the selected EDCs were achieved using an ELISA Kit (Takeda Chemical Industries, Japan). Briefly, plate wells were coated with a monoclonal antibody, which binds exclusively with antigen (EDC). The EDC derived from samples and an antigen-enzyme conjugate were premixed and added into each microplate well for a competitive assay. Unbound EDC and excess antigen-enzyme conjugates were washed out and the chromogenic substrate was added to develop colour in conjunction with the conjugate's enzyme. The amount of antigen-enzyme conjugate remaining with the antibody determines colour intensity. Standard solutions were prepared according to the Takeda protocols just before the test was performed. The standard curve, a dose-response curve from known concentrations of standard EDCs, was determined from the absorbance at 450 nm. The EDC concentration in each sample was accurately calculated from the response intensity of absorbance by extrapolating to the standard curve.

5.2.2.3. Experiment Design

Four fiber-glass (1m³) tanks (Fig. 5.2.1) were set up in the open-air at Chelas-Lisbon STP, with one of a series of treated sewage effluent dilutions produced by mixing different percentages of effluent with clean water (tap water previously dechlorinated). The nominal concentrations of treated sewage effluent were 0% (tap water), 25%, 50% and 100%. Each tank was supplied with a constant flow rate of 8 Lmin⁻¹. The tanks were aerated and the temperature, chlorine and pH were monitored weekly. Mortality was also recorded.



Figure 5.2.1: Fiber-glass tank set-up at the STP.

Sixty mirror carp (*Cyprinus carpio*), male and female (aged 2-3 years), obtained from a local producer, were randomly distributed (15 in each tank) and fed daily with commercial pellets. Carp were exposed to the water-flow at different concentrations of treated sewage effluent over 28 days in two different seasonal periods: winter (November) and spring (April).

5.2.2.4. Vitellogenin determination

At the end of both experimental periods, fish were sampled. Blood was collected from the caudal vein using a 2 or 3-cc heparinized syringe treated with aprotinin (10 TIUml⁻¹) and then transferred to 2ml Eppendorf tubes, chilled on wet ice, and centrifuged for 15 min. at 4,000xg (4°C). The plasma was then pipetted into Eppendorf tubes again and immediately frozen at – 80°C to await further analysis. Vtg concentration in plasma was achieved by direct Enzyme Linked Immunosorbent Assay (ELISA) under a protocol adapted from Denslow et al. (1999). A specific monoclonal antibody against carp Vtg was supplied by the Laboratory of Immunology and Microbiology (LIM/ESE at the University of Metz, France). Standard

curves were constructed by adding serial dilutions of purified carp Vtg, provided by the same laboratory, to a range of between 10 and 1000 ngml⁻¹. A standard female plasma sample diluted to the concentration range of the standard curve was chosen for interassay control as described by Denslow et al. (1999).

5.2.2.2.5. Somatic and total condition indices

The gonadosomatic and hepatosomatic index, GSI and HSI respectively, were calculated as a percentage of total body weight, to provide information on gonad and liver condition. The total fish weight was used to determine the total condition index ($K = 10^5 \times \text{total weight} \times \text{length}^{-3}$) and the somatic condition index (KI) ($(\text{total weight} - \text{gonad weight}) \times \text{length}^{-3} \times 10^5$).

5.2.2.2.6. Histology

After the blood sampling, the fish were sacrificed, weighed and measured and then dissected for organ removal. Gonad, liver and kidney samples were fixed in Bouin's solution for 48hrs and then placed in 70% ethanol for further histological processing. Samples embedded in paraffin were sectioned (5 µm) and stained with hematoxylin and eosin.

Male and female gonads were classified according to the stages of maturation, using a classification scale proposed by Gupta (1975), and compared with control fish in order to complement the evaluation of sewage effects on fish gonads.

5.2.2.2.7. Statistical analysis

The statistical analysis of the data was performed using one-way ANOVA, whereas the Tukey post-test was used for multiple range comparisons of means (Statistica version 5.0; Statsoft Inc., USA). When necessary, data was transformed to achieve homogeneity of variances. Probability of significance was set at $P < 0.05$. Vtg was quantified using the Microplate Manager 4.0 (BioRad Software), with linear-log regression extrapolation of absorbances to the standard curve.

5.2.3. Results

Quantitative results of the selected EDCs, show that they are present both in the STP influent and effluent (Table 5.2.1).

Table 5.2.1: Alkylphenols, BPA and E2 measured in the STP (influent and effluent).

Sample points	Influent	Effluent
APEs ($\mu\text{g l}^{-1}$)	>1d	5.77
BPA ($\mu\text{g l}^{-1}$)	1.02	0.55
17 β -estradiol (ng l^{-1})	1.12	0.91

The water quality and rainfall data observed in the two seasons periods are presented in Table 5.2.2. The results show that the highest mean daily flow and rainfall were observed in the spring experiment.

Table 5.2.2: water quality parameters and rainfall registered in November and April.

Water Quality parameters	Winter	Spring
Temperature	12.00 $\pm$ 1.63	17.2 $\pm$ 0.97
pH	7.28 $\pm$ 0.91	7.17 $\pm$ 0.02
Dissolved oxygen (mg l^{-1})	5.50 $\pm$ 0.36	5.09 $\pm$ 0.28
Mean daily flow (m^3)	37,500	50,000
Mean rainfall (mm)	10	44

The plasmatic Vtg measured in fish exposed to the serial dilutions of treated sewage effluent show significant ($P < 0.05$) induction. The highest levels of the protein were found in both male and female fish (242.58 $\pm$ 21.83 $\mu\text{g ml}^{-1}$ and 1345.60 $\pm$ 21.83 $\mu\text{g ml}^{-1}$, respectively) exposed to the non-diluted treated sewage effluent in the winter (Figure 5.2.2, a and b).

During the two seasons periods of the experiment, the cumulative mortality recorded in the winter experiment was 6.7% for control fish (0%) and 20% for fish exposed to 25%, 50% and 100% sewage effluent; in the spring experiment, only fish exposed to 25% treated sewage effluent registered a mortality rating (20%).

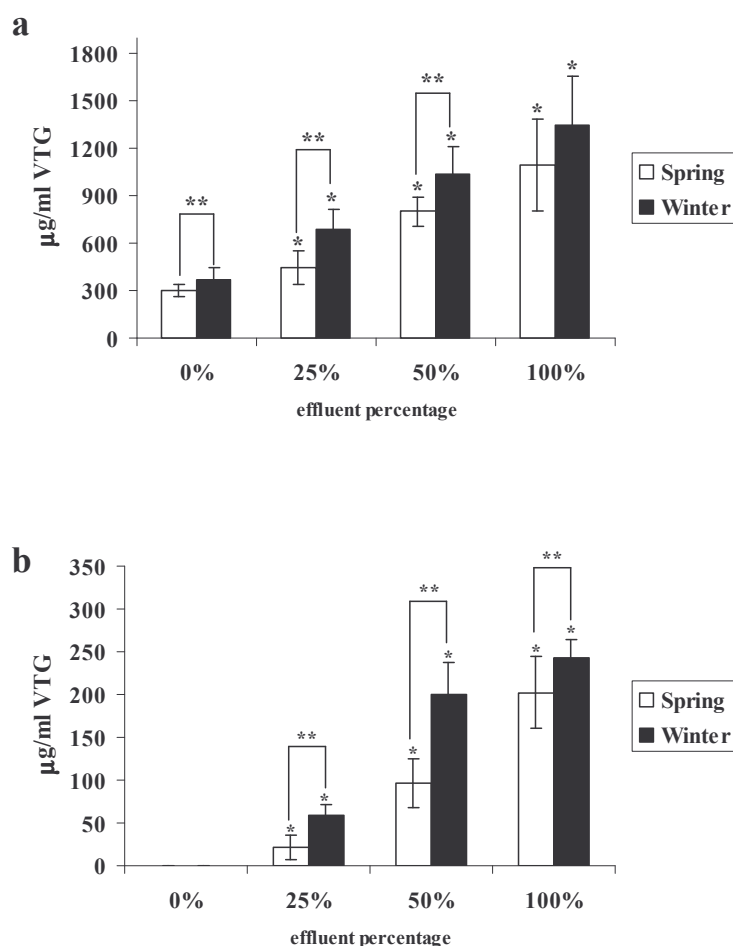


Figure 5.2.2: Vitellogenin levels in females (a) and males (b) exposed to graded treated sewage effluent.: Different from controls if *($P<0.05$); seasonal differences if **($P<0.05$).

Vtg levels showed a significant increase ($P<0.05$) in male and female fish exposed to different sewage dilutions in both seasonal periods (Fig. 5.2.2 a and b). Statistical analysis also revealed significant differences between seasonal periods (winter/spring) for males exposed to graded treated sewage effluent. Moreover, significant differences were found in females exposed to 25% and 50% sewage effluent concentrations in the two seasons periods of the experiment.

Statistical analysis of the gonadosomatic index revealed a significant decrease ($P<0.05$) in male fish exposed to 50% and 100% of treated sewage effluent in the winter period in comparison with the controls (0%), while females showed significant differences ($P<0.05$) in fish exposed to 100% treated sewage effluent in both seasonal periods (Table 5.2.3).

The results show that higher GSI values were observed in male and female controls (spring) reflecting the maturing condition. Additionally, statistical analysis revealed significant differences between spring and winter controls in both males and females exposed to 100% treated sewage effluent.

The hepatosomatic index (HSI) shows significant differences in males exposed to 100 percent effluent in the spring experiment. In winter significant differences were found in male fish exposed to the different effluent percentages in comparison with controls. Seasonal differences were reported in male fish exposed to 25% and 50% treated sewage effluent. A rising trend was observed in female HSI values, over the different treatments. However, no significant differences were detected on the basis of exposure or the season (Table 5.2.3).

Table 5.2.3: Gonadosomatic index (GSI), hepatosomatic index (HSI), total condition index (K), and somatic condition index (KI).

Indices	Males							
	0 %		25%		50%		100 %	
	Winter	Spring	Winter	Spring	Winter	Spring	Winter	Spring
GSI	1.92 ± 0.31	2.65 ± 0.33**	1.85 ± 0.36	2.51 ± 0.33	1.70 ± 0.28	1.99 ± 0.18*	1.58 ± 0.29	1.68 ± 0.47*
HSI	1.62 ± 0.23	1.84 ± 0.32	3.08 ± 0.41	2.06 ± 0.21*,**	3.17 ± 0.63	2.09 ± 0.45*,**	2.89 ± 0.16*	2.64 ± 0.51*
K	2.52 ± 0.66	2.07 ± 0.41	2.55 ± 0.25	2.54 ± 0.35	2.97 ± 1.24	2.61 ± 0.72	2.35 ± 0.25	2.36 ± 0.27
KI	2.47 ± 0.65	2.02 ± 0.41	2.51 ± 0.25	2.47 ± 0.34	2.92 ± 1.22	2.55 ± 0.71	2.31 ± 0.25	2.32 ± 0.29

Indices	Females							
	0 %		25%		50%		100 %	
	Winter	Spring	Winter	Spring	Winter	Spring	Winter	Spring
GSI	3.30 ± 0.45	4.01 ± 0.27**	3.26 ± 0.44	3.33 ± 0.34*	3.12 ± 0.42	3.31 ± 0.42*	2.36 ± 0.47*	2.85 ± 0.48*
HSI	2.22 ± 0.84	2.22 ± 0.44	2.80 ± 0.52	2.15 ± 0.17	2.61 ± 0.63	2.24 ± 0.38	2.54 ± 0.55	2.50 ± 0.40
K	2.51 ± 0.52	2.60 ± 0.31	2.72 ± 0.66	2.03 ± 0.55	2.92 ± 0.39	2.78 ± 0.30	2.19 ± 0.22	2.69 ± 0.30
KI	2.43 ± 0.51	2.50 ± 0.29	2.63 ± 0.63	1.96 ± 0.53	2.82 ± 0.38	2.69 ± 0.30	2.14 ± 0.22	2.46 ± 0.32

*differences from comparison with controls; ** differences from comparison between seasonal periods

At the end of both the periods for the experiment, male and female gonads were evaluated by histological observation and classified according the scale proposed by Gupta (1975).

Testes and ovary development in control fish (0%) were considered normal in appearance, with spermatogenesis and oogenesis characteristically related to the corresponding period of the season. In the winter experiment, control males were classified as having maturing testes (Stage II and III) with clumps or cysts of spermatogonial cells on the lobule periphery and the primary and secondary spermatocytes and spermatids towards the interior (Fig 5.2.3 a).

The testes of control fish exposed in the spring experiment were classified as being at Stage IV, which means there are few spermatogonial cells around the periphery of the lobules, which are filled with spermatozoa (Fig 5.2.3 b).

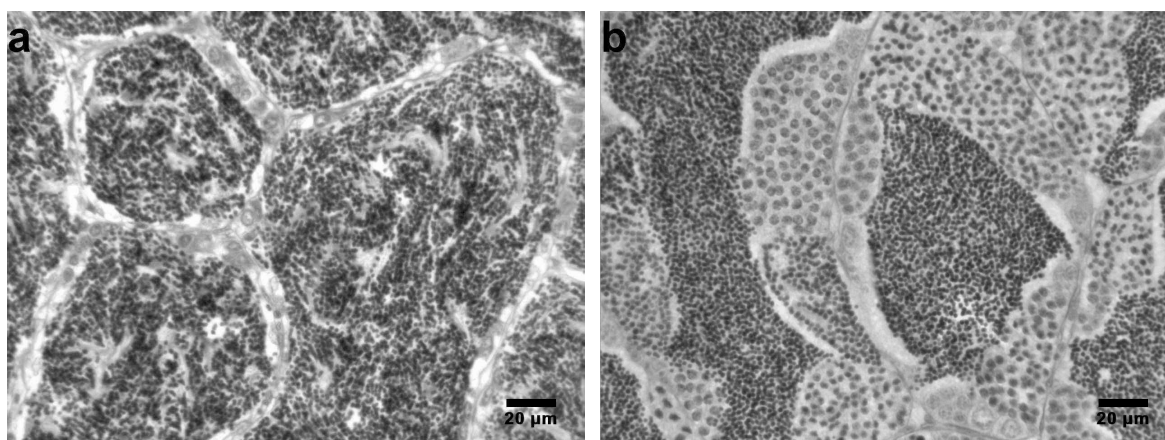


Figure 5.2.3: Control testis histology in spring (a) and winter (b): Scale bar: 20 μm; Section cuts: 5 μm; coloration: H&E.

Only some testes of fish exposed to 25%, 50% and 100% treated sewage effluent in the winter period were classified at stages II and III. However, most of the gonads revealed spermatogenesis restriction or in some cases total inhibition with gonadal recrudescence and some very reduced lobules containing only spermatogonia. Degeneration of the structures of the seminiferous lobules was also observed in a few testes. These effects seemed to be more intense in fish exposed to 50% and 100% treated sewage effluent, where it was also possible to detect the intersex condition (presence of oocytes) (Fig. 5.2.4 a) in 20% and 30% of the fish, respectively. In the spring experiment, testes of fish exposed to 25%, 50% and 100% were classified at stage IV but spermatogenesis was also restricted, showing degeneration of the structures of the seminiferous lobules. In some cases, spermatogenesis was completely inhibited, with extensive degeneration and necrosis of sperm cells in seminiferous tubules. Intersex condition was only found in fish exposed to 100% treated sewage effluent (Fig. 5.2.4b).

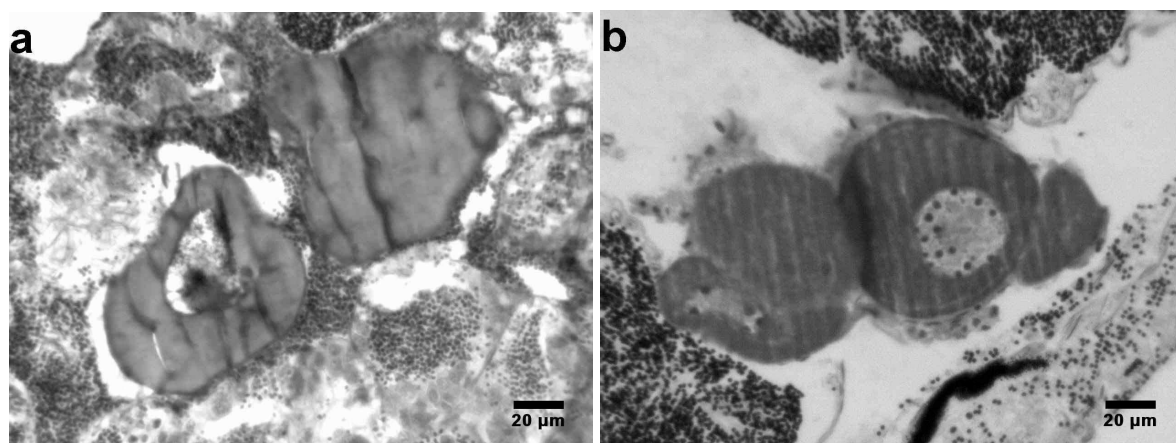


Figure 5.2.4: Testis histology in winter (a) and spring (b): showing growing oocytes (arrowhead) within testis of fish exposed to 100 percent effluent. Scale bar: 20 μm ; Section cuts: 5 μm ; coloration: H&E.

Control females exposed during the winter experiment were classified as being mostly in early stage II, showing maturing oocytes, although some females showed immature ovaries, developing young oocytes. In the spring experiment, control females were classified in advanced stage II and III.

The winter gonads of females exposed to 25%, 50% and 100% treated sewage effluent were classified mainly in stage II, although the gonads of females exposed to 25% treated sewage effluent seem to be at early stage II, similarly to those of the controls. Stroma follicular cell degeneration with the presence of cell types such as macrophages was also observed. In all treatments follicular atresia was observed. Spring female gonads exposed to 25%, 50% and 100% treated sewage effluent were generally classified at stage IV, showing predominantly mature oocytes and some oocytes at all stages of development, although females exposed to 25% seem to be at late stage III or early IV. Follicular atresia was detected in fish exposed to 50% and 100% treated sewage effluent.

5.2.4. Discussion

Large populated areas and industrialized regions may produce sewage effluents containing estrogenic chemical compounds. Consequently, the endocrine system of aquatic organisms exposed to the sewage effluent can be disrupted. The STP chosen to perform the present study is located in a highly populated city and receives large amounts of domestic sewage and

variable volumes of industrial effluents, mainly from small companies located within the urban area. Previous screening of Chelas STP effluent showed the presence of some endocrine compounds, natural and synthetic, such as alkylphenols, bisphenol A and E2 (Table I), which are known to be estrogenic and capable to disrupt endocrine system of fish (Sumpter, 1997). The concentrations of selected EDCs fall into the range of data reported by other authors (Rudel et al., 1998; López de Alda and Barceló, 2001; Spengler et al., 2001; Petrovic et al., 2002). The impact of the natural steroid E2 is highlighted by the fact that concentrations as low as 1 ng l^{-1} of E2 have been shown to be estrogenic to fish (Svenson et al., 2003; Aerni et al., 2004).

The vitellogenic response in exposed male fish revealed a dose-dependence related to STP treated effluent concentrations. Hence, Vtg induction in male fish clearly indicates that EDCs are present in quantities that are capable of eliciting estrogenic responses in both the seasons periods of the experiment. Other authors have reported that estrogenic chemicals present in the aquatic environment cause adverse physiological responses usually associated with high circulating concentrations of estrogens, which in turn induces the synthesis of Vtg in the liver of male fish (Sumpter and Jobling, 1995). Furthermore, several reports have shown evidence of endocrine disruption where Vtg induction and histological abnormalities were observed in various male fish species from freshwater and marine ecosystems contaminated with xenoestrogens (see review in Jobling et al., 1998; Tyler et al., 1998; Vos et al., 2000; Lavado et al., 2004).

The present work reveals significant increases in plasma Vtg induction in all male and female fish exposed to the final treated effluent during the winter and spring periods of the experiment, when compared with controls (clean water). Moreover, the results suggest seasonal differences in estrogenic responses in fish. Seasonal variations in Vtg induction have also been reported by other authors. Hemming et al. (2004) reported increases in Vtg induction in March and December but not in August or June in adult male fathead minnows (*Pimephales promelas*), attributing these results to weather factors such as rain and temperature and to a variable human population. Studies developed by Harries et al. (1996, 1999) have shown a marked seasonal difference (winter/summer) in the potency of discharged STP effluent in the receiving waters. Again, variations were attributed to increased rainfall in winter resulting in a more diluted effluent and it was also pointed out that seasonal variations in dilution will have marked effects on the estrogenic potency of effluent for the organisms exposed to it. Tilton et al. (2002) also reported variations in Vtg induction in fall and spring in

male channel catfish (*Ictalurus punctatus*) but a clear seasonal pattern was not observed. Research in the U.K. has shown temporal variations in the estrogenic response of adult male roach (*Rutilus rutilus*) while the variability in Vtg has also been attributed to the influence of rain and the efficiency of sewage treatment works (Rodgers-Grey et al. 2000). However, Peters et al. (2001) reported little or no seasonal variations in eel plasma Vtg (*Anguilla anguilla*) sampled from U.K. estuaries. Higashitani et al. (2003) investigated Vtg production in male carp (*Cyprinus carpio*) exposed to treated sewage effluent in different seasons (early spring, summer and winter 2000 and early spring 2001). Vtg induction in male fish was observed only in early spring (2000). The results were attributed to the presence of females, in the first experiment, and to the fish physiological status that differ depending on the season.

It should be also highlighted that increased temperatures can contribute to faster degradation of some EDCs present in sewage, resulting in lower estrogenicity (Hemming et al., 2004).

Results show more potent estrogenic responses in fish exposed in the winter experiment than in those exposed in the spring experiment. Although the results are different from other studies reporting that sewage effluents were less potent in winter due to the greater rainfall (Harries et al. 1999; Rodgers-Grey et al. 2000), they can be attributed to similar factors such as differences in the rainfall registered in those periods of the seasons which also influenced the mean daily flow of the treated sewage effluent (Table II). Thus, the present data can be related to the level of precipitation registered in winter, which was lower than in the spring (Table II). This was unusual for the periods of the seasons under consideration (INM, 2003). Furthermore, high temperatures resulting in increased degradation of steroids, for example, may have led to reduced estrogenicity, while degradation of alkylphenolic surfactants (resulting in estrogenic products) may have enhanced that effect. Variations in treatment efficiency may also affect the microbial activity and estrogenic composition of STP effluents (Rodgers-Grey et al., 2000).

Therefore, the amount of EDCs available in sewage effluents is the result of a complex group of environmental, and physical and chemical factors that can act individually or in combination, making it difficult to establish a connection with estrogenic effects in fish and ascertain which factor(s) make the greatest contribution to the seasonal differences observed. Furthermore, effluent toxicity may affect liver and gonad condition but be caused by other parameter(s) not being measured (Hemming et al., 2001). Moreover, it is not clear if the seasonal differences observed in the present study are influenced by the reproductive status

and general physiological condition of the fish, characteristics of both seasonal periods of the experiment, which might affect their sensitivity to EDCs. Nevertheless, the real significance of the variability found in the estrogenic potency of STP effluents is still unclear (Hemming et al., 2004).

An analysis of histopathological changes is often used to assess the estrogenic effects of effluents when associated with information given by biomarkers of exposure. Lye et al. (1997) reported abnormalities in the reproductive health of a wild population of flounder (*P. flesus*) which was consistent with other reports (Harries et al., 1999; Flammarion, 2000; Ashfield et al., 1998; Hashimoto et al., 2000) and were related to the presence of EDCs. The development of oocytes is, then, the major evidence of fish feminization as the result of effluent estrogenicity. Although, oocyte development occurred only in male fish exposed to 50% (winter) and 100% effluent (winter and spring), other histopathological changes showed the toxicological effects of EDCs on fish gonads and complement information on a dose-dependent correlation.

Histopathological analysis of fish testes suggests a dose-dependent correlation according to the different effluent concentrations, in the periods in both seasons, with a general progressive reduction of the normal gonad condition (GSI). Histological observations of females gonads showed different maturation stages according to the respective season. Most probably, the higher levels of circulating Vtg observed in the winter were associated with low incorporation of Vtg into the yolk of developing oocytes and related to the respective stage of seasonal maturation. In the spring experiment, the more advanced stage of maturation led to greater Vtg incorporation in the oocytes.

Gonadosomatic and hepatosomatic indices are useful indicators of the effects of exposure to sewage effluent contaminants at the tissue level. A fish liver, particularly that of a female, contains high concentrations of estrogen receptors, which accounts for its ability to synthesize large amounts of vitellogenin when stimulated by estrogen (Sumpter and Jobling, 1995). The increases in the HSI observed in females exposed to treated sewage effluent have already been reported. They occur during female Vtg synthesis but can also be induced in the liver of males in response to xenoestrogen exposure (Lye et al., 1997).

Although significant differences were detected in Vtg induction, the GSI and the HSI in exposed males, no effects were observed regarding survival, growth, or the total and somatic

condition index, which is in accordance with the results recorded by Rodgers-Grey et al. (2000). Although seasonal variations were observed, a similar trend was detected in GSI and HSI data in both seasons, which could be related to spermatogenesis inhibition. Consequently lower GSI and HSI rises have been associated with liver hypertrophy indicating possible estrogenic effects from STP treated effluent (Hemming et al., 2001). However, Vtg induction does not always correspond to abnormal testis development (Allen et al., 1999) and endocrine disruption can occur without resulting in elevated plasma Vtg levels (Kime et al., 1999).

5.2.5. Conclusions

The present work demonstrates that treated sewage effluent from an STP located in Lisbon (Chelas), possesses a relevant level of estrogenicity for the fish (*C. carpio*) exposed to it, which may lead to endocrine disruption and consequent reproductive dysfunction. The results suggest that the effluent has estrogenic activity in both the periods of the experiment (winter and spring), though the estrogenic response seems to be more potent in the winter period. This is supported by the induction of the Vtg as well as by the histopathological changes observed, such as the development of oocytes in male gonads. Moreover, the differences identified between winter and spring can be partially explained by climatic variations, such as rainfall levels, which were unusual for the periods of the seasons when the experiments were carried out. This study clearly shows that STP discharges into the aquatic environment can induce significant estrogenic changes in the biota, particularly in fish, depending on the dilution and, possibly, the synergistic and/or additive effects of other discharges from industry or agriculture.

Acknowledgments

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Crucian carp (*Carassius carassius*) exposure to treated waste water effluent: evidence of size influence on endocrine disruption?

5.3. Crucian carp (*Carassius carassius*) exposure to treated wastewater effluent: evidence of size influence on endocrine disruption?

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Abstract

The purpose of the present study was to assess the estrogenic ability of a sewage treatment plant (STP) treated sewage effluent, using vitellogenin (Vtg) induction as exposure biomarker and histopathology to evaluate effects in gonad tissues. A correlation with size, vitellogenin induction and exposure effects on fish was investigated. Results show Vtg induction in all male exposed fish suggesting that sewage effluent possess estrogenic activity and have the capability to disrupt endocrine system of fish. In addition, Vtg levels suggest to be related with the different experimental sewage dilutions pointing out the sewage effluent estrogenic potential. Moreover, Vtg concentrations and fish size are not significantly correlated. However, histopathological evaluation and condition indices suggest that the size class 3 to 7 cm is more susceptible to effluent estrogenic activity.

Key words: endocrine disruption, carp, size, vitellogenin

5.3.1. Introduction

There is a strong growing public awareness concerning a group of chemicals, usually termed as endocrine disrupting compounds (EDCs) which are present in the aquatic environment mainly as the result of Sewage Treatment Plants (STP) effluents and other non-treated discharges. Therefore, a large number of chemicals that are in daily use (domestic, industry and agriculture) have been found to have estrogenic effects. These include natural (e.g. 17 β -estradiol, estrone, and estriol) and synthetic steroids such as those used in contraceptive pills (e.g. 17 α -ethynylestradiol), some organochlorine pesticides such as 4,4-dichloro-diphenyl-trichloroethane (DDT), some polychlorinated biphenyls (PCBs), alkylphenolic chemicals widely used in industrial detergents, paints and cosmetics, bisphenol A and some phthalates,

and all of them have been found in effluents of sewage treatment plants (STPs) and in the aquatic environment (Lye et al., 1997; Desbrow et al., 1998; Petrovic et al., 2002, 2004).

The synthetic and natural estrogens are very potent and therefore of particular concern in affecting human and wildlife health. The identification and determination of EDCs, often found at very low environmental concentrations, require sophisticated analytical techniques for their measurement. However, chemical analysis may give a quantitative measurement but miss biological effects (Sumpter, 1997).

Treated domestic sewage discharges have been identified as a major cause of estrogenic effects on fish in the UK and USA freshwater environments (Purdom et al., 1994; Harries et al., 1999; Tilton et al., 2002). For instance Harries et al. (1996, 1997) using caged rainbow trout, have shown that some UK rivers downstream of sewage treatment works STW discharges are oestrogenic for several kilometres although the effect usually declines rapidly with distance due to dilution and other processes. Moreover, Jobling et al. (1998) showed in wild roach (*Rutilus rutilus*) that estrogenic exposure is accompanied in most instances by the presence of intersex conditions. Laboratory experimentation also confirmed the estrogenic ability of some EDCs to disrupt the endocrine system of several species of fish, e.g. wild roach (*Rutilus rutilus*), carp (*Cyprinus carpio*), trout (*Oncorhynchus mykiss*) (Sumpter, 1997; Tyler et al., 1998).

The vitellogenin (Vtg) induction is one of the most sensitive responses and has been used as an *in vivo* biomarker for estrogenicity in oviparous vertebrates (Sumpter and Jobling, 1995; Heppell et al., 1995). Vtg is an estrogen-inducible phosphoprotein used as a useful biomarker of xenobiotics exposure in fish and other organisms. Normally it is synthesized in the liver of female oviparous vertebrates during oogenesis and it is precursor of egg yolk proteins. Studies reported the Vtg synthesis in male common carp in streams impacted by sewage effluents (Solé et al., 2002; Lavado et al., 2004). Therefore, the use of biomarkers of exposure to EDCs can give the first indication that endocrine disruption is occurring in fish (Harries et al., 1999).

Furthermore, condition indices (e.g. gonadosomatic) provide valuable information useful to complement biomarker concentrations. Histology also offers a powerful tool in the study of reproductive health of fishes. Gonad histology, in conjunction with hormone and vitellogenin measurements, morphological and fecundity studies, can provide insights into the effects of various environmental stressors on reproductive health (Blazer, 2002).

The aim of the present study was to assess the estrogenicity of a treated sewage effluent, located in Lisbon (Portugal), on exposed male crucian carps. Furthermore, possible effects related with different size classes were also examined in order to investigate a possible size-dependent susceptibility. Various methods of assessing estrogenic effects were applied in order to a better understanding of EDCs mode of action and effects in fish.

5.3.2. Materials and Methods

The Chelas (Lisbon) STP was chosen as study model as it receives large volumes of urban sewage but also variable amounts of industrial effluents, namely from companies located within the urban area. Moreover, the STP possess tertiary treatment, with activated sludge treatment and treated effluent is discharged to the Tagus estuary after Ultra-Violet treatment.

In the present study, four fiber-glass tanks (1m³) were set up in the selected STP (Fig. 5.3.1), with one of a series of treated sewage effluent dilutions produced by mixing different percentages of effluent with clean water (company water previously dechlorinated). Each tank was aerated and received a continuous water flow (8 Lmin⁻¹). Every week temperature, pH, conductivity and cumulative mortality were registered.

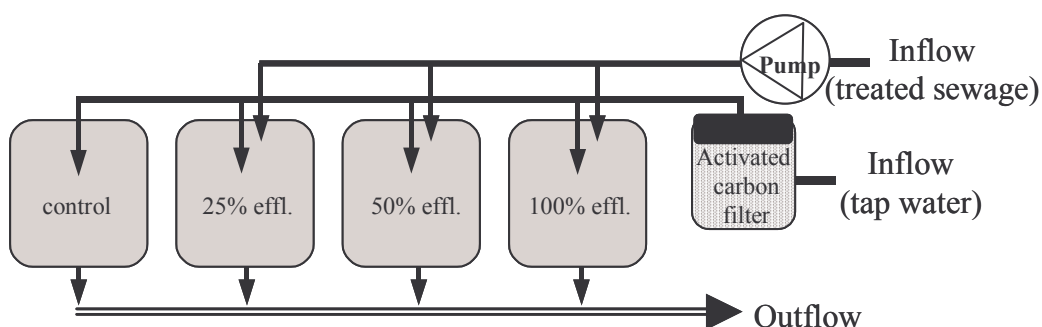


Figure 5.3.1: Flow diagram of experimental design set up in the STP.

Before the start of the exposure, crucian carps (*Carassius carassius*) purchased from national producers (“Estação Aquícola da Azambuja”) were acclimated in the laboratory for two weeks and fed with commercially available pellets (Alpis). This species was selected since is representative of national and European streams and lagoons but also because it is easy to obtain and maintain.

After the pre-acclimation period, eighty crucian carps divided in three different size classes (10-15 cm; 7-10 cm e 3-7 cm) were randomly distributed in each tank and daily fed with commercial pellets. In this study only the males were considered. Fish were exposed to 100%, 50% and 25% STP treated effluent mixed with tap water. A reference tank received only tap water.

At the end of the exposure period (60 days) fish were sampled and blood was collected from the caudal vein of each fish in every treatment, using heparinized syringes (2cc) treated with aprotinin (10 TIUml⁻¹). After a centrifugation at 4,000 g (15 min. at 4°C), blood plasma was stored at - 80°C until further analysis. Vtg levels were analyzed using a previous established direct ELISA protocol (Denslow et al., 1999), using a monoclonal anti-carp vitellogenin antibody (G10/5/1), developed by the “Laboratory of Immunology and Microbiology (LIM/ESE; University of Metz, France)”. To perform the ELISA, microplate (Nunc-Roskilde, Denmark) wells were coated with 50 µl of diluted carp plasma samples, at least 1:200 (for males) and 1: 1000 (for females) in phosphate buffered saline (PBS) solution to avoid interference from concentrated plasma. Each sample was plated in triplicate on the plate. The microplate was incubated overnight at 4°C and subsequently the microplates were washed three times (PBS in Tween- 0.05%) with a squirt bottle and the washes removed by flicking the plate over a sink. After incubation, for 2 h at room temperature with blocking buffer (10% BSA in PBS with 0.02% sodium azide) the plates were washed again. The primary antibody diluted to the appropriate concentration (0.1-5 µgml⁻¹) in blocking buffer was added to each plate well and incubated overnight at 4°C. After washing, the labeled secondary antibody (goat-antimouse immunoglobulin-IgG conjugated to alkaline phosphatase, SIGMA-Aldrich) diluted to the appropriate concentration (1:1000) in blocking buffer was added to wells and incubated for 2 h at room temperature. Then, the microplates were washed and the substrate (p-nitro-phenylphosphate – PNPP, SIGMA-Aldrich) was added (100 µl) to each microplate well and incubated at room temperature (10-30 min.). Finally, 50 µl of stop solution (3N NaOH) was added to stop the development process (positives appear bright yellow) and read at 405 nM in a microplate reader (BioRad-Benchmark,USA).

To determine the amount of Vtg in the plasma sample a calibration curve was constructed preparing standards by serially diluting the carp Vtg standard provided by LIM/ESE (University of Metz, France) in PBS to give a range from 10 to 1000 ngml⁻¹. For interassay control, it was used e a standard female plasma sample and diluted to the required

concentration to fall within the standard curve. This sample was used in every assay at the desired concentration to ensure that the assay is operating correctly. The coefficient of variation was calculated for each triplicate sample and, if it exceeded 15 percent, samples were runned again. Standard curves fit by log-linear regression were used to quantify Vtg concentration, with R² values of 0.96 to 0.99.

After the blood has been collected, length and weight of fish were measured. The gonadosomatic index (GSI) was calculated as percentage of total body weight. Fish were dissected; gonads were removed and placed in Bouin-Hollande fixative for 48 h, washed and placed in 70% alcohol. Samples were then processed to paraffin wax blocks using conventional techniques as described in Martoja and Martoja (1967). Sections (5-7 µm) were stained with haematoxylin and eosin (H & E). Male gonads were evaluated by histological observation, using a microscope (Leica-ATC 2000, Germany), and classified as being in one of four maturation stages according to the scale proposed by Gupta (1975).

Statistical analysis of the results was performed using one-way ANOVA, after checking data for assumptions of normality and homogeneity and log-transformation of data if necessary. To compare pairs of means it was used the post-hocTukey test in order to detect significant differences ($p < 0.05$). The software *Statistica 5.0* (Statsoft Inc., USA) was used in data analysis. To construct standard curve and determine vitellogenin concentrations it was used the *Microplate Manager 4.0* (BioRad Software) by extrapolating absorbances to standard curve.

5.3.3. Results

The results show that plasma Vtg levels (Fig.5.3.2) were significantly elevated ($P < 0.05$) in fish from all size classes, exposed to the different concentrations of treated sewage effluent, relative to control values. Data analysis also suggests a dose-dependent correlation in all size classes. However, correlation between size and Vtg values was not statistically significant ($r = 0.77$; $P > 0.05$) in exposed fish.

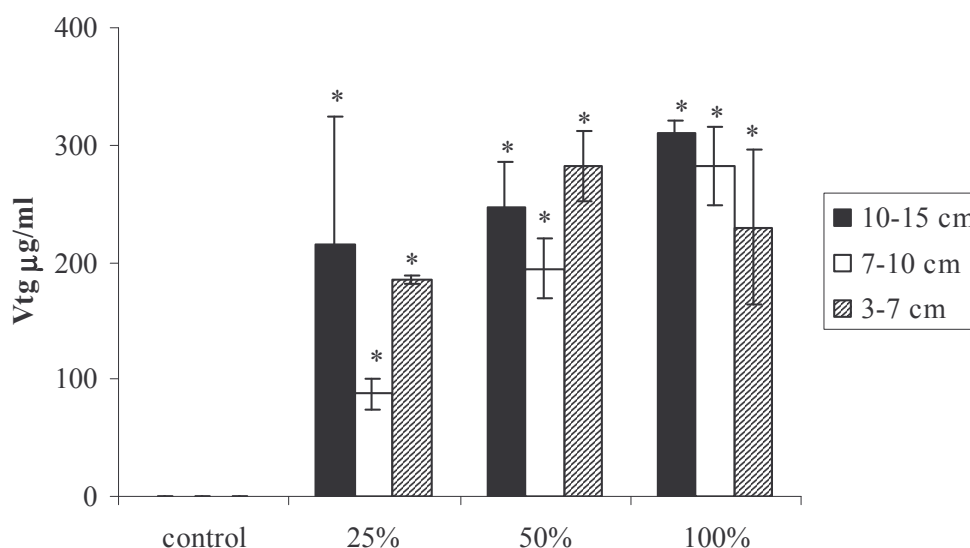


Figure 5.3.2: Vitellogenin levels in different fish size classes exposed to graded treated sewage effluent.

The lowest GSI (Fig. 5.3.3) values were found in fish exposed to 100% effluent treated effluent. The data show a significant reduction ($P < 0.05$) in GSI in all treatments and size classes in comparison with controls, which suggest a exposure effect to EDCs or/and other chemicals present in treated sewage, affecting growth and gonad development.

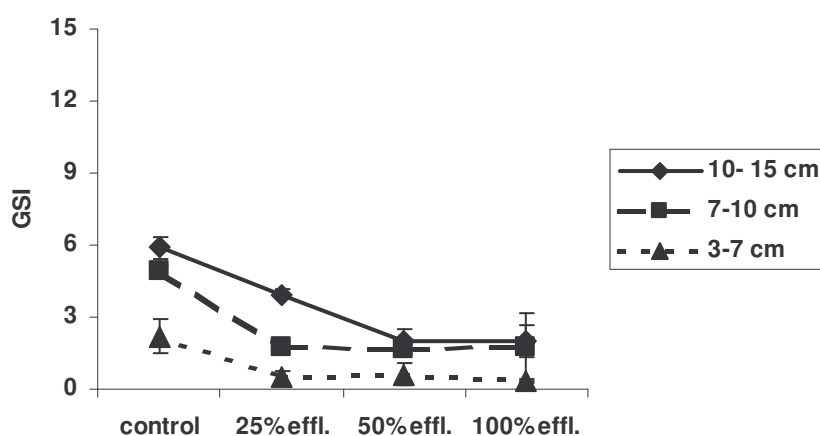


Figure 5.3.3: Gonadosomatic index (GSI).

Histological observation show that control gonads from size classes 10-15 cm and 7-10 cm were classified in stage 2 and 3 (maturing) (Fig.5.3.4 a) showing a normal appearance. Size class 3-7 cm were classified in stage 1 and 2 (immature and maturing respectively).

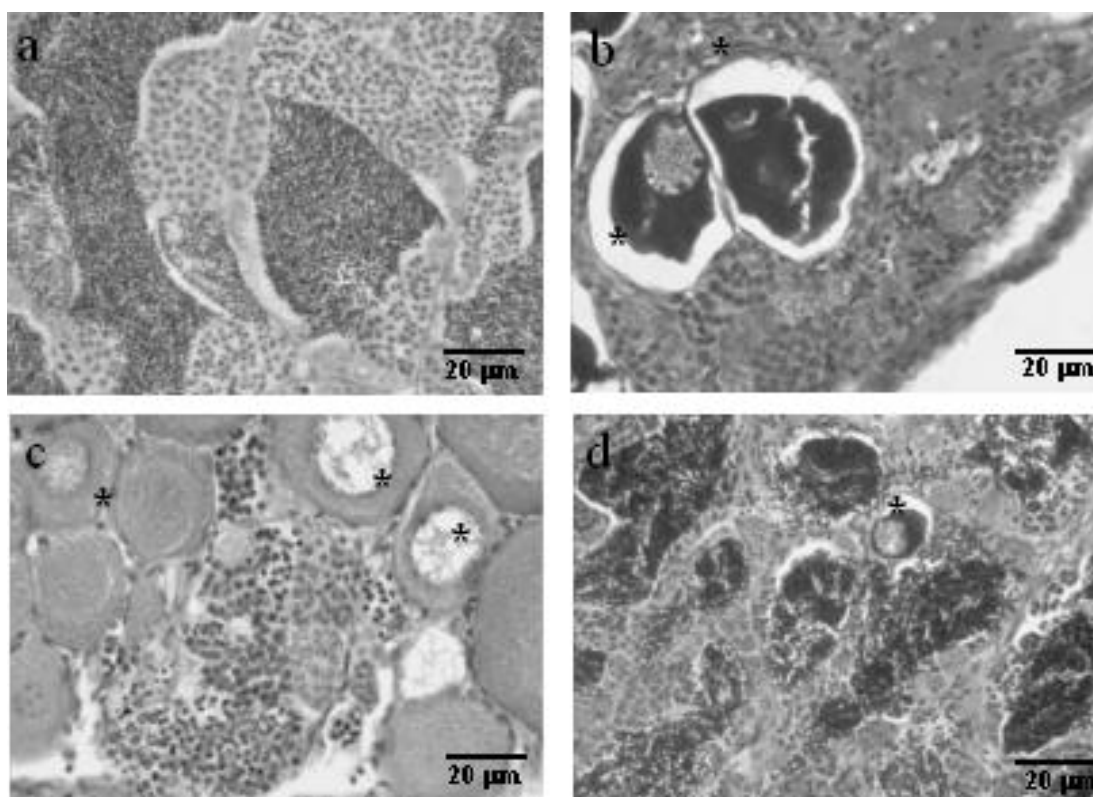


Figure 5.3.4: Morphological effects in fish testis (*Carassius carassius*) exposed to 100% treated sewage effluent; a- control testis (10-7 cm); b- developing oocytes (10-7 cm); c- massive oocyte development (7-3 cm). d- small developing oocyte (15-10 cm): Developing oocytes (*).

Histological analysis show that treated sewage effluent caused spermatogenesis inhibition and germinative cells hypertrophy in all size classes. These adverse effects seems to increase with increasing effluent concentrations. In addition, developing oocytes within testis were detected (Fig. 5.3.4 b,c,d) from fish exposed to the different effluent concentrations.

Table 5.3.1, show intersex percentages, detected in each size class exposure treatments. The highest percentages of ovo-testis were observed in the smaller size class (3-7 cm).

Table 5.3.1: Intersex percentages.

Size class/Effluent dilution	15-10 cm	10-7 cm	7-3 cm
Control	0%	0%	0%
25% effluent	25%	25%	33%
50% effluent	25%	25%	33%
100% effluent	50%	50%	75%

5.3.4. Discussion

The treated domestic effluent possess estrogenic activity, as shown by the Vtg induction in all size classes of fish exposed. Similar studies also reported Vtg induction in male fish as consequence of sewage effluent exposure and can be indicative of endocrine disruption (Routledge et al., 1998; Harries et al., 1999; Tilton et al., 2002).

The reduction in GSI and histological changes detected in exposed fish are often referred in endocrine disruption reports as a consequence of exposure effects to environmental chemicals present in sewage (Tyler et al., 1998; Hemming et al., 2001). Furthermore, studies from other authors show similar effects on gonads, such as spermatogenesis inhibition and sometimes the development of oocytes inside testis (Folmar et al., 2001; Cardinalli et al., 2004).

It is known that sex reversal of gonochoristic fishes by exposure to sex steroids highlights both the plasticity of their gonads and their vulnerability. Studies developed with eels (*Anguilla anguilla*) and flounder (*Verasper moseri*) treated with high doses of estradiol showed that, male feminization can occur even after testicular development had been initiated (Devlin, 2002). Furthermore, Gimeno et al. (1998) demonstrated that exposure of mature male carp to the endocrine disruptors can result in degeneration of the testis and intersex.

The intersex condition is the result of exposure to high estrogenic environments (Tyler et al., 1998), such as STP effluents, clearly showing a reproductive effect of the endocrine disruption action. However, the results from intersex percentages suggest that smaller fish are apparently more susceptible to the treated sewage effluent than bigger fish. This can be related with the possibility that smaller fish have not yet completed gonadal differentiation. Additionally, several experiments indicated that redetermination and redevelopment of the gonad from residual tissue (gonadal duct) can proceed in a direction opposite to the original

genetic sex. This concept is also supported by the observation that PGCs (Primordial Germ cells) can be found in differentiated ovaries, testes, and ovotestes of *Liza aurata*, *Serranus hepatus*, *Coris julis* and *Xiphophorus sp.* The presence of PGCs at many stages of gonadogenesis suggests as well that potential for sex reversal and redifferentiation may be maintained throughout the reproductive lifespan of many fish. This can also explain the occurrence of intersex in higher size classes which are completely differentiated. Moreover, the source of cells involved in the redifferentiation of gonads is of considerable interest. It is not clear whether individual differentiated cells (spermatogonia or oogonia) are able to switch developmental fates during sex reversal, or whether undetermined germ cells within the gonad are recruited to initiate a new course of differentiation (Devlin and Naghama, 2002).

As a concluding remark, this study also shows that there is a strong need of a better knowledge of the EDCs effects, present in the discharged effluents, at the population level to have more knowledge concerning negative impacts on aquatic ecosystems.

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PART VI

CHAPTER 6 - EUROPEAN LEGISLATION AND POLICY ACTION ON ENDOCRINE DISRUPTORS

6. European Legislation and Policy action on Endocrine Disruptors

6.1. Introduction

The EU is the largest chemical producing area in the world, accounting for 32% of an estimated global turnover for chemical production of EUR 1 632 billion in 2001 (CEFIC, 2002). During much of the 1990s, the EU chemical industry grew faster than GDP (Gross Domestic Product), with total chemical production growing more strongly than other EU industry sectors over the past 10 years (3.2% per year), and faster than the chemical sectors of the US (2.4% per year) and Japan (1.4% per year) (CEFIC, 2002). The driving force behind this growth is the stimulation of consumer demand for products based on new uses of chemicals and the availability of many feedstocks (ethylene, benzene, propylene etc.) that are produced by the petrochemical industry (EEA, 1998). Figure 6.1 shows the volume of APEOs imported by EU countries (Eurostat, 2004).

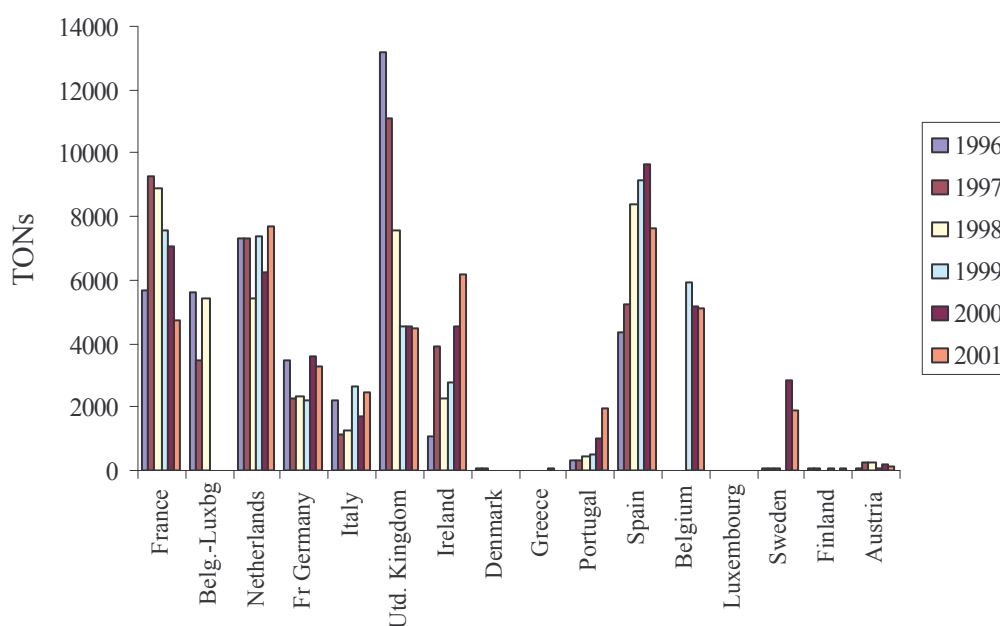


Figure 6.1: Volumes of APEOs imported by EU countries.

The chemical industry supplies a vast range of chemicals to virtually all sectors of the economy although the exact number of substances marketed within Europe is not known. The European inventory of existing chemical substances (EINECS) compiled by industry in 1981 identified 100 195 chemicals that year (although it is uncertain how many were actually marketed). Approximately 3 000 ‘new’ substances have been brought onto the European

market since that time (European Commission, 2001). A large proportion (about 30%) of manufactured chemical products are consumed or further processed within the chemical industry itself. Basic chemicals undergo further treatment to be converted into chemical additives suitable for a variety of industrial, agricultural and consumer products. These include products with high added value such as medicines, adhesives, paints, dyes, plastics, fertilisers, pharmaceuticals, cosmetics and household products.

The use and disposal of products containing hazardous chemicals has been linked to a range of potential impacts on the environment and human health. Concern is greatest for highly persistent chemicals, which may remain in the environment for many years, in particular those that can bioaccumulate in wildlife and humans. Table IX.1 (Annex IX) provides some examples of environmentally persistent chemicals and their main uses. Despite these concerns and the availability of a certain amount of key data within the chemical industry, there is still very little robust and detailed information on pan-European production and import/export volumes of 'hazardous' chemicals, which is accessible to policy-makers and the public. However, EU production volumes of selected toxic chemicals (i.e. those classified as carcinogenic, teratogenic, mutagenic and reprotoxic, or CMR substances, according to EU Directive 67/548/EEC) increased during the 1990s, together with total chemical production. Figure IX.2 (Annex IX) shows the volumes of chemical contraceptives imported by EU countries (Eurostat, 2004).

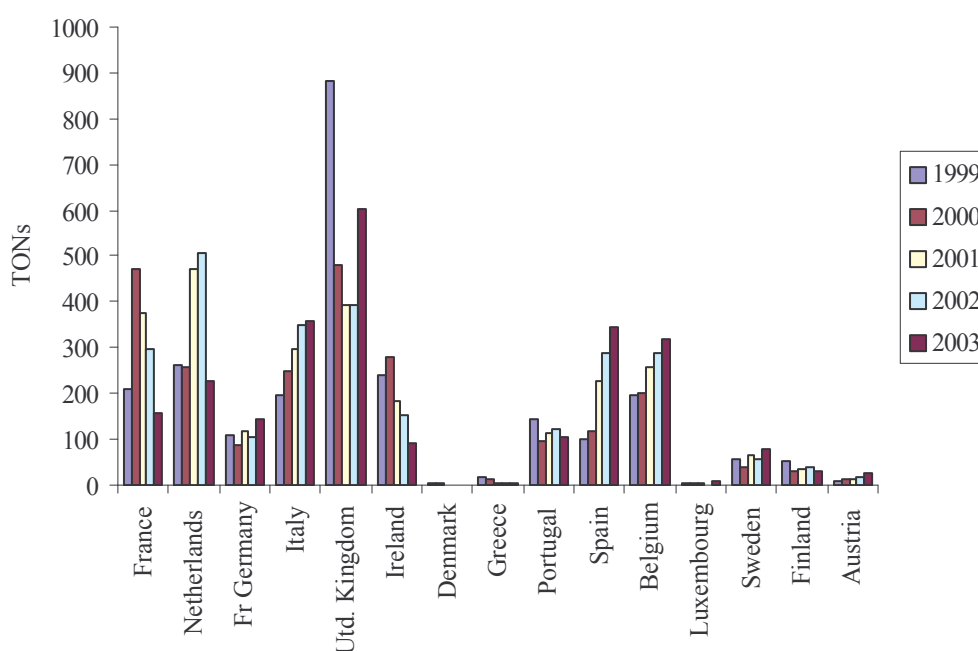


Figure 6.2: Volumes of chemical contraceptives imported by EU countries.

In receiving waters, sewage effluent is subjected to dilution and any EDCs present are susceptible to transformation processes including microbial activity, sorption, and photocatalysis. Currently, the dilution and self-purification processes of the receiving waters are greatly depended upon for the removal from the hydrological cycle of the EDCs which sewage treatment Plants (STPs) have failed to remove (Gomes and Lester, 2003).

The European Parliament has called for a precautionary approach to the endocrine disrupting issue. There is a wide legislative framework that can be amended to control EDCs (Annex IX; Table IX.3) and certain recent directives include direct references to them. The Integrated Pollution Prevention and Control Directive (96/61/EEC), includes a special reference to substances that “may effect reproduction in or via the aquatic environment”. Table IX.2 (Annex IX) shows some of the ecological impacts of chemicals and possible associations with them. EC directives are not legally enforceable within member states and require implementation through national legislation, no less stringent than agreed in the directive (Gomes and Lester, 2003).

6.1.1. The European Community Approach

Under existing EU legislation, assessment of the toxic potential of a chemical is dependent on the type of chemical. For example, pesticides and biocides have separate legislation governing their testing and assessment.

In general, prior to marketing, chemicals are required to undergo a very specific programme of testing and assessment in order to ensure that once in use or released into the environment, they pose no danger to people or wildlife. Under this approach, chemicals undergo testing (hazard identification), the results of which are used in a risk assessment. The third level determines whether restrictions are needed on the use of the chemical (risk management).

Prior to adoption by the Commission in the form of directives, those test results are validated at the international level by the OECD.

While taking account of detrimental endocrine-related effects on reproduction or in diseases such as cancer, this existing legislation does not use disruption to the endocrine system as an endpoint *per se*.

Under the existing EU legislation:

Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances requires that: -Dangerous substances must be classified into one or several of 15 defined categories of adverse effects; -The packaging must be adequate to protect people and the environment from the known dangers; -The substance must be labelled with the dangers and with safety information ([http:// Europa-Environment-Endocrine disrupters-strategy5.htm](http://Europa-Environment-Endocrine-disrupters-strategy5.htm)).

Currently, there are 15 danger categories under which chemicals can be classed. A battery of tests that are specified under this directive define these categories and include tests for carcinogenicity, reproduction and ecotoxicity ([http:// Europa-Environment-Endocrine disrupters-strategy5.htm](http:// Europa-Environment-Endocrine-disrupters-strategy5.htm)).

However endocrine disruption is not addressed under the classification system nor are there (yet) any agreed test methods to detect whether a chemical has endocrine disrupting properties ([http:// Europa-Environment-Endocrine disrupters-strategy5.htm](http:// Europa-Environment-Endocrine-disrupters-strategy5.htm)).

Directive 88/379 EEC addresses the classification, packaging and labelling of dangerous preparations for various groups of chemicals. This has now been amended to include pesticides. Such preparations must be labelled using risk phrases, which denote a range of biological effects such as irritation, cancer and effects on reproduction ([http:// Europa/Environment-Endocrine disrupters-strategy5.htm](http://Europa/Environment-Endocrine-disrupters-strategy5.htm)).

There is insufficient data to conduct a basic risk assessment for 86% of EU high production volume chemicals (ECB, 1999). The threat that chemical releases may pose to humans and the environment cannot, in many cases, be assessed. It must be remembered, too, that the absence of evidence (of ill effects) is not the same as evidence for the absence of such effects (EEA, 2001). A number of wider questions remain that Wallström (2002) and others have raised, e.g.: - How can risks be combined to reflect different types of exposures and cumulative impacts?; - How can we account for interactions between host and exposure factors (including genetic, lifestyle and host susceptibility)?; - What options are there for developing policies that address mixtures of chemicals and the ‘cocktail effect’?; - What are the current research priorities: exposure pathways and low dose impacts, or mechanisms of action?

6.1.2. Legislative Action

The first recognized action from the European Union (EU) on the issue of endocrine disruptors was the workshop ‘The impact of endocrine disruptors on human health and wildlife’ that was held in Weybridge, UK, on 24 December 1996. At this workshop a definition of endocrine disrupter was endorsed by the EU: “An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism or its progeny, consequent to changes in endocrine function”.

The relevant chemicals identified in this first workshop were classified in several groups: (a) organohalogens (e.g. dioxins, polychlorinated biphenyls (PCBs) etc.), (b) bisphenol A, phthalates and alkylphenol, (c) steroids, (d) pesticides and (e) phytoestrogens. In a review of current research and monitoring activities, the priorities established were the analysis of STP effluents (domestic and industrial) and of the effects on freshwater fish and the analysis of sediments.

The Commission and Member States continue to participate in the OECD Endocrine Disrupter Testing and Assessment Task Force, which was set up in 1998 with the goal of developing agreed test methods for endocrine disrupters ([http://: Europa-Environment-Endocrine disrupters-documents3.htm](http://Europa-Environment-Endocrine-disrupters-documents3.htm)).

In October 1998, the European Parliament adopted a Resolution calling upon the Commission to take action on the issue of endocrine disrupters (EDs). Among the aims of the Resolution were: - an improvement in the legislative framework, - reinforcement of research efforts and - an increased effort to make information available to the public ([http://: Europa-Environment-Endocrine disrupters-documents3.htm](http://Europa-Environment-Endocrine-disrupters-documents3.htm)).

In March 1999, the Scientific Committee for Toxicity, Ecotoxicity and the Environment (SCTEE) issued a report, “Human and wildlife health effects of endocrine disrupting chemicals with emphasis on wildlife and on ecotoxicology test methods”. The report identified a 'potential global problem' for wildlife. It also stated that “impaired reproduction and development causally linked to endocrine disrupting substances are well-documented in a number of wildlife species and have caused local and population changes” ([http://: Europa-Environment-Endocrine disrupters-documents3.htm](http://Europa-Environment-Endocrine-disrupters-documents3.htm)).

Following the Weybridge workshop, the EU prepared a strategic document entitled: “A Community Strategy for Endocrine Disruptors”, published in December 1999 and adopted in March 2000. This document was implemented by the Commission following the adoption by the Commission of a Communication to the Council and European Parliament (COM (1999)706). In this document the need to develop and validate appropriate environmental monitoring tools was recognized. The need to establish monitoring programs was also indicated.

During 2000, a list of candidates containing 553 man-made substances and 9 synthetic/natural hormones was compiled. The list was divided into three separate groups of substances, depending on the level of information available, and a list of priorities for action was drawn up to further evaluate the role of these substances in endocrine disruption (COM(2001)262).

Two major classes of substances were identified as the most important in causing endocrine disruption: (a) natural hormones, like progesterone, testosterone, and phytoestrogens, and (b) synthetically produced hormones, like oral contraceptives, and synthetic chemicals designed for industrial use. Part of the legislative action proposed in this document was that the Water Framework Directive (WFD) should include EDCs in the list of priority substances. Basically this document urges adoption of a strategy based on the precautionary principle. The new priority list of 32 substances, which are part of the WFD compounds now includes EDCs like nonylphenol, di-2-ethylhexylphthalate (DEHP) and polybrominated diphenyl ethers (PBDEs) (<http://Europa-Environment-Implementation of the Community Strategy for endocrine disruptors.htm>).

The Commission also held meetings with WHO and the US EPA in 2000 with a view to enhancing international cooperation.

Regarding other short-term action, the Commission held a European workshop on endocrine disruptors with the sponsorship of the Swedish Ministry for the Environment, Swedish National Chemicals Inspectorate (KEMI), OECD, WHO and European Environment Agency. The workshop took place from 18-20 June 2001 in Aronsborg - Balsta (Sweden) and focused on: - monitoring, particularly the need to study “hot spots”, especially near STPs; - research and development, by developing integrated analytical chemistry/toxicity identification evaluation, and the need to develop analytical methods for the detection and quantification of EDCs in complex matrices; - strategy and international cooperation.

In June 2001, the Commission adopted a Communication on the implementation of the "Community Strategy for Endocrine Disrupters - a range of substances suspected of interfering with the hormone systems of humans and wildlife". The COM (2001)262 was adopted in 2001.

Under the 5th Community Framework Programme for R&D (1999-2002), research into endocrine disruption has been prioritised in the latest revisions of the relevant work programmes. In addition, a *dedicated call for research proposals* on the health and environmental implications of endocrine disruptors was published in May 2001 with a budgetary envelope of 20 MEURO (<http://Europa-Environment-Implementation of the Community Strategy for endocrine disruptors.htm>).

Finally, regarding *legislative action*, the proposed revision of the General Product Safety Directive proposes, *inter alia*, a simplification of conditions and procedures for urgent measures at Community level. In addition, the issue of endocrine disruptors is addressed specifically in the context of new and existing legislation covering water policy and in the recent White Paper on a strategy for a future chemicals policyb(<http://Europa-Environment-Implementation of the Community Strategy for endocrine disruptors.htm>).

Table 6.1.1 summarizes the most important EU legislative action on the endocrine disruptor issue.

Table 6.1.1: summary of legislative action on EDCs.

Date	Policy and document reports
October 1998	European Parliament adopted Resolution A4-0281/98 on endocrine-disrupting chemicals
4 March 1999	The European Commission Scientific Committee for Toxicity, Ecotoxicity and the Environment (SCTEE) presented its Opinion "Human and Wildlife Health Effects of Endocrine Disrupting Chemicals, with emphasis on Wildlife and on Ecotoxicology test methods" (SCTEE 1999)
December 1999	The Commission adopted a Communication to the Council and to the European Parliament on a Community Strategy for Endocrine Disrupters: a range of substances suspected of interfering with the hormone systems of humans and wildlife (COM (1999) 706)
30 March 2000	Council of Ministers (Environment) adopt Council Conclusions on the Commission Communication COM(1999)706
July 2000	Report (A5-0197) of the Committee on the Environment, Public Health and Consumer Policy of the European Parliament on the Commission Communication to the Council and to the European Parliament on a Community Strategy for Endocrine Disrupters (COM (1999) 706 final)
26 October 2000	European Parliament adopted Resolution A5-0197/2000 on endocrine disrupters, emphasising the application of the precautionary principle and calling on the Commission to identify substances for immediate action
June 2001	The Commission adopted a Communication to the Council and to the European Parliament (COM(2001)262) on the implementation of the Community Strategy for Endocrine Disrupters: a range of substances suspected of interfering with the hormone systems of humans and wildlife (COM (1999) 706)

The EU wants to connect its EU Environmental Policy with its EU Research Policy in the field of water EDC pollution. Thus, the new precautionary strategy will contribute to building a strong global position for Europe in the field of water quality (Petrovic et al., 2001).

6.1.3. EU strategy on endocrine disruption

6.1.3.1. Short-term strategy

One of the first key short-term actions identified in the European Commission's Communication (COM(1999)706) is the establishment of a list of priority substances for further evaluation of their role in endocrine disruption. The priority list was to be established in two steps: firstly an independent review of evidence relating to the issues of endocrine-disrupting effects and human/wildlife exposure and secondly a priority-setting exercise in consultation with stakeholders and the Commission's scientific committees. The establishment of this list is managed by the Environment DG of the European Commission and is ongoing ([http:// Europa-Environment-Endocrine disrupters-Strategy3.htm](http://Europa-Environment-Endocrine%20disrupters-Strategy3.htm)).

Short-term action also encompasses international co-operation and the need to inform the public.

Informing the public: Increased activities in the field of research, sponsored by the European Commission's Research DG, will considerably increase the visibility of endocrine disruption, as project results are made available to the public through leaflets, press releases, websites and workshops. The European Commission has also created two websites: the endocrine disrupter website and the present endocrine disrupter research website ([http:// Europa-Environment-Endocrine disrupters/](http://Europa-Environment-Endocrine%20disrupters/)).

International co-operation: International initiatives have been established under the short- and medium-term activities of the Commission's strategy. Under the short-term action, the exchange of information and international co-ordination is essential if the scientific community is to remain abreast of research results and avoid duplication, as well as being necessary to ensure that international trade agreements are not infringed when policy actions are being considered ([http:// Europa-Environment-Endocrine disrupters-strategy3.htm](http://Europa-Environment-Endocrine%20disrupters-strategy3.htm)).

The EU-US Science and Technology Agreement: Under this agreement, a joint meeting was held in Italy in 1999. Common research priorities were identified and, subsequently, funding for research was made available by the Commission and the US Environmental Protection Agency (US EPA) ([http://: Europa-Environment-Endocrine disrupters-Strategy2.htm](http://Europa-Environment-Endocrine%20disrupters-Strategy2.htm)).

Through the International Programme for Chemical Safety (EU/WHO/IPCS) the European Commission and the World Health Organisation (WHO) have co-operated on the maintenance of a global research inventory, which is housed at the Commission's Joint Research Centre, and on the compilation of a "Global Assessment of the State-of-the-Science of Endocrine Disruptors", published in 2002.

Both the European Commission and the WHO are committed to supporting the efforts of the Organisation for Economic Co-operation and Development (OECD) in developing agreed test methods for endocrine disrupters. The European Commission works closely with EU Member States to coordinate EU input at the OECD.

In December 2003, the Japanese Ministry of the Environment and the WHO/UNEP/ILO International Programme on Chemical Safety hosted a joint workshop in Tokyo on "Endocrine Disruptors: Research Needs and Future Directions". The final report is available. It followed an International Symposium on Environmental Endocrine Disrupters held in Sendai, Japan.

In 2003 the Commission finalised a study on "Information Exchange and International Coordination on Endocrine Disrupters" carried out by the MRC Institute for Environment and Health (UK), with the remit *to assess the global initiatives on Endocrine Disruptors, concerning regulatory, testing and research activities, and make recommendations on ways to improve coordination and the sharing of the workload*. The study comprised a number of interrelated elements. Information on the activities, opinions, approaches and outputs of various countries and stakeholder organisations was obtained through interviews, supplemented with data from websites. The information obtained was subjected to detailed critical analysis to compare and contrast the EC approach with those of its Member States and the US and Japanese governments.

6.1.3.2. Medium-term strategy

6.1.3.2.1. Medium-term action

Medium-term action includes:

Identification and assessment of EDCs. The availability of agreed test strategies/methods to identify and assess endocrine-disrupting chemicals is a basic requirement for comprehensive legislative action aimed at protecting people and the environment from the potential dangers posed by these chemicals.

Research and development. Further research is needed on test methods and testing strategy; the effects on humans/wildlife; the ED mechanism; the effects at key stages of a life cycle; models to estimate exposure; and the development of monitoring tools).

Studies should focus on the links between exposure to endocrine disrupters and the specific potential effects on human health (decrease in semen quality and increase in hypospadias, testicular and prostate cancer in males and breast and ovarian cancers in females). A more detailed assessment of the extent to which humans are exposed to endocrine disrupting chemicals is needed. Regarding wildlife, further studies are needed on the effects of endocrine disrupters on amphibians, reptiles and birds. For fish, a European comparison of endocrine disruption effects is needed, though methods need to be harmonized beforehand. Test methods for invertebrates are also needed. Studies need to look at the full range of possible endocrine disruptive effects and not just oestrogenic action, e.g. the effects on immune systems, behaviour and neurological development. Greater use should be made of genomics, proteomics and metabonomics. Finally, analytical methods and procedures need to be improved to allow an assessment of exposure to endocrine disruptors ([http://: Europa-Environment-Endocrine disrupters-documents2.htm](http://Europa-Environment-Endocrine-disrupters-documents2.htm)).

Critical windows of exposure in humans and wildlife need to be identified and methods are needed to identify sensitive individuals within populations.

Low dose effects (where unusual dose-response curves occur) also need to be investigated. Furthermore, the extent to which exposure to multiple chemicals in mixtures can lead to

unexpected effects requires more investigation. Finally, there is still a need to decide what level of change is actually important.

As a result, the Research DG of the European Commission has substantially increased spending on research projects linked to endocrine disruption ([http://: Europa-Environment-Endocrine disrupters-documents2.htm](http://europa-environment-endocrine-disrupters-documents2.htm)).

6.1.3.3. Long-term strategy

Long-term action focuses on **legislative action**. Policy action is needed to further substantiate suspected harmful effects and then control the production of harmful substances. Independent advice, e.g. that of the SCTEE or committees replacing it in the near future, will be sought and legislative instruments will be adapted to cover the hazard identification, risk assessment and risk management stages. In this context, both the REACH and the SCALE initiatives will provide new legislative support. Further information on the policy relevance of endocrine disruption can be found at http://europa.eu.int/comm/environment/endocrine/index_en.htm.

6.1.3.3.1. International development and validation of tests and test strategies

EU/OECD/US: the Commission participates in the Task Force on Endocrine Disrupters Testing and Assessment (EDTA), which was set up to co-operate with other national and international bodies in developing and validating new tests and test strategies that are complimentary and will be recognised and accepted by different international and national organisations.

EU-US Science and Technology Agreement: Under this agreement the Commission and the US EPA have agreed to share information on priority-setting for chemicals, screening and testing development and validation, and research activities.

6.1.3.3.2. Funding and policy issues

As the endocrine disrupter issue will require long-term studies, the question of how to fund such work needs to be resolved. Table 6.1.2 summarizes a set of needs:

Table 6.1.2: Summary of policy issues and needs regarding the endocrine disrupter issue.

Policy issues/Needs
Need to check whether changes in regulation of chemicals actually have a measurable effect in the environment
Development of test methods and testing strategies
There are outstanding needs for changes in the testing process:
Include whole animal tests for all possible endocrine disrupting effects
Focus on test methods assessing reproductive function and developmental effects
Refine the existing chronic mammalian designs and improve the endpoints to better cover sensitive periods of the life cycle
OECD activities do not yet cover all of the issues
Tests are needed for the adrenal system, pheromone signalling and the neuro-endocrine/immune systems
Tests on a wider range of invertebrates are needed
Alternative (but carefully validated) techniques, such as SARs and in vitro systems, should be developed
The testing strategy needs to consider not only chemical production volume to define what tests are needed, but should also consider patterns of exposure, persistence, bioaccumulation and toxicity
Homologous series of chemicals (i.e. ones having similar structures and properties) need to be tested
A simple framework to determine testing needs should be developed
Discussions should begin on developing an EU strategy for testing endocrine disrupters
The Annex V Committee to 67/548 should coordinate EU views and activities on test methods and strategies to feed into the OECD

6.1.3.3.3. Establishment of monitoring programmes

In humans, large-scale epidemiology studies are needed to look at altered reproductive function (e.g. sperm counts, semen quality, fertility rates), malformations, female breast cancer and endometriosis and adult male testicular cancer. Besides, further use should be made of registers of reproductive effects and malformations to look for time and place trends in incidence. Thus, extensive, but cost-effective, schemes are needed to monitor changes in reproductive parameters.

For environmental monitoring, "hot spots" should be targeted for study to identify causative agents. Moreover, programmes are needed to provide background effect levels and exposure levels and models are needed to predict where 'hot spots' may occur in the environment.

6.1.3.3.4. Risk assessment

The Risk Assessment process assesses the probability that a chemical may harm human health or the environment. Data from tests carried out in hazard identification exercises are used with the calculated environmental levels of the chemical to find out whether the proposed use of the chemical will pose a threat to human health or the environment. In 2001, the Commission presented its 'Strategy for Chemicals', which when adopted will supersede risk assessment, as it now exists ([http://: Europa-Environment-Endocrine disrupters-strategy4.htm](http://Europa-Environment-Endocrine-disrupters-strategy4.htm)).

The Commission has several regulations and directives in place that govern the risk assessment of specific types of chemicals and provide a framework by which all chemicals are assessed according to a uniform/similar standard (Table 6.1.3). The regulations and directives are as follows:

Table 6.1.3: Risk assessment regulation and directives from the Commission.

Commission regulations and directives on risk assessment	
Risk assessment of existing substances	Regulation (EEC) No. 793/93
Restrictions on marketing and use of certain dangerous substances and preparations	Directive 76/769/EEC
Placing of plant protection products on the market	Directive 91/414/EEC
Marketing of Biocidal products	Directive 98/8/EC
Materials and articles intended to come into contact with food stuffs	Directive 89/109/EEC and its specific directives

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Endocrine Disruptor Compounds Website. <http://Europa/Environment-Endocrinedisrupters-strategy5.htm>, (2003); [http://Europa/Environment-Endocrine disrupters-strategy3.htm](http://Europa/Environment-Endocrine%20disrupters-strategy3.htm), (2003); [http:// Europa/Environment-Endocrinedisrupters-strategy2.htm](http://Europa/Environment-Endocrinedisrupters-strategy2.htm), (2004); [http:// Europa/Environment - Endocrinedisrupters-strategy4.htm](http://Europa/Environment-Endocrinedisrupters-strategy4.htm), (2003); [http:// Europa/Environment-Endocrine disrupters - documents2.htm](http://Europa/Environment-Endocrine%20disrupters%20documents2.htm), (2003); [http:// Europa/ Environment-Endocrinedisrupters-documents3.htm](http://Europa/Environment-Endocrinedisrupters-documents3.htm), (2003); [http://Europa/Environment-Implementation of thecommunity strategy for endocrine disruptors.htm](http://Europa/Environment-Implementation%20of%20thecomunity%20strategy%20for%20endocrine%20disruptors.htm), (2003);

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PART VII

CHAPTER 7 - GENERAL DISCUSSION AND CONCLUSIONS

7. General Discussion and Conclusions

The presence of endocrine-disrupting chemicals in the freshwater environment is regarded as a matter of concern by many scientists and water quality regulators (Johnson and Sumpter, 2001). Treated and non-treated wastewaters are major contributors of pollutants to aquatic ecosystems. It is also known that the effluents which STPs discharge into rivers can contain estrogenic contaminants at sufficiently high levels to induce vitellogenin biosynthesis in male fish (Jobling et al., 1998). Among these, natural and synthetic estrogens are already effective at the lower ngl^{-1} level (Purdom et al., 1994; Routledge et al., 1998), while most other chemicals with an estrogenic effect are biologically active at the μgl^{-1} level (D'Ascenzo et al., 2003). Several studies have shown that birds, reptiles and mammals in polluted areas also undergo alterations in the endocrine–reproductive system (Preziosi, 1998).

It is also to be noted that the efficiency of modern sewage treatment is such that the overall organic loading of receiving waters is low. Indeed significant improvements in water quality have been achieved over the past 50 – 100 years, particularly with the introduction of the activated sludge process. Together with other improvements, this has permitted the return of a range of previously absent flora and fauna to many freshwater systems (Johnson and Sumpter, 2001).

Thus, major improvements have been made in environmental science and technology with regard to sewage treatment plant processes and, consequently, the efficiency of removing chemical compounds. On the other hand, information on the removal of EDCs in sewage treatment plants is very scarce or not completely understood, a circumstance that hinders the calculation of mass balance estimates, which is essential to any forecast of environmental impacts on aquatic systems.

Sewage treatment plants receive a large spectrum of chemical compounds from domestic and industrial wastes, and possibly many more compounds that are not known to have estrogenic activity. Many of these EDCs are not totally eliminated during the treatment processes (Ternes, 1998; Ternes et al., 1999a). In the effluents of the STPs, a complex mixture of molecules including both the remainder of the partially eliminated wastewater molecules and the metabolites formed during treatment processes are finally discharged into the rivers. In this context, STP discharges are considered as a major source of estrogenic surface water pollution that may play a significant role in environmental contamination.

In addition to this, there is little information available on the effects of large wastewater discharges into the environment and our knowledge of effluent estrogenic potential is still very limited, particularly in aquatic systems.

Accordingly, to ensure that the environmental risk associated with these compounds is correctly interpreted, we must adopt an integrated approach involving, on the one hand, mass flow from its source to the STP, to soils and to ground water reserves and, on the other, the estrogenic responses of the organisms exposed.

Many environmental chemicals may act as endocrine disruptors (Sumpter, 1995; Jobling et al., 1998; Kime, 1998). These studies have included endpoints such as levels of circulating steroid hormones (Monosson et al., 1996; Parrott et al., 1999), circulating vitellogenin levels (Denslow et al., 1999; Hemming et al., 2001) and morphological changes such as those reflected in the gonadosomatic index and histopathology (Blazer, 2002).

This study focused on semi-field and laboratory assays for EDCs, providing a better understanding of the causes and effects of observed changes. It also focused on understanding the meaning of many of the histological observations made in wildlife studies, in terms of the reproductive success of fish populations.

7.1. Presence of Endocrine Disruptor Compounds in a Sewage Treatment Plant

The present study was carried out at the Chelas STP in Lisbon, which treats a mean flow of approximately 50,000 m³ of wastewater per day and processes mainly urban waste waters from about 211,000 inhabitants, though also variable amounts of industrial waste waters. In addition, the STP includes a tertiary treatment stage with filtration and disinfection by U.V. radiation.

It is very difficult or even impossible to identify all of the EDCs present in the STP effluent. Thus, numerous authors have attempted to detect and quantify the estrogenic potency of water samples either by targeting their research on specific chemicals, such as natural (17 β -estradiol) and synthetic (17 α -ethynylestradiol) hormones and/or alkylphenols (Solé et al., 2000), or by evaluating estrogenic activity (Korner et al., 1999).

Therefore, in this case, since identifying all the EDCs present in the STP was impracticable, a target-group of chemical compounds was chosen: alkylphenol ethoxylates (NP and OP), bisphenol A (BPA) and 17 β -estradiol (E2). They were selected on the basis of their known estrogenic potential, which has already been demonstrated in several studies.

Although different analytical techniques were used to evaluate the presence of the selected endocrine compounds, it was shown that the compounds screened were present in the effluent in varying concentrations throughout the treatment process and are discharged into estuarine waters at levels that may cause physiological effects in wildlife, as shown by both the ELISA and LC-MS-MS data. However, the estuarine waters may also have a diluting effect, reducing EDC concentrations and consequently the availability to their absorption by aquatic organisms.

The highest APE (NP and OP) levels were found in the incoming sewage and the lowest were recorded in the effluent. With respect to the final effluent, the APE ELISA kit results showed that data were overestimated on comparison with LC-MS-MS results. However LC-MS-MS results after filtration were higher than those with ELISA, a fact that cannot be easily explained. Nevertheless, the experiments carried out by Goda et al. (2000) also suggest that, because of the broader cross-reactivity, ELISA may be subject to interference that gives an overestimated value. Thus, different APEO concentrations recorded by the analytical techniques used to screen the WWTP effluent can be explained to some extent by the cross-reactivity phenomenon (Oubina et al., 1997).

The highest E2 level was detected after the primary sedimentation and slight variations in E2 values were detected along the WWTP stages. They are not significantly different and could be related in part to the wastewater microbiological activity and also the cross-reactivity with other steroids. Although, LC-MS-MS did not detect E2 (above the detection limit) that does not mean that the compound is not present in the STP effluent. A possible explanation is the low sample volume collected for the LC-MS-MS analysis for steroids, which was suitable for ELISA testing but insufficient for LC-MS-MS.

The highest BPA levels were found in the primary sludge (above detection limits) and concentrations underwent a significant reduction in the course of the treatment stages, indicating that BPA is efficiently removed from wastewater. Although the methods are not

comparable, ELISA and LC-MS-MS results showed similar levels of the compound, suggesting that the BPA kit is reliable and can be used safely.

We can also conclude that ELISA assays used in this study are suitable for routine screening of the selected chemicals and can be applied to EDC monitoring programs. However, care should be taken if the results are intended to provide absolute values or are to be used to assess the kinetics of EDC bio-removal.

Finally, the treated effluent is discharged into the River Tagus estuary. Considering that there are many river and estuarine organisms of commercial value that may bio-accumulate EDCs and be consumed by humans and, also, that river water is used in the production of drinking water, public health problems may arise from human exposure to estrogenic chemicals.

It can be suggested that the effluent is sufficiently diluted within the estuarine waters and no effects are expected to occur in wildlife or humans. On the other hand, it should be considered that, from its source, the River Tagus receives many treated and untreated effluents.

7.2. Laboratory Assays

7.2.1. Exposure to single chemicals

Laboratory experiments were carried out to assess the estrogenic potency of each individual EDC previously selected, by evaluating the estrogenic effects in fish. To carry out this evaluation several endpoints were used, such as vitellogenin induction, steroid analyses and histological changes.

All the chemicals that were tested showed the capacity to induce Vtg synthesis in males in a dose-dependent manner, indicating that all the selected chemicals are estrogenic. However, the results indicated that they possess different estrogenic potencies and suggested that E2 and EE2 are the most potent EDCs, which confirms other studies that presented similar findings (Rodgers-Gray et al., 2000; Bjerselius et al., 2001).

Regarding the female fish, the presence of abnormal concentrations of Vtg has also been suggested as a reliable and complementary biomarker of potential reproductive disturbances (Folmar et al., 2001). However, the present studies show that in general no significant

variations were observed in female plasmatic Vtg, with the exception of females exposed to NP and high doses of BPA. This is consistent with similar studies that exposed fish to different concentrations of STP-treated effluent (Rodgers-Gray et al., 2000; Solé et al., 2003).

In general, treatment with NP, OP, BPA, E2 and EE2 resulted in significant reductions in the male gonadosomatic index (GSI) compared with the respective control values and can be the result of the estrogenic action of the chemicals, which affects gonad growth. Moreover, the GSI decrease seems to be related in a dose-dependent manner. The correlation analysis also showed a significant negative relation between Vtg and GSI with regard to males in all treatment groups, which can mean a direct association between Vtg induction and negative effects in male testes.

With regard to the females, treatment with NP, OP and E2, BPA and EE2 also resulted in significant reductions in comparison with controls.

However, no significant differences in the male hepatosomatic index (HSI) were observed for any of the tested chemicals in all treated groups compared with controls, although a rising trend can be observed. Regarding female HSI, significant differences were observed for the fish exposed to OP and E2.

Histological examination of the testes revealed a similar dose-dependent pattern of the observed effects though it varied in intensity according to the exposure chemical. The most severe effects on testicular structure were detected in fish exposed to steroids (E2 and EE2). Less pronounced effects were observed in fish exposed to APEOs (NP and OP) suggesting that these compounds possess a weaker estrogenicity. In addition, testes from fish exposed to BPA showed identical changes comparatively to those exposed to alkylphenols (NP and OP).

For example, there was a reduction in the diameter of the seminiferous lobules and, in some, cases it was only possible to observe remains of spermatozoa in fish exposed to each chemical in a dose-dependent manner. The effect also varied in intensity according to the respective compound. Moreover, in fish exposed to the highest concentrations spermatogenesis seemed to be inhibited. In a few cases it was also possible to observe the absence of lumen in the lobules, which were composed mostly of spermatogonia. However, ovo-testis was only detected in male fish exposed to both steroids (E2 and EE2).

The results thus provide a better understanding of the action of EDCs in the endocrine system of fish involved in the experiment. An evaluation of the histological abnormalities caused by the exposure to EDCs will allow later comparison with histological changes found in wildlife exposed to environments polluted with EDCs.

As a concluding remark, the laboratory evidence of the estrogenic potency of these chemicals provides valuable information for comparing and understanding the estrogenicity of municipal STPs effluents that have been shown to contain natural estrogens and other estrogenic substances that disturb the reproduction of fish. The information can be also used for comparison purposes in field studies when it is suspected that endocrine disruption is occurring in aquatic ecosystems.

7.2.2. Exposure to a mixture of chemicals

It is known that aquatic biota is exposed to a complex mixture of chemicals rather than to a single one. These studies therefore show the relevance of studying mixtures as well as single chemicals, an approach which more realistically reflects the aquatic environment. The results enable the comparison with the effects found in field studies where chemical mixtures occur.

In some of these studies, the same target EDCs were chosen and mixed together. The results showed that Vtg was significantly induced in male carp exposed to all treatments of a chemicals mixture containing NP, OP, BPA, E2 and EE2. Moreover, Vtg induction occurred even at the lowest mixture concentration ($0.1 \mu\text{g l}^{-1}$).

Experiments using each chemical isolately showed that most probably the steroids were the most potent chemicals, which is in agreement with studies by other authors (Gimeno et al., 1998; Folmar et al., 2001; Van den Belt et al., 2003, 2004). It can also be suggested that most probably the major estrogenic effects were also caused by the action of steroids (E2 and EE2), the most potent estrogens present in the chemical mixture.

Most of the studies that use Vtg as a biomarker have been restricted to males. It has, however, been shown that females are also likely to be affected by xenoestrogens and therefore Vtg measurement can be used as a reliable and complimentary biomarker of potential endocrine disturbance (Kime et al., 1999). With regard to females, no significant differences were detected in Vtg induction, which may suggest that females are apparently, less affected by

exposure to a xenoestrogen mixture or, perhaps, that the duration of the experiment was too short.

As with the experiments performed with the chemicals singly, the chemical mixture caused a significant decrease in the male GSI for all treatments. In addition, significant differences were also found in the male GSI between fish exposed to 100 $\mu\text{g l}^{-1}$ and 0.1 and 1.0 $\mu\text{g l}^{-1}$. With regard to females no significant differences were found, probably because they are less affected by xenoestrogen exposure or need a longer exposure for the xenoestrogens to produce adverse effects.

The steroid 11-KT concentrations decreased in male plasma and showed a significant dose-dependent decrease in relation to the chemical mixture treatments. At the same time a significant increase was registered in male E2 plasma concentrations. Significant differences were also found in female E2 concentrations, which increased in a dose-dependent manner. Depressed values for 11-KT or T have also been found by other authors (Bjerselius et al., 2001; Folmar et al., 2001; Nichols et al., 2001), which suggests that it is the result of treatment blocking and is also related to the effects of E2 on the testes.

In addition, regressed testes and spermatogenesis inhibition and/or the intersex condition observed in fish exposed to the chemical mixtures confirmed findings from other studies that used different species of fish exposed to EDC (singly or binary mixtures) treatments which observed severe effects in testes, such as spermatogenesis inhibition (Gimeno et al., 1998; Panter et al., 1998; Gray et al., 1999).

Thus, the results obtained show that, for concentrations usually found in the STP effluents, significant effects can be observed in fish, a fact that emphasizes the importance of the treatment systems. On the other hand, it is important to assess the effects of substantially lower concentrations of the compounds, to simulate the conditions of the aquatic environment that receives the discharges of treated effluents.

7.3. Semi-field Studies

The semi-field studies were carried out by exposing different species of cyprinids to different concentrations of the treated sewage effluent. In addition, the influence of size, season and short and long-term exposure on fish exposed to treated effluent were also assessed. In the

current scientific literature, several reports show a correlation between reproductive abnormalities in fish and exposure to STP effluents, even several kilometers downstream of outfalls (Purdom et al., 1994; Harries et al., 1996; Jobling et al., 1998; Harries et al., 1999).

In all the experiments that exposed fish to the treated effluent of the Chelas STP, plasma Vtg was significantly induced in males and females. Furthermore, the vitellogenic response in exposed male fish revealed a dose dependence related to the STP treated effluent concentrations. Significant increases in plasma Vtg induction in all male and female fish exposed to the final treated effluent were also found during the winter and spring periods of the experiment, suggesting seasonal differences in estrogenic responses in fish. Hence, Vtg induction in male fish clearly indicates that EDCs are present in quantities that are capable of eliciting estrogenic responses in males.

The differences in the estrogenic responses in different seasons (winter and spring) can be attributed to factors such as differences in the rainfall registered in those periods, which also influenced the mean daily flow of the treated sewage effluent. Furthermore, the influence of climatic factors, such as rain and temperature, on the sewage effluent estrogenic potency has also been pointed out by other authors (Harries et al., 1999; Rodgers-Grey et al., 2000).

The experiments exposing fish of different sizes also resulted in a significant increase in Vtg levels. This occurred in fish from all size classes exposed to the different concentrations of treated sewage effluent. Moreover, analysis of the GSI revealed a fall for both males and females, with a more pronounced effect in fish exposed to 100% effluent. Therefore, the gonad growth seems to be affected by exposure to the effluent, suggesting that sewage compounds may have a negative effect on fish reproduction. In fact, the reduction in GSI and histological changes detected in exposed fish are often mentioned in endocrine disruption reports as a consequence of exposure to environmental chemicals in sewage (Tyler et al., 1998; Hemming et al., 2001).

Gonadal histology, in conjunction with hormone and vitellogenin measurements and morphological and fecundity studies, can provide insights into the effects of various environmental stressors on reproductive health (Blazer, 2002). Histopathological analysis of fish testes suggests a dose-dependent correlation, i.e. according to the different effluent concentrations, with a general progressive reduction in the normal gonad condition (GSI). The season and size studies indicated similar results. However, with respect to HSI, no significant

differences were detected. Therefore, sewage effluent estrogenicity may not in itself explain GSI and HSI data and it should be considered that effluent toxicity and other environmental parameters that were not measured but which could affect fish physiology.

In general, the histological analysis showed that treated sewage effluent caused spermatogenesis inhibition and germinative cell hypertrophy in all size-classes. Those adverse effects seem to increase with increasing effluent concentrations. Most of the fish exposed to 100% effluent showed inhibited spermatogenesis and in some cases the development of oocytes. This suggests a strong effect resulting from the sewage chemicals, which disrupt the reproductive functions of male fish. Fish exposed to 25 and 50 percent effluent dilutions present a progressive reduction in spermatogenesis and fewer spermatozoa in lobule lumina in comparison with controls. The estrogenic response seems to be less potent than in fish exposed to 100 percent effluent but may be enough to compromise reproductive success. These findings are also consistent with other authors who have shown similar effects on gonads, such as spermatogenesis inhibition and, sometimes the development of oocytes inside testes (Folmar et al., 2001; Cardinalli et al., 2004).

The intersex condition is the result of exposure to highly estrogenic environments (Tyler et al., 1998), e.g. STP effluents, a fact that very clearly exemplifies the reproductive effects of endocrine disruption. However, the intersex percentages involved suggest that smaller fish are more vulnerable to the treated sewage effluent, which can be related to the possibility that smaller fish have not yet completed gonadal differentiation. Additionally, several experiments indicated that redetermination and redevelopment of the gonad from residual tissue (gonadal duct) can proceed in the opposite direction to the original genetic sex. This concept is also supported by the observation that PGCs (primordial germ cells) can be found in the differentiated ovaries, testes, and ovotestes of some species (e.g. *Liza aurata*, *Serranus hepatus*, *Coris julis* and *Xiphophorus sp*). The presence of PGCs at many stages of gonadogenesis also suggests that the potential for sex reversal and redifferentiation may be maintained throughout the reproductive lifespan of many fish. This may also explain the occurrence of intersex in higher size-classes, which are completely differentiated. Moreover, the source of cells involved in the redifferentiation of gonads is of considerable interest. It is not clear whether individual differentiated cells (spermatogonia or oogonia) are able to switch their developmental destiny during sex reversal or whether undetermined germ cells within the gonad are recruited to initiate a new course of differentiation (Devlin and Naghama, 2002).

With respect to females, atretic oocytes and in some cases oocyte degeneration were observed which could be the result of exposure to EDCs or other unknown chemical compounds, with the toxicological effects on gonads affecting the normal development of oogenesis. Kime (1995) also noted that several studies on the effects of pollution in the teleost ovary show that exposure to pollutants leads to less-developed oocytes and fewer large, mature oocytes and, also, to an increase in the number of atretic follicles. Nevertheless, it was not possible to establish a dose-dependent pattern or an evident correlation between adverse effects in females and EDCs.

In another semi-field experiment, carried out in two periods and in two different seasons, no adverse effects were detected in females and female gonads showed different but typical maturation stages according to the respective season. These variations in female estrogenic responses make it difficult to establish an association with the estrogenic potency of the treated sewage.

As a concluding remark, this work shows that treated domestic effluent from an STP located in Lisbon (Portugal) possesses significant estrogenic activity. This is demonstrated by the gonadal reduction and the histological abnormalities (e.g. presence of oocytes) observed in the testes of male fish exposed to it and is also shown by the induction of Vtg, all of which provide evidence that sewage effluent contains chemical compounds capable of affecting the endocrine system of fish. Estrogenic potency can also be influenced by climatic factors, such as rainfall and temperature, which affect the dilution levels of the effluent and biodegradation rate of the chemicals in the sewage, respectively.

This study also shows that STP discharges into the aquatic environment can induce significant estrogenic changes in the biota, particularly in fish, depending on the dilution, and possibly synergistic and/or additive effects when combined with other discharges from industry or agriculture. The possibility that EDCs may contaminate drinking water when produced from surface water downstream of STP effluents has also to be considered.

7.4. Future Directions

Wildlife and human studies: There is insufficient information about the extent to which humans and wildlife are exposed to EDCs. For most chemicals there is also a lack of data on

geographical differences and temporal trends in concentration levels. Therefore, more wildlife studies are needed at the population and ecosystem level.

Methods of EDC risk assessment: New and improved methods are required for comparing and ranking EDCs within and between compound classes exhibiting the same type of hormone disrupting effect. There is also a need for new and improved methods of comparing and ranking mixtures of EDCs that exhibit the same and different types of hormone disrupting effects. In addition, systematic evaluation and comparison of data on EDC effects, taking chemical and physical properties into consideration, is needed.

Laboratory and (semi) field studies: more research, both (semi) field and laboratory, will be necessary to identify specific contaminants and mixtures that cause individual changes at the cellular level and also to determine how specific changes affect fish populations and their reproductive health.

Another recommended areas for future research activities include: development of *in vitro* and *in vivo* models for endocrine disruption; experimental effect studies and determination of dose-response relationships and threshold levels identified; chemical identification of EDCs present in the environment; identification and monitoring of endocrine disruption in wildlife and humans; basic research about hormone systems and elucidation of the mechanisms of action of endocrine disrupters; the kinetics of potential EDCs and their metabolites; additive, synergistic and antagonistic interactions between chemicals in mixtures have to be examined using both *in vitro* and whole animal systems.

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Annexes

Annex I – GENERAL INTRODUCTION

Table I.1 - Summary of Morphological effects found by other authors

Species	Location	Assoc. chemicals	Effects	References
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Switzerland Laboratory study	NP	Increased Vtg expression in trout liver occurred at 1.05 µg/l NP, whereas Vtg mRNA levels, were not significantly elevated. ZRP contents were significantly higher at 10.17 µg/l NP.	Ackermann et al. 2002
wild cyprinid fish Gudgeon (<i>gobio gobio</i>)	UK field study	Near STP discharges	Elevated concentrations of plasma vitellogenin both in male and in intersex fish indicated that fish had been exposed to estrogen(s).	Van Aerle et al. 2001
Fathead Minnow (<i>Pimephales promelas</i>)	U.K. Laboratory study	EE2	Exposed to EE2 in early life stages. Vtg induction in males and disruption in duct development. Spermatozoa reduction. Short exposures of embryos/early life stage to environmental relevant concentrations of EE2 lead to alterations in gonadal development.	Van Aerle et al. 2002
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Five Switzerland STPs Semi-field study	EDCs mixture (e.g. E1, E2, EE2, alkylphenols)	At two sites the male fish have significantly increased Vtg blood plasma concentrations after the exposure.	Aerni et al. 2004
Flounder (<i>Platichthys flesus</i>)	U.K. Field study Scotland estuaries	Sewage and industrial waste that enters estuaries	Vitellogenin levels in male blood plasma were found to be significantly elevated. At most locations, ovotestis conditions in male flounder were entirely absent but 9% of male Mersey fish and 7% of male Tyne fish contained ovotestis. Most testes did not show gross morphological abnormalities related to oestrogenic exposure.	Allen et al. 1999
Eelpout (<i>Zoarces viviparus</i>).	Denmark Laboratory study	E2 and OP	Estrogen binding activity was revealed in the cytosolic fraction of hepatic extracts from adult male and female. The binding was specific to known estrogens (E2) but not to other tested steroids.	Andreassen and Korsgaard, 2000
juvenile atlantic salmon (<i>Salmo salar</i>).	USA Laboratory study	o,p'-DDT, NP	Vtg and Zrp respond significantly stronger to NP treatment alone and in combination with o,p'-DDT compared to control and o,p'-DDT treatment alone. However, a slight reduction in NP-induced Zrp levels was indicated when NP was injected in combination with o,p'-DDT.	Arukwe et al. 1998
Rainbow trout (<i>Oncorhynchus mykiss</i>)	USA Laboratory study	E2 and NP	Induction of Vtg and Zr-protein gene expressions at the early time intervals after exposure are not dependent on increase in the transcriptional activity of the ER gene and Vtg and Zr-protein gene transcriptions require only basal or minimal ER concentration, in addition to other mechanisms.	Arukwe et al. 2001
female juvenile rainbow trout (<i>Oncorhynchus mykiss</i>)		OP, NP, NP1EC	Reduction in growth and GSI	Ashfield et al. 1998
Guppies (<i>Poecilia reticulata</i>)	Denmark Laboratory study	vinclozolin, p,p'-DDE, flutamide	Juveniles were fed with sublethal doses of chemicals from birth to adulthood. All caused caused a reduction in the orange display coloration, inhibited gonopodium development, reduced the sperm count and suppressed courtship behaviour, in a manner consistent with antiandrogen action.	Bayley et al. 2002
Male Mosquitofish (<i>Gambusia affinis holbrooki</i>)	Australia semifield study	River contaminated by sewage	The GP was reduced in length in males sampled downstream from a sewage treatment plant discharge point compared to GP fin length in males upstream or from other comparison sites.	Batty and Lim 1999

		effluent		
Juv. Rainbow trout (<i>Oncorhynchus mykiss</i>) and zebrafish (<i>Danio rerio</i>)	Belgium Laboratory study	OP, NP, E2, EE2, BPA, dibutylphthalate	All these compounds, except for DBP, were found to be estrogenic to both fish species. The results demonstrated a difference in species sensitivity for NP with the zebrafish being about 5 times less sensitive. Increase in plasma Vtg.	Van den Belt et al. 2003
zebrafish embryos (<i>Danio rerio</i>)	Belgium Laboratory study	EE2	The results demonstrated a significant reduction in total body length, body weight, and an increase in morphological abnormalities for fish exposed to 25 ng/l EE2 as a function of exposure time. An increase in total body VTG content was observed for fish exposed for a period of 3 months down to levels of 1 ng/l EE2. At	Van den Belt et al. 2003
female zebrafish (<i>Danio rerio</i>)	Belgium Laboratory study	E1, E2, EE2 and NP	All compounds were found to be estrogenic in both in vitro assays and were able to induce VTG and to reduce the ovarian somatic index in female zebrafish.	Van den Belt et al. 2004
goldfish (<i>Carassius auratus</i>)	Sweden Laboratory study	E2	Exposure to physiological levels of E2 was shown to severely affect the male goldfish reproductive behaviour and physiology. Reduction in milt and tubercles.	Bjerselius et al. 2001
sea bass (<i>Dicentrarchus labrax</i> L.),	Spain Laboratory study	E2, EE2	Were fed estrogens and resulted in a females (126-226 DPF) ratio increase.	Blázquez et al. 1998
zebrafish (<i>Danio rerio</i>)	Belgium Laboratory study	EE2	Zebra fish at different developmental stages (embryos, juveniles, and adults). Significantly higher VTG concentrations (compared to controls) were measured in adults	Versnennen, and Janssen, 2004
Guppies (<i>Poecilia reticulata</i>)	Italy Laboratory study	NP	Treated males displayed anomalous transcription of vitellogenin in correlation with a significant reduction of the gonadosomatic index (GSI); however, an increase of vitellogenin gene transcription, correlating directly with an increase in GSI, was observed in NP treated females. The hepatosomatic index (HSI) was significantly increased in exposed males and females.	Cardinalli et al. 2004
Carp (<i>Cyprinus carpio</i>)	Italy Laboratory study	E2, NP	Vtg induction in order to validate an ELISA	Casini et al. 2002
flounder (<i>Platichthys flesus</i>)	Denmark Laboratory study	NP	Male flounders received intraperitoneal injections. Increase in Vtg and a significant increase of plasma GPT-concentrations indicated that 4-NP also elicited toxic effects on the fish.	Christensen et al. 1999
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Denmark Laboratory study	NP, BPA, BBP and DBP	Increase in Vtg synthesis (except for DBP)	Christiansen et al. 1998
Eelpout (<i>Zoarces viviparus</i>).	Denmark Laboratory study	NP and E2	Increase in male Vtg synthesis. Severe damages in testis (lobules degeneration, squamous sertoli cells, containing phagocytized sperm cells.	Christiansen et al. 1998b
Eelpout (<i>Zoarces viviparus</i>).	Denmark Laboratory study	NP and E2	A highly significant reduction in the gonadosomatic index was observed concomitant with the increase in the plasma vitellogenin concentration. The seminiferous lobules were degenerated (May) or were filled with numerous spermatozoa and the Sertoli cells appeared very squamous (June). Great numbers of phagocytized spermatozoa in these Sertoli cells.	Christiansen et al. 1998c
Carp (<i>Cyprinus carpio</i>)	USA	Urban STP	VTG induction and testosterone reduction in male fish	Folmar et al. 1996
walleye (<i>Stizostedion vitreum</i>)	USA Field study	STP estrogenic effluent	All male and female walleye collected from the effluent channel contained measurable levels of VTG in their blood. Males from that location also exhibited depressed serum T concentrations and elevated serum estradiol-17 β (E2) concentrations compared with males from the Snake River reference site. Females collected at the STP site had greatly elevated serum E2 concentrations, but serum T concentrations were not different from	Folmar et al. 2001

			females collected in the Snake River.	
juvenile rainbow trout (<i>Oncorhynchus mykiss</i>)	?	NP	An increased Vtg protein concentration was detected at 1 µg/l NP, Vtg mRNA and Zrp content were significantly increased at 10 µg/l NP.	Fent et al. 2000
chub (<i>Leuciscus cephalus</i>)	Laboratory and field	E2	Increase in plasma Vtg in both male and female chub, indicating that vitellogenic response in the chub is sensitive to estrogen(s). Some alterations in the testis compared with wild fish from a reference site	Flammarion et al. 2000
Male sheepshead minnows (<i>Cyprinodon variegatus</i>)	USA Lab study	E2, EE2, diethylstilbestrol	VTG synthesis in male	Folmar et al. 2000
walleye (<i>Stizostedion vitreum</i>)	Field study	River locations below the St. Paul metropolitan ST	Increase in levels of VTG in blood. Males exhibited depressed serum T concentrations and elevated serum estradiol-17 beta (E2) concentrations compared with males from the reference site. Males obtained from Mississippi River, 3-20 miles downstream of the STP also exhibited reduced serum T concentrations, but showed no alterations in E2 concentrations or the presence of VTG in the serum. Females collected at the STP site had greatly elevated serum E2 concentrations, but serum T concentrations were not different from females collected in the Snake River.	Folmar et al. 2001
flounder (<i>Paralichthys dentatus</i>)	USA Field study	E2	Males were injected. Excessive hyalin material accumulated in the livers, kidneys and testes of the treated fish. A portion of that material was identified as VTG by immunohistochemistry. The accumulation of VTG, and possibly other estrogen-inducible proteins, resulted in hepatocyte hypertrophy, disruption of spermatogenesis, and obstruction or rupture of renal glomeruli.	Folmar et al. 2001b
Japanese medaka (<i>Oryzias latipes</i>)	USA Laboratory study	NP, E2	Increased VTG in males exposed to NP and increased VTG in both males and females exposed to E2	Foran et al. 2000
Juv.Carp (<i>Cyprinus carpio</i>)	The Netherlands Laboratory study	TPP and E2	The average number of primordial germ cells (PGCs) per gonadal section was significantly reduced. Vitellogenin levels were significantly elevated in the plasma of the carp exposed to both 17β-estradiol concentrations, but only at the highest 4-tert-pentylphenol concentration. Formation of oviduct.	Gimeno et al. 1998
fathead minnows (<i>Pimophales promelas</i>)	USA lab study	NP water borne exposure	In the first experiment, Vtg concentration generally increased with NP concentration, whereas the second experiment showed a negative correlation. Plasma E sub(2) concentrations in both males and females were significantly affected by NP. The concentration of total estrogen equivalents in the plasma increased 900% because of exposure to NP. Most of this increase resulted from increased plasma E sub(2) concentrations, with only a 4% increase resulting from the estrogen agonist activity of NP.	Giesy et al. 2000
goldfish (<i>Carassius auratus</i>)	USA semifield study	STP effluent	There were no significant differences in plasma VTG of either male or female goldfish among locations or between sites at WWTPs and reference sites. Concentrations of E2 in the plasma of female goldfish were similar among WWTP sites, all of which were less than in females at a pond reference location. Concentrations of E2 in the plasma of male goldfish were similar at all WWTP locations, except for one, where they were greater. No consistent trends in hormone concentrations or gonadal histology could be attributed to putative endocrine disrupter exposure in WWTP effluents.	Giesy et al. 2003
Carp (<i>Cyprinus carpio</i>)	The Netherlands	TPP and E2	A reduced diameter of the seminiferous lobules. Sperm decrease. The occurrence of early	Gimeno et al. 1998b

	Laboratory study		ovo-testes in some fish with completely regressed testes suggests that oogenesis may proceed in testes which have first been inhibited (demasculinised) following estrogenic stimulus.	
Japanese medaka (<i>Oryzias latipes</i>)	Canada Laboratory study	OP	Reduction in some courtship activity and overall reproductive success. Eggs produced by matings of exposed males and females demonstrated various developmental problems, including circulatory system difficulties, incomplete eye development (i.e., anisophthalmia), and failure to inflate swim bladders upon hatch. One male fish with an intersex gonad was able to fertilize the eggs of an unexposed female.	Gray et al. 1999
Japanese medaka (<i>Oryzias latipes</i>)	Canada Laboratory study	OP	Developmental abnormalities observed in embryos and larvae ranged from circulatory problems to failure to inflate swim bladders.	Gray and Metcalfe, 1999
rainbow trout (<i>Oncorhynchus mykiss</i>)	UK semiField study	treated sewage effluent	After 3 weeks exposure, caged fish held up to 15 km downstream of inputs showed an increase in plasma vitellogenin concentration, with statistically significant elevations up to 4.5 km downstream.	Harries et al. 1996, 1997
rainbow trout (<i>Oncorhynchus mykiss</i>)	UK Field study	treated sewage effluent	Increases in plasma vitellogenin concentrations occurred in fish held in affluent from Harpenden STW at concentrations of 50% and above. At Chelmsford STW, the effluent appeared to be more potent. causing induction of vitellogenin in fish at concentrations as low as 25% (compared with 50% for the Harpenden effluent).	Harries et al. 1999
adult male gruppies (<i>Poecilia reticulata</i>)	Lab study	TBT, BPA	Significant declines (by 40-75%) in total sperm counts for male fishes exposed to tributyltin and bisphenol A compared with controls. Possibly the spermatogenesis was inhibited through in vivo interference with normal Sertoli-cell function	Haubruge et al. 2000
Male fathead minnows (<i>Pimephales promelas</i>).	USA semifieldstudy	treated sewage effluent	The VTG was elevated. The GSIs were significantly less at the inflow site. At wetland sites closest to the inflow, secondary sexual characteristics, tubercle numbers, and fatpad thickness were less than in laboratory controls. The HSIs and density of male breeding stripes were not significantly different from those of laboratory controls. However, elevated HSIs were found at the inflow site. Hematocrit and condition factors were both in effluent-exposed fish at wetland sites closer to the inflow than in control fish or fish further downstream	Hemming et al. 2001
Fathead minnows (<i>Pimephales promelas</i>).	USA Field study	treated sewage effluent	Significant increases in plasma vitellogenin concentrations were detected in March and December, but not in August or June. Additionally, the magnitude of expression was variable. Variability in the spatial scale was also evident during the March and June exposure months.	Hemming et al. 2004
Male carp (<i>Cyprinus carpio</i>)	Lab study	NP, OP	Nonylphenol and octylphenol were administered to male carp by injection, feeding and immersion. Vitellogenin levels increased significantly after 14 and 28 days. When transferred to clean water, the elevated plasma vitellogenin levels in carp exposed to 4 microg/L octylphenol for 42 days returned to control levels within 28 days.	Huang and Wang, 2001
goldfish (<i>Carassius auratus</i>)	Japan Laboratory study	BPA	There was no significant induction of VTG at the concentrations of 1 and 10 µg/l. Exposure to 100 µg/l and 1000 µg/l BPA for 28 days significantly elevated plasma VTG concentrations in males.	Ishibashi et al. 2001
flounder (<i>Platichthys flesus</i>)	The Netherlands Semifield study	dredged spoil that contained a mixture of contaminants	Female and males were exposed in large mesocosms for up to 3 years. Premature vitellogenesis, resulting in a high number of oocytes in the yolk granule stage in spring, normally the previtellogenic period of the year. VTG was significantly elevated in the plasma of these females relative to the concentrations in fish from the reference	Janssen et al. 1997

		representative of pollution concentrations found in the natural environment	mesocosm. The high concentration of plasma VTG in females from the polluted mesocosm coincided with significantly elevated concentrations of testosterone and 17 beta -oestradiol. The in vitro ovarian production capacity of these steroids, however, was not altered. In feral and pollution-exposed male flounder, no VTG was detected.	
(fathead minnow; <i>Pimaphales promelas</i> , rainbow trout (<i>Oncorhynchus mykiss</i>); carp; <i>Cyprinus carpio</i>).	UK and Germany laboratory study	Single (EE2, BPA and OP) and mixed	Plasma vitellogenin concentrations in two species of male fish (the rainbow trout and the carp) increased over the same time period.	Jobling et al. 2003
Japanese medaka (<i>Oryzias latipes</i>)	Japan Laboratory Study	E2	The number of egg produced and fertility of the paired medaka exposed to 463 ng/l E2 were significantly less compared with those of the control fish. Males in all treatment groups had developed testis-ova. Males treated with E2 concentrations 55.7 ng/l contained relating great concentrations of hepatic Vtg.	Kang et al. 2002
Japanese medaka (<i>Oryzias latipes</i>)	Korea Laboratory Study	Di-2-ethylhexyl phthalate (DEHP)	First, blood vitellogenin levels in all treated test subjects markedly decreased. Gonado Somatic Index (GSI) decreased Female fish in the control treatment had completely matured oocytes in their ovaries, but only 37%, 0% and 22% of female fish matured to the last stage in the 1, 10 and 50 mug/l treated test subjects, respectively.	Kim et al. 2002
male guppies (<i>Poecilia reticulata</i>)	Denmark laboratory study	E2, OP, bisphenol A, .utamide, p; p0-DDE, or vinclozolin	Reduced number of spermatogenetic cysts and an increased number of spermatoeugmata in the ducts. Indicative of a blocked spermatogonial mitosis. 17b-Estradiol produced effects different from those of the other compounds studied. The effects were seen as an increased number of hypertrophied Sertoli cells/efferent duct cells. The mammalian antiandrogen vinclozolin had no observable effects on the testis structure	Kinnberg and Toft 2003
platyfish (<i>Xiphophorus maculatus</i>)	Denmark laboratory study	E2 and NP	Vitellogenin increase in all fish exposed to nonylphenol or 17(beta)-estradiol. Exposure to 17(beta)-estradiol resulted in a significant reduction in the gonadosomatic index. A tendency for a dose-dependent reduction in the gonadosomatic index in the nonylphenol exposed groups was observed. Histological examination revealed dose-dependent effects of nonylphenol on the testis structure. The testes of control fish contained numerous cysts with spermatogenetic cells. The testes of fish exposed to nonylphenol or 17(beta)-estradiol showed a decrease in the number of cysts concomitant with an increase in the amount of hypertrophied Sertoli cells present.	Kinnberg et al. 2000
platyfish (<i>Xiphophorus maculatus</i>)	Denmark laboratory study	NP	A tendency of a concentration-dependent decrease in the gonadosomatic index was observed after 28 days of treatment with nonylphenol. Histological examination revealed marked effects of nonylphenol on the testis structure.	Kinnberg et al. 2000
guppies (<i>Poecilia reticulata</i>)	Denmark laboratory study	E2, OP	Indications of blocked spermatogonial mitosis in the testis structure of adult males after exposure to octylphenol or 17b-estradiol. The post-parturition ovaries of adult females exposed to 17b-estradiol showed effects suggesting an inhibited yolk deposition.	Kinnberg et al. 2003b
Japanese medaka (<i>Oryzias latipes</i>)	Germany laboratory study	OP, E2	Increase in mortality. Decrease in growth. Sex ratio changes. Testes-ova development. Low fertilization rate.	Knorr and Braunbeck 2002

Fathead minnows (<i>Pimephales promelas</i>)	USA laboratory study	E2	The alteration of the timing of recrudescence including vitellogenin production. Spawning was inhibited. Vitellogenin induction in male fathead minnows was strongly correlated with E2 exposure.	Kramer et al. 1998
Crucian Carp (<i>Carassius Carassius</i>)	Semifield study	Pond of Secondary Treated Pulp Mill Effluent	Higher liver somatic index (LSI), lower gonad somatic index (GSI), lower haemoglobin and haemocrit, but higher T ₃ than in the reference fish. Increased plasma testosterone concentration and decreased thyroxin (T ₄) levels were found in the exposed male fish. No vitellogenin production was found in the exposed male fish and some females showed decreased vitellogenin levels. The histological structure of the gonads in the exposed males was normal.	Kukkonen et al. 2000
fathead minnow (<i>Pimephales promelas</i>).	Lab study	EE2	A fish full life-cycle (FFLC) study. Influence on male exposed to EE2 at 4.0 ng/L failed to develop normal secondary sexual characteristics. Females exposed to this level of EE2 were able to breed when paired with males that had not been exposed to EE2. After 172 d posthatch, no testicular tissue was observed in any fish exposed to EE2 at 4.0 ng/L. At the same time point, plasma vitellogenin levels were significantly higher in fish exposed to EE2 at 16 ng/ L. A lack of sexual differentiation occurred in males at concentrations greater than or equal to 4.0 ng/L. Taking into account these data, the overall no observed-adverse-effect concentration was considered to be 1.0 ng/L.	Lange et al. 2001
Feral carps (<i>Cyprinus carpio</i>)	Field study	River downstream a STP (Spain)	Low gonadosomatic index (GSI), plasmatic vitellogenin (VTG), depressed levels of testosterone, and histological alterations in gonads. Important alterations (viz. delayed maturation in females, indications of arrested spermatogenesis in males) were detected. Low ovarian P-450 aromatase and reduced glucuronidation of testosterone and estradiol in males	Lavado et al. 2004
rainbow trout (<i>Oncorhynchus mykiss</i>)	Lab study	BPA	A significant induction of the VTG synthesis was obtained at 500 µg BPA/l exposure, although an increase in the ratio of responding animals was observed already between 40 and 70 µg BPA/l.	Lindholm (2000).
flounder (<i>Platichthys flesus</i>)	Scotland estuaries	Treated STP effluent	Increase in VTG in all sites. Testis abnormalities. Increase in HSI.	Lye et al. 1997
flounder (<i>Platichthys flesus</i>)	Denmark laboratory study	OP	Increase in plasma VTG in males.	Madsen et al. 2003
flounder (<i>Platichthys flesus</i>)	Denmark laboratory study	OP, E2	Increase in plasma VTG in males.	Madsen et al. 200, 2002
flounder (<i>Platichthys flesus</i>)	UK field study	Contaminated estuaries	Elevated VTG and ovo-testis up to 20% in males	Matthiessen et al. 1998
Flounder (<i>Platichthys flesus</i>) blenny (<i>Zoarces viviparus</i>); Sand goby (<i>Pomatoschistus minutus</i> and <i>P. lozanoi</i>)	UK field study	Collected samples from estuaries-combined sewage/industrial discharges	VTG and intersex induction in males (flounder and blennies); the urogenital papilla appeared partially feminised,	Matthiessen et al. 2002
fathead minnow (<i>Pimephales promelas</i>)	USA laboratory study	NP	Changes in the number and size of Sertoli cells and germ cell syncytia. Necrotic aggregates of various stages of germ cells in the spermatogenic sequence were observed in the testes. Presence of phagocytic cells in the lumina of seminiferous tubules. The	Miles-Richardson et al. 1999

			cytoplasm of some Sertoli cells was distended with myelin figures and necrotic spermatozoa. No significant effects on the stages of follicular development were observed in females exposed to NP. The histologic effects caused by NP were similar to, but not exactly the same as those caused by exposure of fathead minnows to 17 beta -estradiol	
fathead minnow (<i>Pimephales promelas</i>)	USA laboratory study	E2	Reduction in size of the prominent male secondary sex characteristics, the fatpads and nuptial breeding tubercles. Histological lesions observed in the testes included proliferation of Sertoli cells and degenerative changes. Females had ovaries in which most of the follicles were in the primary stage of development. There were also more atretic follicles and fewer secondary and Graafian follicles than in unexposed females.	Miles-Richardson et al.1999
juvenile male summer flounder (<i>Paralichthys dentatus</i>)	USA laboratory study	DDT, DDE and octylphenol	17b-Estradiol-treated flounder exhibited decreased gonadosomatic index (GSI), altered hepatosomatic index (HSI), elevated plasma estradiol, reduced plasma testosterone, and high levels of plasma vitellogenin. Fish treated with <i>o,p</i> %-DDT showed lower GSI, no change in HSI or plasma estradiol, depression of plasma testosterone, and induction of vitellogenesis. Octylphenol treatment resulted in lower GSI, no change in HSI, initially increased plasma estradiol and decreased testosterone, and no vitellogenin production. <i>p,p</i> %-DDE treatment did not significantly alter any indicator relative to controls. In experiments using combinations of chemicals, flounder receiving <i>o,p</i> %-DDT plus octylphenol had lower GSI after 8 weeks and elevated plasma estradiol after 15 weeks exposure. Fish treated with <i>p,p</i> %-DDE plus octylphenol for 8 weeks exhibited a significantly lower GSI.	Mills et al. 2001
Immature catfish (<i>Ictalurus punctatus</i>)	USA laboratory study	E2, EE2, mestranol and DES, NP, methoxychlor.	Intraperitoneal injection. Increase in VTG in plasma.	Nimrod &Benson, 1996
Juvenile salmon (<i>Salmo salar</i>)	Sweden Lab study	E2, DEHP, nonylphenol and PCB	Juvenile salmon fed diets containing E2 differentiated into 88 and 100% phenotypic females, respectively. A significantly increased liver somatic index was detected for groups exposed to diets. Intraperitoneal injections of nonylphenol, di-2-ethylhexyl phthalate, or polychlorinated biphenyls did not induce synthesis of vitellogenin.	Norrgren et al. 1999
male Killifish (<i>Fundulus heteroclitus</i>)	USA lab study	OP, BPA, E2	Plasma vitellogenin production occurred in a dose dependant manner in males exposed (injection). There was some indication that the effect on GSI may be influenced by the season in which the experiments are carried out.	Pait and Nelson, 2003
wild fathead minnows (<i>Pimephales promelas</i>)	Canada lab study	EE2	Increase in VTG in males. VTG in females was induced 8- to 80-fold and was sustained beyond the normal window of vitellogenesis in Lake 260. Histological examination of tissues from EE2-exposed male fatheads in Lake 260 showed widespread fibrosis and inhibition of testicular development. Enlargement of liver cells, edema in the interstitium between kidney tubules, and eosinophilic deposits in the kidney tubule lumen were also evident in male fatheads from Lake 260.	Palace et al. 2002
male fathead minnows (<i>Pimephales promelas</i>)	UK lab study	E2, E1	Increase in plasma vitellogenin levels accompanied by an inhibition of testicular growth. The results from this study indicated that although oestrone induced significant effects at a lower concentration, oestradiol was more potent than oestrone at higher concentrations, causing greater vitellogenin synthesis and testicular inhibition.	Panter et al. 1998
juvenile rainbow trout (<i>Oncorhynchus mykiss</i>)	Sweden semifield study	EE2, fish placed	Increase of Vtg in the plasma of caged fish two km downstream from the effluent outlet.	Parkkonen et al. 2000

		downstream STP		
Juvenile Rainbow trout (<i>Oncorhynchus mykiss</i>)	Sweden semifield study	EE2	All estrogens indicated above were present in the bile of the caged fish, and the estrogen-inducible protein, vitellogenin, was found in large amounts in the plasma	Parkkonen et al. 2000
fathead minnow (<i>Pimephales promelas</i>)	USA laboratory study	EE2	Significant decrease in GSI, condition factor, and number of batches of eggs and their fertilization rate at EE2 exposure concentrations between 10 and 100 ng/L. A reduction in the extent of parenchymatic areas in ovaries and ultrastructural changes in the livers of females could be detected at EE2 concentrations > 3 ng/L.	Pawlowski et al. 2004
Juvenile Rainbow trout (<i>Oncorhynchus mykiss</i>)	Denmark laboratory study	t-OP, t-NP, n- OP, n-NP	Significant vitellogenic response after 12 days	Pedersen et al. 1999
Carp (<i>Cyprinus carpio</i>)	Spain field study	river water downstream of STPs	Increase in VTG in feral carp	Petrovic et al. 2002
male fathead minnows (<i>Pimephales promelas</i>)	UK lab study	4-tert- nonylphenol (4-NP),	Plasma vitellogenin induction vtg. mRNA induction This study indicate that a chemical such as 4-NP has a higher oestrogenic potential when it enters the bloodstream via the gills/skin of a fish compared with exposure through the diet.	Pickford et al. 2003
longear sunfish (<i>Lepomis megalogotis</i>)	USA semifield study	sewage effluent	Significant increases in condition factor, hepatosomatic index, serum testosterone, and plasma vitellogenin concentrations were observed in male sunfish collected from the study stream in comparison to fish collected from the reference stream.	Porter, And Janz, 2003
Roach (<i>Rutilus rutilus</i>)	UK field study	Polluted rivers	High incidences of intersex gonads have been observed in fish in the lagoons of sewage treatment works	Purdum et al. 1994
roach (<i>Rutilus rutilus</i>),	UK semifield study	Sewage treatment works (STW) effluents	Long-term exposure of maturing adult roach to a graded concentration of this effluent demonstrated that the vitellogenic response was both dose and time dependent. The functional significance and/or ecological consequences of induction of vitellogenin resulting from exposure to STW effluent has yet to be determined in wild fish.	Rodgers-Grey et al. 2000
adult male rainbow trout (<i>Oncorhynchus mykiss</i>) and adult roach (<i>Rutilus rutilus</i>)	UK lab study	E1, E2	The results indicate that environmentally relevant concentrations of natural steroidal estrogens are sufficient to account for the levels of vitellogenin synthesis observed in caged male fish placed downstream of certain STW effluent discharges in British rivers.	Routledge et al. 1998
Carp (<i>Cyprinus carpio</i>)	Germany lab study	NP, EE2	Histopathological alterations in the kidney, the liver and the spleen occurred exclusively after treatment with EE2, whereas NP-exposed fish did not show any tissue lesions.	Schwaiger et al. 2000
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Germany lab study	NP	NP, in an environmentally relevant concentration range, acts as a weak estrogen in directly exposed adult male rainbow trout as indicated by elevated plasma vitellogenin levels. Reproduction success was reduced as indicated by decreased hatching rates. Hormonal imbalances detected in the offspring of exposed fish indicate a transgenerational effect mediated by the endocrine system.	Schwaiger et al. 2001
mature goldfish (<i>C. auratus</i>)	Semifield and lab study?	undiluted effluent from a major metropolitan STP	Exposure to effluent in both the summer and winter trials induced small, but nonsignificant, decreases in the frequency of male spawning behaviors but had no effect on sperm production. Exposure to E2 caused a clear reduction in spawning behavior in one trial while causing reduced sperm production in both trials.	Schoenfuss et al. 2002

Zebra.fish, (<i>Danio rerio</i>)	UK lab study	EE2, BPA	Vitellogenin Significant induction. Juvenile growth reduction. Fertilization success reduction	Segner et al. 2003
male medaka (<i>Oryzias latipes</i>)	Japan lab study	EE2	A significant decrease in fecundity was observed only at the highest EE2 concentration, whereas hepatic VTG concentration in males was increased at concentrations of 63.9 ng/L and greater. In addition, an intersex condition (testis-ova) of the gonad was observed in male medaka exposed to EE2 concentrations of > or = 63.9 ng/. Thus, the physiological and histological measurements were approximately eightfold more responsive to the EE2 exposure than were overt reproductive effects. This suggests that the elevated VTG levels and testis-ova may not be definitely responsible for reproductive impairment of the fish	Seki et al. 2002
male medaka (<i>Oryzias latipes</i>)	Japan lab study	NP, DEHP	E2 caused a significant decrease in the number of eggs and hatchings. Also, the highest concentrations of bisphenol-A and NP caused a decrease in the number of hatchings, but no decrease in hatchings was observed in DEHP treatments.	Shioda, and. Wakabayas 2000
Male Flounder (<i>Platichthys flesus</i>)	UK Field study	Contaminated estuary	Hepatic tubular vacuolation, foci of cellular alteration, and hepatocellular tumours were seen. Renal and gonadal pathology included the presence of two Mersey fish with enlarged and abnormal glomeruli, three phenotypic male Mersey flounder with unilateral intersex gonads and one male with bilateral, abnormal ovarian follicular components in the testis.	Simpson et al. 2000
fathead minnow (<i>Pimephales promelas</i>).	UK lab study	BPA	VTG induction. Inhibition of gonadal growth (as measured by the gonadosomatic index) occurred in both males and females. Inhibition of spermatogenesis. Egg production was inhibited. The results demonstrate that BPA acts as a weak estrogen to fish when administered via the water, with effects on breeding at and above 640 µg/L.	Sohoni et al. 2001
carp (<i>Cyprinus carpio</i>)	Spain field study	River streams near STP	VTG increase being more evident at the vicinity of the treatment plants	Solé et al. 2000
male and female carp (<i>Cyprinus carpio</i>)	Spain lab study	EE2 injection	Vtg induction. Significant inhibition of P450 (CYP1A), EROD	Solé et al. 2000
male and female carp (<i>Cyprinus carpio</i>)	Spain field study	Contaminated rivers	Elevated levels of VTG in males and the presence of some intersex individuals. In the Cardener, no intersex fish were found and male plasmatic VTG was not so highly elevated. However presence of hepatic VTG, in up to 54% of the male fish analyzed, proved exposure to xenoestrogens. In females, VTG fluctuated according to the biological cycle with a plasmatic peak in May and an earlier maximal in the liver. However, this pattern was altered in the locations with higher xenoestrogens presence.	Solé et al. 2002
male and female carp (<i>Cyprinus carpio</i>)	Spain field study	Contaminated rivers	Highly variable Vtg content in all fish groups; (2) an increase in sex hormones content in March for the female group; and (3) an enhanced xenobiotics metabolism in the affected fish group, measured as total cytochrome P450, EROD activity in the January survey and cytosolic GST in March. The observed increase in VTG, sex hormones and in most of the enzymatic activities from January to March that could also be attributed to higher water temperature.	Solé et al. 2003
male and female carp (<i>Cyprinus carpio</i>)	Spain field study	Contaminated rivers near STP	Low plasma VTG content in females collected 4 km downstream of an effluent discharge suggested possible estrogenic disturbances; however, hepatic VTG content and sex hormone levels did not mirror this. In fact, E2 and T largely fluctuated among fish, with individual variations even greater than among sampling stations or periods. Hepatic biotransformation enzymes, such as EROD, increased with increasing water temperature	Solé et al. 2003

			while, in contrast, GST was maximal at the lowest water temperature.	
hermaphroditic <i>Rivulus marmoratus</i> (Osteichthyes, Cyprinodontiformes),	USA lab study	NP	Oogenesis was also significantly inhibited by NP. Dysplasia of the gonadal lumen also occurred in fish. These changes, including testicular agenesis.	Tanaka and Grizzle, 2002
Japanese medaka (<i>Oryzias latipes</i>), sunshine bass (<i>Morone saxatilis</i> _ <i>Morone chrysops</i>) and channel catfish (<i>Ictalurus punctatus</i>)	USA lab study	E2	Plasma VTG induction in effluent-exposed fish ranged from 14 to 82% above reference values depending on species. Extrapolation of field responses with laboratory- exposed fish indicate catfish and bass were exposed to the equivalent of 27±240 ng E2/l in sewage effluent.	Thompson et al.2000
male channel catfish (<i>Ictalurus punctatus</i>)	USA semifield study	STP effluent	Serum Vtg of animals exposed at WWTP-A was induced 220% above reference values in the Fall of 1996 and 480% in Spring of 1997. In animals exposed to effluent of WWTP-B, serum Vtg was elevated 370% in Spring of 1997 and 480% in Fall of 1997 relative to fish held in a reference location. Serum 17-b-estradiol (E2) levels were also significantly elevated 13 and 16-fold in the Fall 1997 and Summer 1998 in the fish exposed to WWTP-A effluent.	Tilton et al. 2002
larval sunshine bass (<i>Morone saxatilis</i> x <i>M-Chrysops</i>)	USA semifield study	STP effluent	VTG and ER levels in sewage-exposed fish were 3-5 times that observed in controls	Todorov et al. 2002
guppies (<i>Poecilia reticulata</i>)	Denmark Lab study	E2, OP	The male sperm count was markedly reduced after exposure to a high concentration of E2. The length of the gonopodium (copulatory organ) was increased when exposed to 100 mg/L OP and at all the tested concentrations of E2. Similar concentrations of OP and E2 reduced the gonad weight of the females.	Toft and Baatrup, 2003
guppies (<i>Poecilia reticulata</i>)	Denmark Lab study	E2, OP	Both compounds increased the number of sperm cells in the ejaculates, reduced the area and color intensity of the sexually attractive orange spots, and inhibited testis growth. In the case of E2, the effects on sperm count and coloration were still evident after 3 months of recovery in clean water. Preliminary results on male reproductive capability demonstrated that treated males produced fewer offspring than untreated fish, indicating an impairment of reproduction itself.	Toft and Baatrup, 2001
Barbel <i>Barbus plebejus</i> , Cyprinidae	Italy field study	Contaminated river	The gonads of the two groups of barbel have been histologically examined, and only the downstream specimens showed histo-morphological alterations that can be related to the Lambro tributary as a source to the main river of endocrine disrupting chemicals, possibly with estrogenic effects. In fact, 50% of the barbel captured 8 of 16 fish in the downstream reach showed intersex gonads.	Vigano et al. 2001
mature male carp (<i>Cyprinus carpio</i>)	USA lab study	NP (<0.05 to 5:4 lg NP/l for 28–31 d)	NP exposure did not significantly increase plasma concentrations of 17b-estradiol (E2), testosterone (T) or vitellogenin (VTG). Excluding outliers, plasma E2 concentrations ranged from <175 to 700 pg E2/ml. T concentrations ranged from 940 to 24,700 pg T/ml plasma. The greatest VTG concentration detected was 52 lg/ml.	Villeneuve et al. 2002
juvenile male summer flounder (<i>Paralichthys dentatus</i>)	USA lab study	17b-estradiol, <i>o,p</i> _-DDT, octylphenol and <i>p,p</i> _-DDE	All chemicals tested, with exception of <i>p,p</i> _-DDE, altered gonadal development, whereas only E2 caused histopathological changes in the liver and kidney. Estrogenic activity induced the liver to produce a vitellogenin (VtG)-like substance and inhibited testicular maturation. As a result, both the lack of target cells for VtG and a continuous supply of VtG from the liver allowed the hyaline material to accumulate in the liver, testis and kidney causing histopathological changes.	Zaroogian et al. 2001

Annex II - Effects of nonylphenol exposure – gonadal histology and vitellogenin induction in crucian carp (*Carassius carassius*)

Effects of nonylphenol exposure – gonadal histology and vitellogenin induction in crucian carp (*Carassius carassius*)

M.S. Diniz, I. Peres and J.C. Pihan (2003) Panel Communication Presented at the 6th National Conference on Endocrine Disruptors, 12nd SESA Technical Jornada, November (26 – 28) Elche (Spain).

Abstract

There is an increasing concern on the potential effects of EDCs (Endocrine Disruptor Compounds) in wildlife. Several studies have shown that sewage treatment plants (STPs) are point sources for these chemicals into the aquatic environment. Alkylphenol polyethoxylates (APEs) are non-ionic surfactants, widely used in many domestic and industrial products. The bio-degradation leads to the formation of several metabolic compounds such as nonylphenol that are more toxic than the parent compound and possess estrogenic activity. In order to investigate the estrogenic potency of nonylphenol, 48 crucian carps were placed randomly in four glass aquaria (30 L). Fish were exposed during 28 days in a semi-static experiment to nominal concentrations of nonylphenol, ranging from relevant to non-relevant environmental concentrations (0, 1, 25 and 100 $\mu\text{g l}^{-1}$). At the end of the experiment the fish were sampled and blood collected for vitellogenin analysis using ELISA. Fish were sacrificed and gonads and liver removed, weighed and expressed as the gonadosomatic (GSI) and hepatosomatic indices (HSI). Samples of the extracted organs were fixed in Bouin's fixative and processed following the usual histological techniques. The results showed that all exposed male fish show significant elevated levels of VTG in blood plasma even at 1 $\mu\text{g l}^{-1}$ concentration. A reduction in the GSI was also observed with more pronounced effect in fish exposed to 100 $\mu\text{g l}^{-1}$. Although no occurrence of testis-ova was observed, exposed fish show a progressive reduction to total inhibition of spermatogenesis. This study gives a valuable contribute to a better understanding of the estrogenic potency of nonylphenol, usually considered a weak xenoestrogen and often present in STPs treated effluent.

Key words: *Carassius carassius*, vitellogenin, nonylphenol, testis-ova

Introduction

Although it is known that since 1930's exogenous substances are capable of interfering with the endocrine system, Endocrine Disruption was only recognized as a environmental issue since about 1992 (Mathiessen, 2000). Thus a variety of natural products and synthetic chemicals, known collectively as endocrine-disrupting compounds (EDCs), mimic or interfere with the mechanisms that govern vertebrate reproductive development and function (Crews, 2000).

Alkylphenol ethoxylates (APEs) are non-ionic surfactants that have been used in a wide range of industrial, domestic and commercial applications like as detergents, plastics emulsifiers, pesticides and consumer cleaning products (Nichols et al., 2001). Since APEs are primarily used in aqueous solutions, they enter into the environment mainly through sewage and industrial wastewater treatment plants where they may undergo biodegradation processes, resulting in less degradable small-chain ethoxylates primarily mono and diethoxylates, carboxylates and alkylphenols which are more toxic than the parent compound (Maguire, 1999; Arukwe, 2000). Nonylphenols (NPs) are widely employed in industry and constitute the predominant components of alkylphenols found in the aquatic environment, possessing estrogenic effects that have been reported in fish, amphibians, birds and mammals (Thibaut *et al.*, 2002).

Vitellogenin (Vtg) is a phospholipo-glycoprotein used as a useful sensitive biomarker of exposure to xenobiotic exposure in fish and other animals. Usually it is synthesized by the liver during oogenesis and is a major precursor of the egg-yolk proteins of oviparous vertebrates (Wallace, 1985). The synthesis of this protein is normally restricted to females but it can be induced in males by exposure to estrogens. Therefore, vitellogenin has been used as a biomarker of exposure to estrogenic compounds (Sumpter and Jobling, 1995; Denslow *et al.*, 1999). NP was shown to bind to estrogen receptor in fish and the induction of circulating Vtg was characterized after *in vivo* exposure of various species to NP (Drèze *et al.*, 2000).

The main objectives of the present study was to assess vitellogenin induction and histological effects in both female and male adult crucian carps, exposed to different concentrations of NP under laboratory conditions in order to a better understanding of the NP effects on reproductive organs and reproduction status of fish. Additionally, this study also provides useful information that can be used for comparison with field studies.

Materials and Methods

The fish were placed randomly in four glass aquaria (30 L). Carp were exposed during 28 days in a semi-static experiment to nominal concentrations of nonylphenol, ranging from relevant to non-relevant environmental concentrations (0, 1, 25 and 100 $\mu\text{g l}^{-1}$).

Sexually mature (1+ year) *Carassius carassius* (N=48; average body mass 12,45g) were obtained from a local producer and placed randomly in four glass aquaria (30 L) distributed in groups of 12 each, after a period of acclimation. Aquaria (Fig. II.1) were supplied with tap water previously dechlorinated. The water in the aquaria was contaminated with nominal concentrations of Nonylphenol: 0, 1, 25 and 100 $\mu\text{g l}^{-1}$, respectively.



Figure II.1: Experiment glass aquarium.

At the end of the experiment the fish were sampled and blood collected for vitellogenin analysis (Fig. II.2). Vtg analysis was performed by quantitative enzyme-linked immunoassay (ELISA) following an adaptation of the method described by Denslow et al. (1999) After blood sample, fish were sacrificed, weighed and measured, and the organs (gonads, liver, kidney) removed and weighed and related to the total fish weight and multiplied by 100 and expressed as the gonadosomatic (GSI) and hepatosomatic indices (HSI). The total fish weight was used to determine the total condition index ($K = 10^5 \times \text{total weight} \times \text{Lenght}^{-3}$); and (total weight – gonad weight) $\times \text{lenght}^{-3} \times 10^5$ give the somatic condition index (KI).

Samples of the extracted organs were fixed in Bouin's fixative for 48h followed by dehydration in graded ethanol and embedding in paraffin following the usual histological techniques. Serial sections (5 μm) were cut and stained with Hematoxylin and Eosin for further histological evaluation.

Results and discussion

Histological analysis showed progressive regressed testis, which is in close relation with NP concentrations used in the experiment. Fish exposed to 25 and 100 $\mu\text{g l}^{-1}$ show inactive spermatogenesis, with few spermatozoa and tissue degeneration (Fig II.2). The significant decrease ($P < 0.05$) of the GSI in males exposed to 25 and 100 $\mu\text{g l}^{-1}$ show that gonad growth is also affected by NP exposure. Data also show that condition index of fish exposed to 25 and 100 $\mu\text{g l}^{-1}$ is significantly different from controls. Fish exposed to 25 and 100 $\mu\text{g l}^{-1}$ show amorphous eosinophilic tissue around or in the seminiferous lobules. We also detected foci of lipidic cells within testis cell structure.

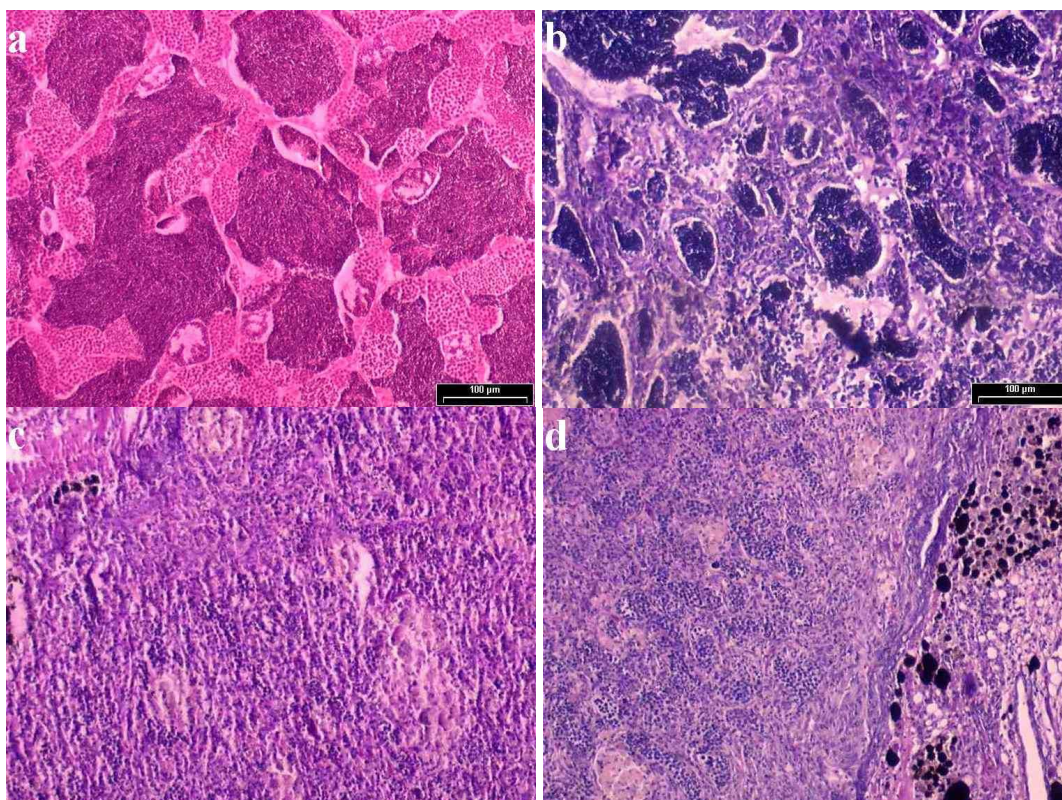


Figure II.2 - Male gonad of fish exposed to 0 (a), 1 (b), 25 (c) and 100 $\mu\text{g/l}$ (d), showing spermatogenesis inhibition, with few spermatozoa within testis tissue (c, d). Sections cut: 5 μm ; staining H&E.

Measured Vtg was significantly higher ($P < 0.05$) in all exposed fish in comparison with controls (Fig. II.3). No intersex condition was noticed in exposed males. However, we can hypothesize that cell foci found in testis tissue could be cells reorienting to develop female typical cell structures because we detected similar structures in some females.

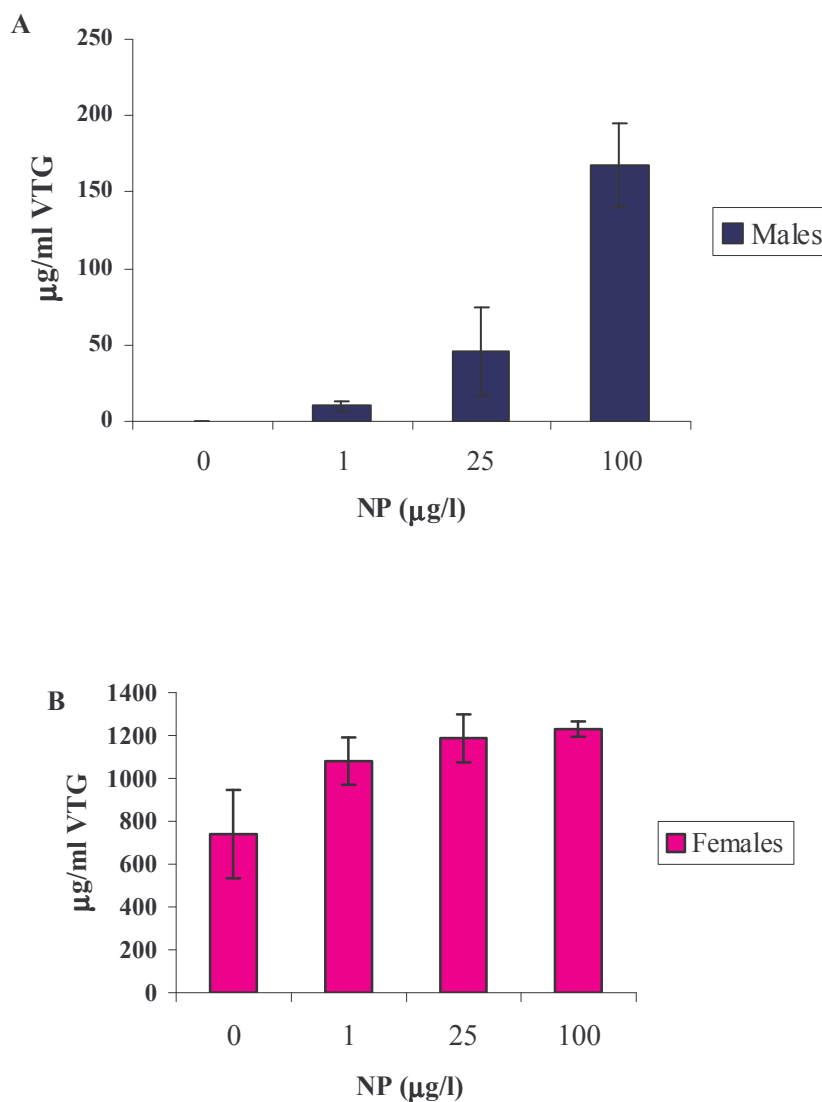


Figure II.3 - Vitellogenin concentration in males (A) and females (B) exposed to different concentrations of nonylphenol.

Table II.1 - Indices: gonadosomatic index (GSI), hepatosomatic index (HSI), total condition index (K), somatic condition index (KI).

Males				
Indices	0 ppb	1 ppb	25 ppb	100 ppb
GSI	1,39 $\pm$ 0,43	0,61 $\pm$ 0,18	0,31 $\pm$ 0,16	0,2 $\pm$ 0,06
HSI	2,32 $\pm$ 0,28	2,62 $\pm$ 0,46	2,81 $\pm$ 0,39	1,81 $\pm$ 0,13
K	2,63 $\pm$ 0,42	2,14 $\pm$ 0,25	1,85 $\pm$ 0,18	1,92 $\pm$ 0,41
KI	2,58 $\pm$ 0,4	1,98 $\pm$ 0,21	1,87 $\pm$ 0,19	1,91 $\pm$ 0,42

Females				
Indices	0 ppb	1 ppb	25 ppb	100 ppb
GSI	4,15 ±1,22	2,69±0,26	2,52±0,3	2,53±0,57
HSI	2,01±0,29	2,6±0,73	3,27±0,31	2,74±0,27
K	2,75±0,21	2,53±0,28	2,34±0,46	1,98±0,16
KI	2,52±0,37	2,36±0,27	2,26±0,45	1,95±0,14

In this study were used environmental relevant levels of NP confirming the estrogenicity of NP to induce Vtg, also documented by other authors, and the adverse effects on gonad condition. Thus, our results can help to a better understanding of NP action on fish endocrine system and histological abnormalities.

Conclusions

This study, provide useful information concerning the estrogenic effects of nonylphenol, a xenoestrogen that is capable to affect physiology and reproduction of fish and other living organisms. Thus, the results can help to a better understanding of nonylphenol mode of action in fish endocrine system. Moreover, the assessment of histological changes caused by the exposure to NP permit further comparisons to histological effects detected in wildlife or semi-field studies.

Acknowledgments

The Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project “*Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater*”, (POCT/36303/MGS/2000) and also the author PhD grant (SFRH/BD/3093/2000).

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Annex III - Vitellogenin induction and histological effects of *Carassius carassius* exposed to 17 β -estradiol under laboratory conditions

Vitellogenin induction and histological effects of *Carassius carassius* exposed to 17 β -estradiol under laboratory conditions

M.S. Diniz, I. Peres and J.C. Pihan (2003) Presented as Poster at 5th Iberian and 2nd Ibero - American Congress of Environmental Contamination and Toxicology (CICTA 2003), Porto, 22-24 Setembro.

Abstract

Xenoestrogens such as 17 β -estradiol have been shown to be present in Sewage Treatment Plants (STP) as the result of the Human waste, in concentrations sufficient to induce estrogenic responses. Controlled laboratory experiments were carried out to a better understanding of the estrogenic effects and potency of this compound. Crucian carp (*Carassius carassius*), were randomly distributed (N= 58) by four 30 L tanks (T°C=15 $\pm$), in a 28 days semi-static experiment. The water was renewed each 48 h and aquarium contaminated with nominal concentrations of 17 β -estradiol, 100, 25, 1 and 0 ppb respectively. At the end of the experimental period all fish were sampled and blood collected for vitellogenin analysis. Organs (Gonad, Liver, Kidney) were also sampled for histological evaluation. The results show that the exposure to 17 β -estradiol induced vitellogenin in male fish. A reduction in GSI was also observed in all exposed fish in comparison with controls. The histological analysis shows that in some fish structures like oocytes developed in male gonads. The results confirm that 17 β -estradiol which is known to be discharged by sewage effluents possess estrogenic potency able to disrupt fish endocrine system.

Key words: vitellogenin, 17 β -estradiol, *Carassius carassius*, xenoestrogens, estrogenic

Introduction

Natural estrogens like as 17 β - estradiol (Fig.III.1) are known to be present in some Sewage Treatment Plants (STPs) as the result of the Human waste, in concentrations sufficient to induce estrogenic responses (Lopez de Alda and Barceló, 2001; Lee *et al.*, 2002). These estrogens are excreted primarily in conjugated forms and the presence of unconjugated forms

in the aquatic environment probably is the result of their deconjugation by the STPs biological processes (Johnson and Sumpter, 2001).

Recently, there has been a focus on 17β -estradiol, estrone, estriol, and their possible involvement in endocrine disruption in wild life. Natural steroid estrogens are very potent hormones and have biological activity at very low concentrations. For example, studies with salmonids exposed to high doses of estradiol in the diet caused deleterious effects on spermatogenesis and even complete regression of the testes. However, different responses were reported according to the administered doses and also the stage of testicular development at the time of treatment (Tyler *et al.*, 1998). Moreover, the significance of the presence of natural estrogens in the environment is presently unknown; once these estrogen reach the river it is still unclear their availability to aquatic organisms (Tyler *et al.*, 1998).

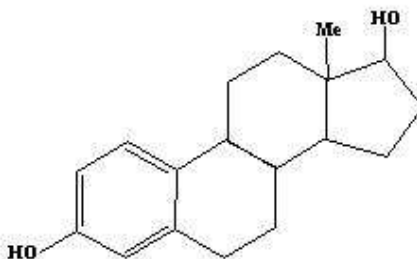


Fig. III.1 Chemical structure of 17β - estradiol

Vitellogenin is a phospholipo-glycoprotein, major precursor of the egg-yolk proteins of oviparous vertebrates (Wallace, 1985), usually restricted to females but it can be induced in males by exposure to estrogens. Therefore, vitellogenin has being used as a sensitive biomarker of exposure to estrogenic compounds (Sumpter and Jobling, 1995; Denslow *et al.*, 1999).

The aim of the present study was to assess vitellogenin induction and histological effects in both female and male adult carps exposed to different 17β -estradiol concentrations under laboratory conditions, and use it as a positive control, for further comparison with other contamination studies.

Materials and Methods

Crucian carps (*Carassius carassius*) (Fig.III.2a; N=48), obtained from a local producer, weighing an average of 15 ± 2 g, were placed randomly in four glass aquaria (30 L) distributed in groups of 12 each. Aquaria (Fig.2b) were supplied with tap water previously dechlorinated. The aquaria were aerated to keep the oxygen content above 6 mg l^{-1} and the water temperature was maintained at $15 \pm 2^\circ\text{C}$ throughout the experiment. The pH values ranged between 7.2 ± 0.3 . During the experimental period, fish were fed with commercial pellets. The fish were not fed for 48 h prior to sampling. The water in the aquaria was contaminated with nominal concentrations of 17 β -estradiol (SIGMA): 0, 1, 25 and 100 mg l^{-1} , respectively. During the experimental period (28 days) the aquaria test conditions were renewed each 48 h.

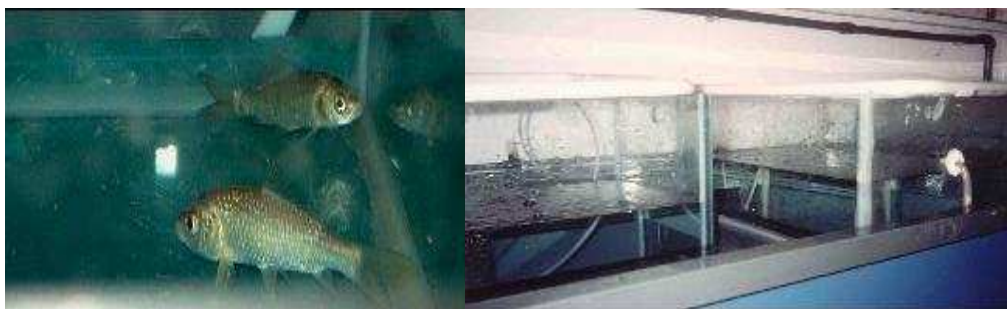


Figure III.2 - carp (*C. Carassius*) and aquarium used in laboratory experiments.

At the end of the experiment the fish were sampled and blood collected for vitellogenin analysis (Fig. III.3). Vtg analysis was performed by quantitative enzyme-linked immunoassay (ELISA) following an adaptation of the method described by Denslow *et al.* (1999) After blood sample, fish were sacrificed, weighed and measured, and the organs (gonads, liver, kidney) removed and weighed and related to the total fish weight and multiplied by 100 and expressed as the gonadosomatic (GSI) and hepatosomatic indices (HSI).

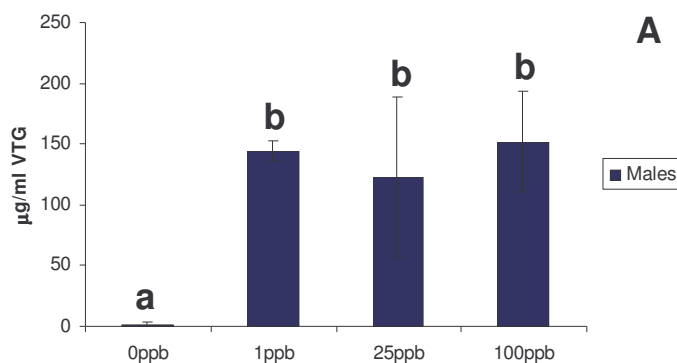


Figure III.3 – ELISA reader device.

Samples of the extracted organs were fixed in Bouin's fixative for 48h followed by dehydration in graded ethanol and embedding in paraffin following the usual histological techniques. Serial sections (5 μ m) were cut and stained with Hematoxylin and Eosin for further histological evaluation.

Results and discussion

Measured Vtg was significantly higher ($P < 0.05$) in fish exposed to the different concentrations, comparing with controls, but no differences were detected between treatments, which can be explained by the high potency of 17β - estradiol (E2) and therefore testing 1 or 100 μ g/l, considering the short time of the experiment (28 days) could induce the same Vtg amounts. Additionally, we also should consider the compound breakdown and microbial degradation of the E2.



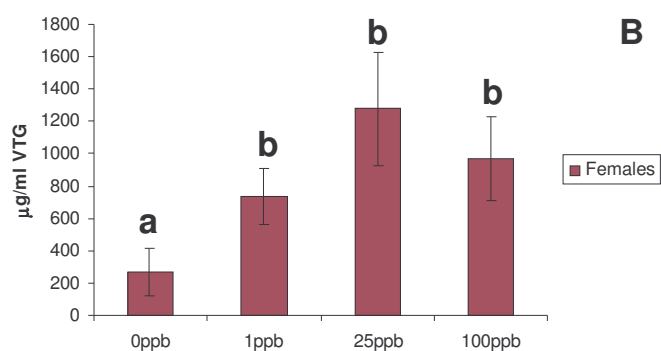


Figure III.4 - Vitellogenin concentration in males (A) and females (B) exposed to different 17β -estradiol concentrations of. Significant differences if letters are different.

The significant decrease ($P < 0.05$) of the GSI in males show that gonad growth is also affected by contaminant exposure (Figure III.5).

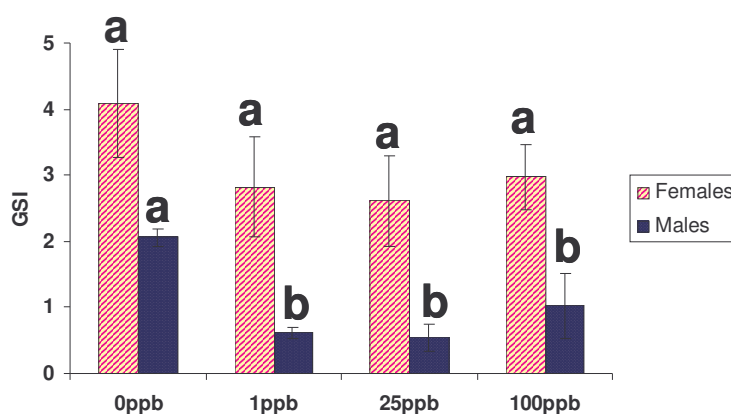


Figure III.5 - Gonadosomatic Index(GSI). Significant differences if letters are different.

Histological analysis revealed progressive regressed testis in fish according with the used E2 concentrations (Fig. III.6).

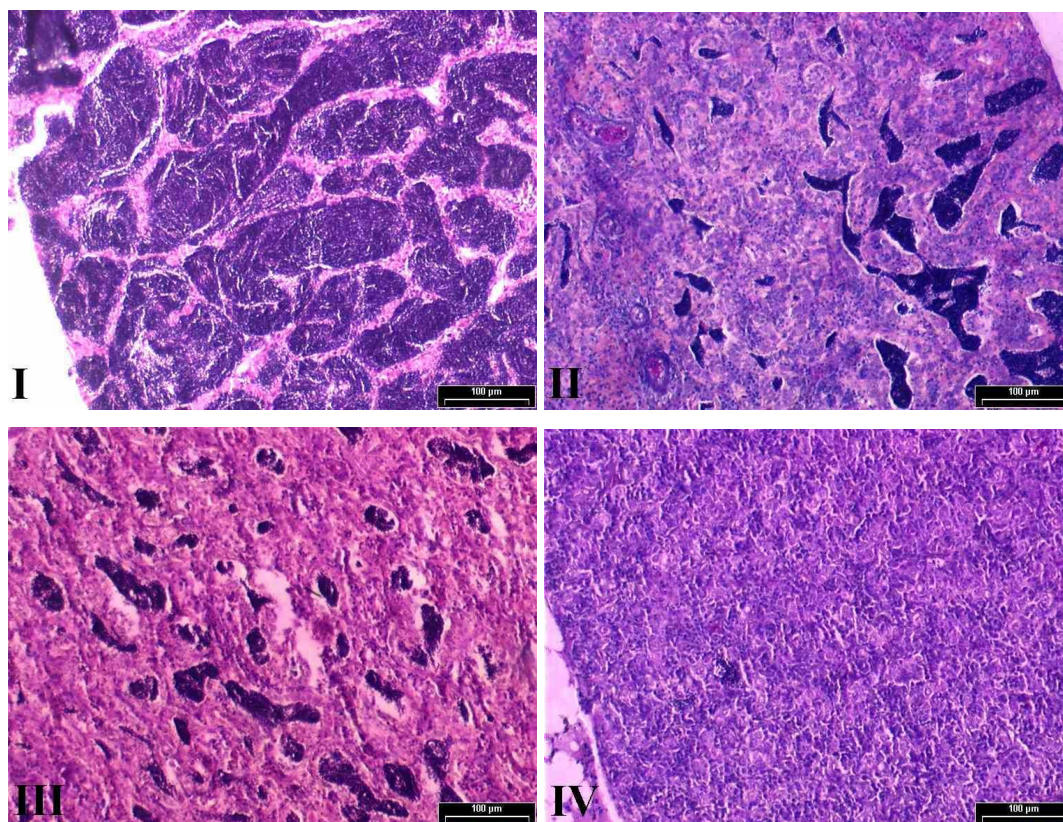


Figure III.6 - Male gonad of fish exposed to 0 (I), 1 (II), 25 (III) and 100 $\mu\text{g l}^{-1}$ (IV), showing spermatogenesis inhibited, with few (II, III) or absence of spermatozoa (IV) and enlarged blood vessels (arrowhead). Sections cut: 5 μm ; staining H&E.

Fish exposed to all different E2 concentrations show inactive spermatogenesis, with few spermatozoa and reduction of the seminiferous lobules size. All exposed fish also show vacuoles and fibrous and amorphous eosinophilic tissue around or in the seminiferous lobules. The vacuoles could be full of lipidic material or even Vtg, once it is known that Vtg is present in blood a long time before it begins to be taken by developing oocytes in females, or the gonad do not contain oocytes (immature ovary or testis) to take up Vtg, then Vtg accumulates and can cause severe histopathological changes in several organs (*in Gimeno et al.*, 1998).

It was also noticed the presence of intersex in two fish exposed to 100 $\mu\text{g/l}$ E2 (Fig. III.7), showing male tissue scattered with oocytes, but we could not say if that is a direct consequence of exposure or an hermaphroditism condition, considering the short experimental period.

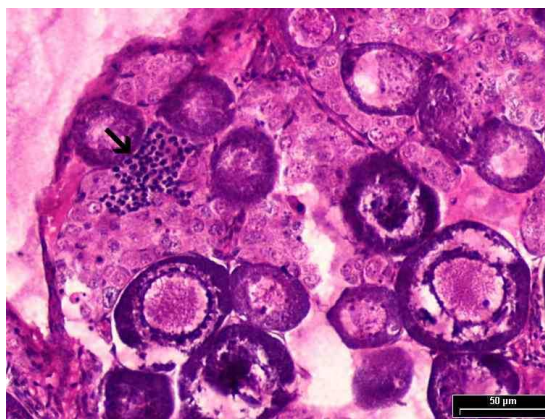


Figure III.7: Hermaphroditism or intersex? Legend: spermatids (arrowhead); Oocyte (Oc).

Although the nominal concentrations of E2 used in the present study were very high and not intended to reproduce a field situation, it is a valuable tool that helps the evaluation of gonadal histopathology. This study also confirms the high potency of E2 to induce Vtg, also documented by other authors.

Acknowledgments

The Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project “*Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater*”, (POCTI/36303/MGS/2000) and also the author PhD grant (SFRH/BD/3093/2000).

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Annex IV - Estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated sewage effluent

Estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated sewage effluent

M.S. Diniz, I. Peres and J.C. Pihan (2003)

Presented as poster at 5th Iberian and 2nd Iberoamerican Congress of Environmental Contamination and Toxicology (CICTA 2003) Porto, 22-24 September

Abstract

To assess the estrogenic potency of a treated domestic sewage effluent from a Sewage Treatment Plant (STP) located in Lisbon (Chelas), sixty mirror carps were exposed to different dilutions of the sewage effluent (0, 25, 50 and 100% sewage effluent) during 28 days. Vitellogenin induction in males has been used as a biomarker of exposure to xenoestrogens. Therefore, at the end of the experiment blood samples were taken for vitellogenin analysis and the fish were sacrificed and dissected. Gonads, Liver and kidney samples were taken for histological evaluation of the sewage effects. The results show a progressive induction of the vitellogenin in exposed fish, both males and females, according to the different dilutions of the sewage effluent considered. The gonadosomatic index show a significant decrease ($P < 0.05$) in fish exposed to 100% effluent in comparison with controls. The Hepatosomatic Index (HSI) was elevated in all exposed fish in comparison with controls, although statistically not significant. Histological abnormalities in fish gonads were evaluated and related with the different percentages of sewage effluent.

Key words: estrogenic, biomarker, vitellogenin, *Cyprinus carpio*, sewage

Introduction

There is an increasing number of anthropogenic chemical compounds that have been released into the environment and possess the potential to disrupt the endocrine system of wild life and humans (Allen *et al.*, 1999; Rodgers-Gray, 2001). There are many documented reports that identified and quantified chemicals with estrogenic activity in STPs (Station Treatment Plants) (Spengler, *et al.*, 2001). Studies developed by Harries *et al.* (1999) showed the estrogenic potency from STPs to rainbow trout. Additionally, Allen *et al.* (1999) identified the presence of xenoestrogens in lagoons and rivers close to U.K. STPs where intersex fish were often found.

Vitellogenin (Vtg) is an estrogen-inducible phosphoprotein used as a useful biomarker of xenobiotics exposure in fish and other animals. Normally it is synthesized by the liver of female oviparous vertebrates during oogenesis and is precursor of egg yolk proteins. Several studies reported the Vtg synthesis in both male common carp (*Cyprinus carpio*) in streams impacted by sewage effluent (Purdom *et al.*, 1994; Folmar *et al.*, 1996). Moreover, the use of biomarkers of potential endocrine disruption in fish are important for detecting and monitoring potential adverse effects of environmental contaminants in aquatic organisms.

The main objective of this study is to evaluate the estrogenic responses of carps exposed to a STP treated effluent located in Lisbon (Portugal), using Vtg as biomarker of exposure and also histopathological analysis.

Materials and Methods

Four fiber-glass (1m³) tanks (Fig.IV.1) was set up in a Lisbon (Portugal) Sewage Treatment Plant (Fig. IV.2), with one of a series of treated sewage effluent dilutions produced by mixing different percentages of effluent with clean water (company water previously dechlorinated). Nominal concentrations of treated sewage effluent were 0% (only clean water), 25%, 50% and 100%. Each tank was supplied with a constant flow rate of water that totaled 8 L/min. Tanks were also aerated and temperature and pH were monitored.



Figure IV.1: Experimental tanks at the sewage treatment plant.

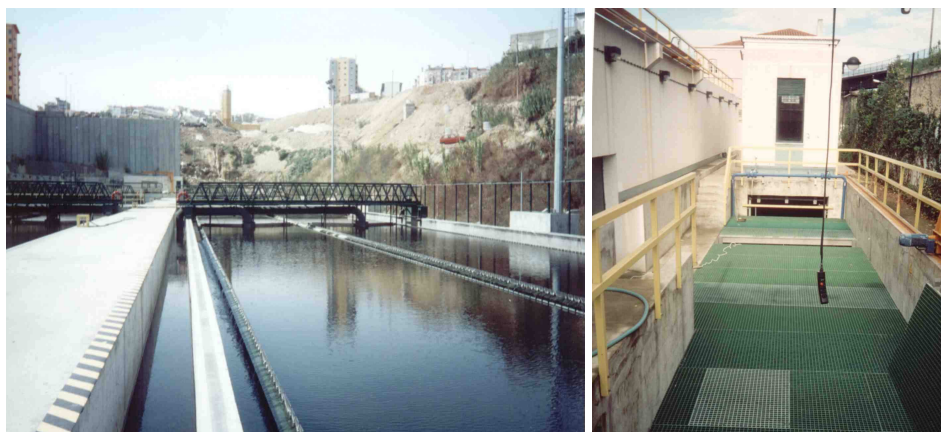


Figure IV.2: View of the Sewage Treatment Plant (A) and sand filters (B).

Sixty sexually mature adult carps (*Cyprinus carpio*) (Fig. IV.3), with an average weight of $12,8 \pm 3,1$ g, obtained in a local producer, were randomly distributed (15 in each tank) and daily fed with commercial pellets. Carps were exposed to the graded concentrations of treated sewage effluent during 28 days. Mortality was also recorded.



Figure IV.3: Carp (*C. carpio*).

At the end of the experimental period, fish were sampled and blood was collected from the caudal vein and centrifuged for 15 min. at 4,000xG (4°C). Plasma was then immediately frozen at -80°C until further analysis. Vitellogenin concentrations in plasma were achieved by Enzyme Linked Immunosorbent Assay (ELISA) under a protocol adapted from Denslow *et al.* (1999).

Fish were sacrificed after blood sampling, weighed and measured, and dissected for organs removal. Gonads, liver and kidney were sampled and fixed in Bouin's solution for 48h and then placed in 70% ethanol until further histological processing. Samples embedded in paraffin were sectioned (5 μm) and stained with hematoxylin and eosin. The gonadosomatic and hepatosomatic index, GSI and HSI respectively, were calculated as percentage of total body weight, in order to provide information of the gonad and liver conditions.

Results and discussion

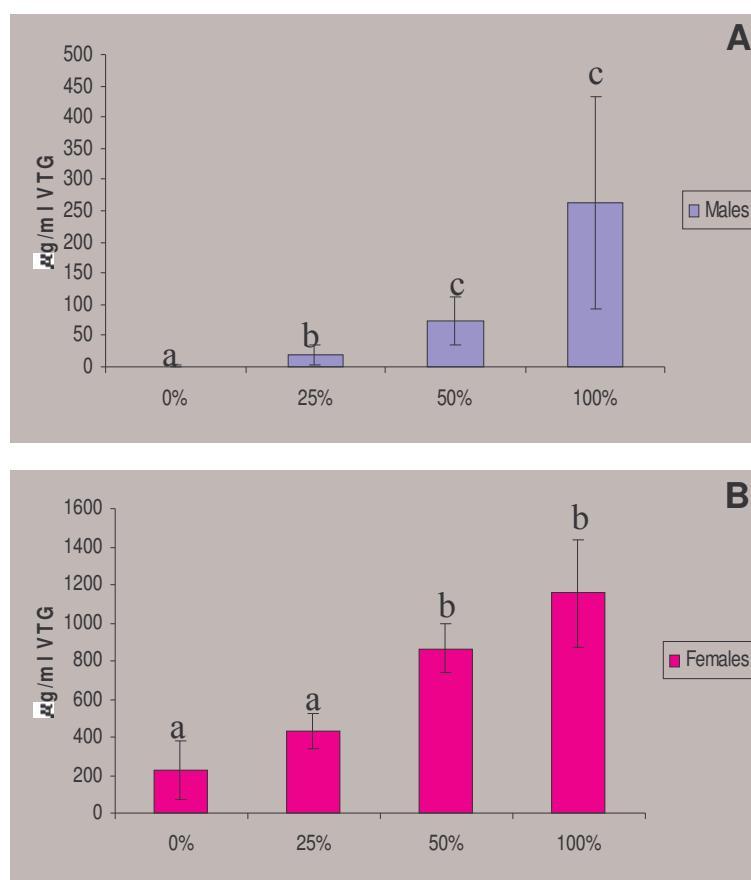


Figure IV.4 - Vitellogenin levels in males (A) and females (B) exposed to different grades of treated sewage effluent. Significant differences if letters are different.

The significantly ($P < 0.05$) higher levels of Vtg measured in the plasma of both carp males and females exposed to 50% and 100% sewage effluent show that mature fish responded to an estrogenic exposure by synthesising Vtg which is in agreement with other similar studies (Gimeno et al., 1999).

The histopathological analysis show that only fish exposed to 100% sewage effluent present visible morphological changes with an extensive tissue degeneration. The others sewage grades show mature testis with active spermatogenesis and spermatogenic cells in different stages of development.

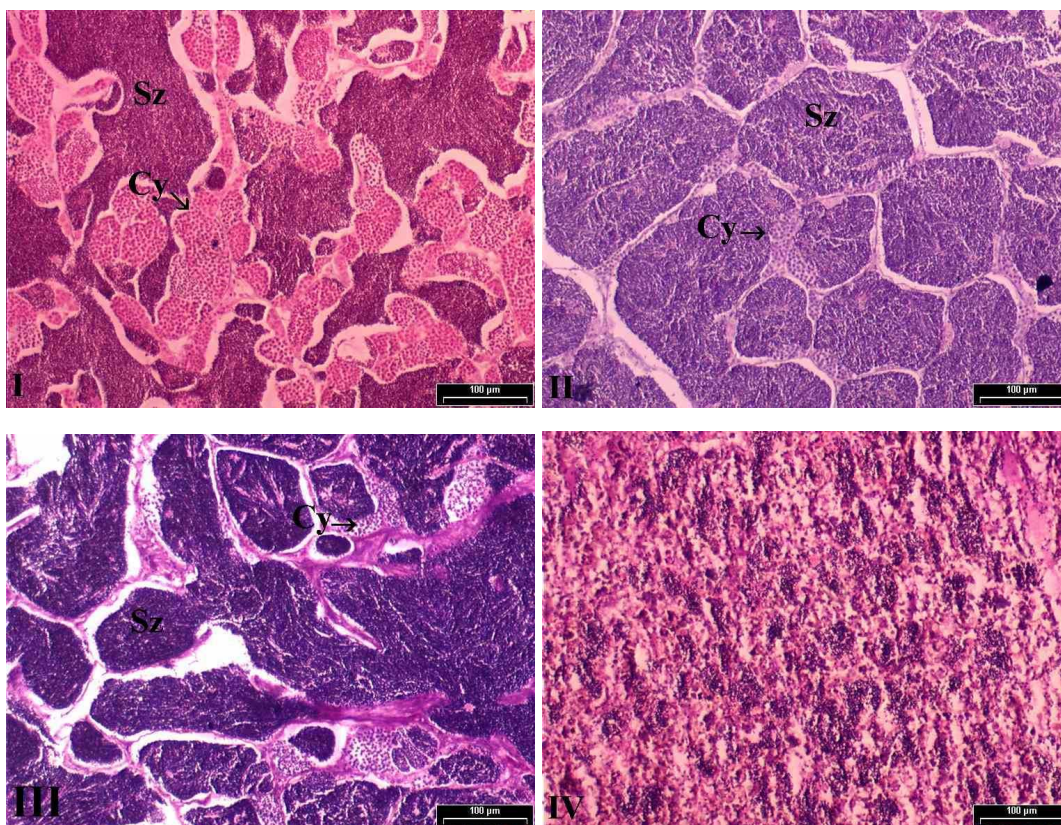
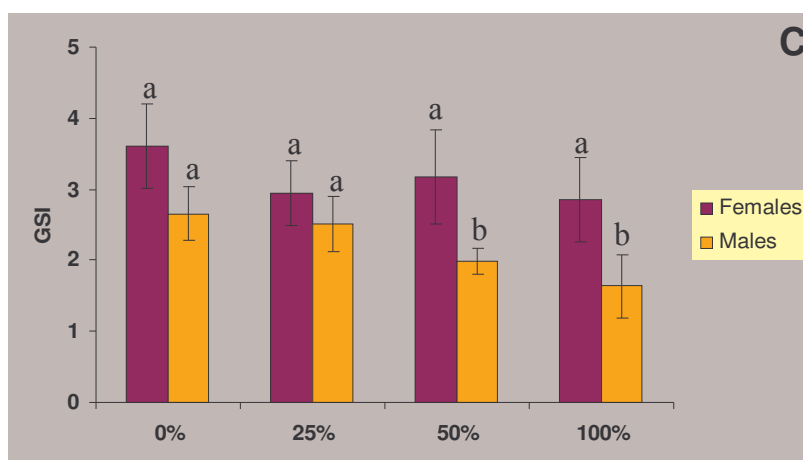


Figure IV.5 - Testis of mature fish exposed to different concentrations of sewage effluent I (0%), II (25%), III(50%), IV(100%), showing active spermatogenesis (I, II and III) and extensive tissue degeneration (IV). Sz (spermatozoa); Cy (spermatogenic cysts). Sections cut: 5 µm; staining H&E.

These findings are in agreement with GSI data, which revealed significant differences ($P < 0.05$) in males exposed to the highest effluent concentration.



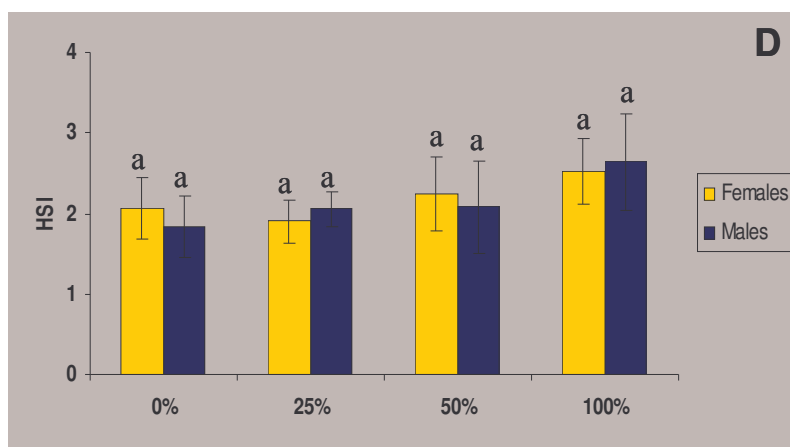


Fig. IV.6 - Gonadosomatic (C) and Hepatosomatic indices (B). Significant differences if letters are different.

The present work show that the studied sewage effluent, possess estrogenic activity although no oocyte development was noticed which can be explained by the short exposure time (28 days).

Acknowledgments

The Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project “*Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater*”, (POCT/36303/MGS/2000) and also the author PhD grant (SFRH/BD/3093/2000). We also thank to J. Martins from SIMTEJO (Chelas- Sewage Treatment Plant) for STP facilities in the set up and maintenance of experimental tanks.

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Annex V - Study of the Endocrine Disruptor Compounds Biodegradation

Study of the Endocrine Disruptor Compounds Biodegradation

R. Maurício, M. Diniz, E. Mateus, L. Amaral, F. Santana (2001)

Presented in Iberian Conference on Environmental Protection, Instituto Piaget – Viseu, 12 a 14 de Julho (Portugal).

Abstract

The negative impact of xenobiotics in the aquatic wildlife has generated serious modifications in the ecosystems, such as the disequilibrium of the populations relatively to its sex ratio composition (males/females), which have been associated to the effects of endocrine disruptor compounds (EDCs). The mechanisms of removal (in sewage treatment plants, STPs) or the routes of transference of EDCs through sources of diffuse pollution into the aquatic environment are not well known, even so these aspects constitute factors key for the reversion of effects in aquatic ecosystems. This project aims at the study of EDC's in urban wastewaters through the knowledge of the capacity of removal associated to the processes of treatment commonly used and the evaluation of potential effects caused by the respective discharges (liquid and solid). To the level of the urban wastewaters treatment it is intended to study the aspects related with kinetic of degradation of EDC's in the biological processes and their removal by solid/liquid separation. With regard to the biological treatment, the kinetics of aerobic and anaerobic degradation will be studied, to the laboratorial scale. The separation promoted by sedimentation process will be evaluated considering the influence of adsorption to particles and the removal of the particulate phase associated to these compounds. The preliminary results showed that the removal of nonylphenol (NP) in the Chelas sewage treatment plant is effective. A significant reduction (> 95%) of the concentration of this compound along the treatment steps of the working station was observed, reducing, therefore, the potential adverse effects that this compound could cause in the aquatic environment.

Introduction

In the last 50 years, some synthetic compounds such as the APEOs and PCB's, were widely used in varied domestic, commercial, and industrial applications, being part of them discharged into the aquatic environment. Some of these xenobiotic compounds, present in sewage effluents and sludge, e.g. NP, OP, PCB's, etc., can cause alterations in the endocrine function of fish and others species, namely the imbalance of the populations relatively to the

sex ratio composition (male / female). Therefore, the main objective of this work is the study of EDCs (nonylphenol, octylphenol, 17 β -estradiol and bisphenol A) in wastewaters, through the knowledge of the capacity of removal associated with the processes of treatment normally used and, the evaluation of potential effects caused by the respective discharges (liquid and solid).

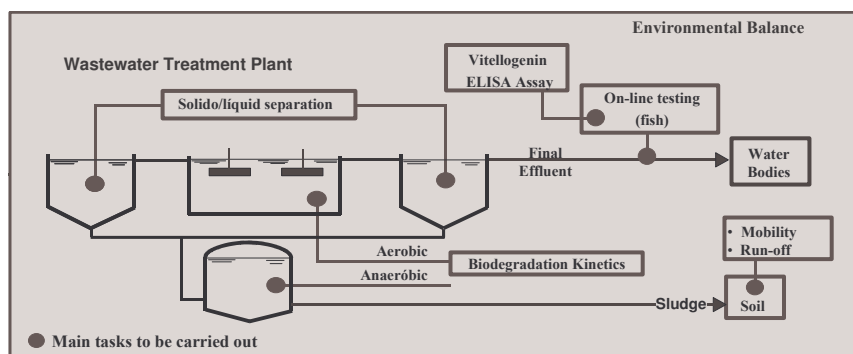


Figure V.1 - Endocrine disruptors: mass balance evaluation in a wastewater treatment plant.

Preliminary Results

Sampling campaign at the ETAR of Chelas

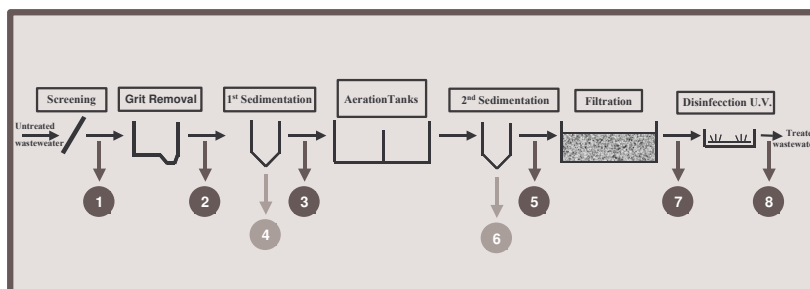


Figure V.2 – Sampling points at the Wastewater Treatment Plant.

Wastewater treatment: - Study of the kinetic of aerobic and anerobic EDCs degradation; - Removal by liquid solid separation; - Removal by adsorption to the particulate phase.

Annex VI - Screening Endocrine Disruptors Compounds in a Portuguese Wastewater Treatment Plant using Enzyme Linked Immunoassay (ELISA).

Screening Endocrine Disruptors Compounds in a Portuguese Wastewater Treatment Plant using Enzyme Linked Immunoassay (ELISA).

Maurício, R., Diniz, M., Amaral, L., Peres, I., Santana, F. (2003)

Panel Communication Presented at the 6th National Conference on Endocrine Disruptors,
12nd SESA Technical Jornada, November (26 – 28) Elche (Spain).

Abstract

Anthropogenic compounds that are able to disrupt the endocrine systems of wildlife species are a major cause for concern and lead to a demand for new screening methods. The presence and quantification of EDCs (Endocrine Disruptor Compounds) at different wastewater treatment stages is of major interest to identify endocrine activity of wastewater treatment plants (WWTPs) discharges to the environment. This study consists in a preliminary survey of concentrations of previously selected EDCs, undertaken to establish environmental concentrations to support a biological program assay exposing freshwater fish, evaluate removal efficiency at different stage wastewater treatment processes and study the kinetics of bioremoval of EDCs under aerobic/anaerobic conditions in order to achieve information for the optimization removal in the WWTPs. Selected endocrine disrupting chemicals (APEs, BPA and 17 beta-estradiol) were measured in samples from a WWTP located in Lisbon (Portugal), using an enzyme-linked immunosorbent assay (ELISA) kit developed by Takeda Industries Ltd. The sample treatment method involved a solid phase extraction step using Oasis HLB (Waters) cartridges from wastewater and sludge samples. Sub-samples were also taken and analyzed by LC-MS-MS. A short consideration is made concerning ELISA and LC-MS-MS methods and results obtained by these two methods are also under discussion. The results show that after treatment EDCs are still present in the effluent and sludge at environmental relevant levels and vary along the different wastewater treatment stages process.

Keywords: EDCs, Wastewater, ELISA, screening, LC-MS-MS

Introduction

In the last decade there have been an increasing concern about a group of chemical compounds with endocrine activity, which are present in aquatic environment, and that are able to cause adverse effects on reproduction of humans and wildlife species (Korner et al., 1998). Many reported effects were related with several classes of chemical compounds such as pesticides, aromatic compounds, alkylphenols, phytoestrogens and natural estrogens present in wastewater effluent. For that reason is of major importance the identification and quantification of these endocrine disruptors compounds (EDCs).

However the identification and quantification using the traditional analytical methods (e.g. GC-MS, LC-MS-(MS), HPLC) are difficult, expensive, time consuming and demand the development of specific procedures to analyse these compounds in complex environmental samples. Therefore, there is a strong need for fast, simple, and cost-effective methods for quantitative analysis of EDCs, such as ELISA (Goda et al., 2000).

The main objective of this study was to test a novel ELISA Kit developed by Takeda Chemical Industries (Japan) in order to identify the presence and quantify a group of previously selected EDCs in a wastewater effluent from Lisbon (Portugal). Additionally, we intended to discuss results with data obtained from sample analysis using LC-MS-MS.

Materials and Methods

Wastewater samples were filtered with a glass-fiber filter of one-micrometer pore size (Macherey – Nagel, Germany). For ELISA analysis, the EDCs were extracted from water using OASIS HLB 3cc solid phase extraction (SPE) column (Waters). Prior to extraction, the columns were conditioned with methanol and water. The EDCs were eluted 2x5ml of dichloromethane or methanol (10%), (depending the EDCs in analysis) and concentrated under gentle stream of nitrogen gas.

The Elisa kit, the technique and the protocols used were developed by Takeda Chemical Industries (Japan). The ELISA test is based in competitive reaction between a specific monoclonal antibody and the analysed compound (Goda et al. 2000). The inner surface of each well is coated with the protein called monoclonal antibody, which binds exclusively with EDC compound (antigen). The EDC derived from samples and an antigen-enzyme conjugate,

i.e. EDC labeled with a coloring enzyme, are premixed and added into each microplate well for a competitive assay, vying for a limited number of antibody binding sites. When the EDCC concentration is higher relative to the enzyme conjugate's, the EDC will predominantly bind antibody and vice versa.

The standard curve, a dose-response curve from known concentrations of EDCC standards, is determined from the absorbance at 450nm. The EDC concentration in each sample is accurately calculated from the response intensity of absorbance.

The LC-MS-MS method used was development by Petrovic et al. (2002). The HPLC system consisted of an HP 1100 autosampler and an HP 1090 A LC pump (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was performed using a reversed-phase C18 analytical column (LiChrospher 100 RP18) of 250 x 4 mm and 5- μ m particle diameter preceded by a guard column (4 x 4 mm, 5- μ m) of the same packing material from Merck (Darmstadt, Germany) The injection volume was set at 25 μ l and the flow-rate was 1 ml / min. Detection was carried out using an HP 1040 Mdiode-array UV-Vis detector coupled in series with an LC-MSD HP 1100 mass-selective detector, equipped with an atmospheric-pressure ionization source and electrospray (ESI) interface. Quantitative analysis was performed in a selected ion monitoring (SIM) mode using external calibration. To check the influence of ion suppression on the MS detection of target compounds, 4-heptylphenol was used as an internal standard for NI mode.

Discussion and Results

Table VI.1 and VI.2 – EDC show results from ELISA (VI.1) and LC-MS-MS (VI.2)

Table VI.1 – EDC Results by ELISA in samples

Sample points	APEs (μ g/l)	BPA (μ g/l)	17 β -estradiol (ng/l)
After - Screening	>ld	1,02	1,12
After - Grit Removal	Int.	0,21	1,12
After - 1 st sedimentation	5,77	1,32	1,73
Primary Sludge	0,724	>ld	1,38
Secondary Sludge	>ld	1,32	0,57
After - 2 nd sedimentation	9,73	>ld	1,38
After - filtration	5,77	0,55	0,91
Final effluent – UV	5,32	0,08	0,91

Table VI.2 – EDC Results LC-MS-MS

Sample points	APEs (µg/l)	BPA (µg/l)	17β-estradiol (ng/l)
After - Screening	78,15	1,55	<ld
After - Grit Removal	55,02	0,15	<ld
After - 1 st sedimentation			
Primary Sludge			
Secondary Sludge			
After - 2 nd sedimentation			
After - filtration	15,53	0,31	<ld
Final effluent – UV	5,20	<ld	<ld

Regarding 17β-estradiol and BPA, ELISA assay showed, in some samples, overestimated results in comparison with data obtained by LC-MS-MS which could be due to the broader cross-reactivity with other analytes, as pointed out by Goda et al. (2000). However, LC-MS-MS results for APE were higher than in ELISA which is not in agreement with other studies and can not be easily explained.

Although ELISA results could not be considered as absolute values, mainly due to the interferences, it can give an idea concerning the presence and quantity of the selected EDCs. Moreover allow quicker procedure and small sample values with low detection limits and acceptable costs (Goda et al., 2000).

Our results also support that ELISA can be used for environmental monitoring of EDCs but caution is need in interpretation and discussion of the results as consequence of several interferences that may occur influencing the final values. This study shows that more tests are needed for a better knowledge of ELISA data and compare it with traditional analytical methods.

Acknowledgments

The Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project “Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater”, (POCTI/36303/MGS/2000) and also the authors PhD grant (SFRH/BD/3093/2000 and SFRH/BD/3098/2000). We also thank to Simtejo the WWTP facilities. Departament de Química Ambiental del Centre d'Investigació i Desenvolupament del Consell Superior d'Investigacions Científiques, em Barcelona speccily to Prof. D. Barceló

and Doutor M. Petrovic for LC-MS-MS sample analysis. Takeda Chemical Industries Lda. – Japan, for giving us the opportunity to test the ELISA Kits.

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Annex VII - Nonylphenol distribution in *Cyprinus carpio* organs. Histology as a marker of visible effects. Preliminary Results.

Nonylphenol distribution in *Cyprinus carpio* organs. Histology as a marker of visible effects. Preliminary Results.

M.S. Diniz, I. Peres, Castro, L. and J.C. Pihan (2001)

Presented as Poster at Biomarkers of Environmental Contamination, 24 – 26 September 2001, Póvoa do Varzim , Portugal.

Abstract

Alkylphenol ethoxylate non-ionic surfactants (APEOs) have been widely used in a variety of industrial, household and commercial applications, being found in many types of products including detergents, plastics, emulsifiers, pesticides and consumer cleaning products. In sewage treatment plants and in natural environments APEOs are biologically degraded, resulting in less biodegradable small-chain ethoxylates, primarily mono- and diethoxylates, carboxylates and alkylphenols which are more toxic than the parent compound. Their degradation products, in particular nonylphenol (NP) are toxic to aquatic organisms and have been associated with the disruption of the endocrine system in fishes, acting as weak estrogen-like properties. Due to its enhanced resistance towards biodegradation, toxicity, estrogenic effects and ability to bioaccumulate in aquatic organisms NP have been regarded as the most critical metabolite of APEs. The aim of this study is to contribute to a better understanding of the NP distribution and tissue effects in fish. In this experiment twelve fish (*Cyprinus carpio*) were randomly distributed between three tanks (30 L), at a constant temperature ($15 \pm 1^\circ\text{C}$). After a period of acclimation fish were exposed to 0, 25 and 100 $\mu\text{g/L}$ of NP, in a semi-static 28 days test. At the end of the assay, NP was determined in fish organs (liver, gonads, kidney and muscle) by fluorescence HPLC. Tissue samples were also collected for histological analysis in order to assess NP effects. Vitellogenin ELISA are also to be performed. Preliminary results show that fish organs bioaccumulate increasing concentrations of NP according to the exposition treatments. An apparent decrease in GSI was observed in fish treated with NP. Histological examination also revealed alterations in testis structure.

Key words: Nonylphenol, bioaccumulation, endocrine disruption, *Cyprinus carpio*

Introduction

Alkylphenol ethoxylate non-ionic surfactants (APEOs) have been widely used in a variety of industrial, household and commercial applications, being found in many types of products including detergents, plastics, emulsifiers, pesticides and consumer cleaning products (Nichols et al., 2001). The use of these surfactants result in their discharges to the aquatic environment via wastewater treatment plants (WWTP) as well as directly from untreated effluents.

After entering the WWTP these compounds may undergo biodegradation, resulting in less biodegradable small-chain ethoxylates, primarily mono- and diethoxylates, carboxylates and alkyl phenols which are more toxic than the parent compound (Maguire, 1999; Ranke, 2000). Their degradation products, in particular nonylphenol (NP) are toxic to aquatic organisms and have been associated with the disruption of the endocrine system of fishes, acting as weak estrogen-like properties. Due to the enhanced resistance towards biodegradation, toxicity, estrogenic effects and ability to bioaccumulate in aquatic organisms NP have regarded as the most critical metabolite of APEOs (Ranke, 2000).

The aim of this study is to provide a major contribute to a better understanding with regard to distribution and bio-accumulation in fish tissues and to evaluate the histopathological effects caused by exposure to different concentrations of NP.

Materials and Methods

Sexually mature carp (*Cyprinus carpio*) (N=16, mean body weight 14.59 ± 2.36 g), were obtained from a state producer ("Aquário Vasco da Gama") at Lisbon district. The fish were transported to the laboratory facilities and randomly separated into four groups. Fish were maintained in four glass aquaria (Fig. VII.1), containing 30 L of tap water (previously de-chlorinated) aerated and at constant temperature $15 \pm 2^\circ\text{C}$. Carp were distributed in groups of four each and submitted to 16h: 8h (L:D) photoperiod. The water in aquaria was renewed each 48h and fish fed daily with commercial dry pellets (Alpis). The fish were acclimated to these conditions one week prior to the experiment.

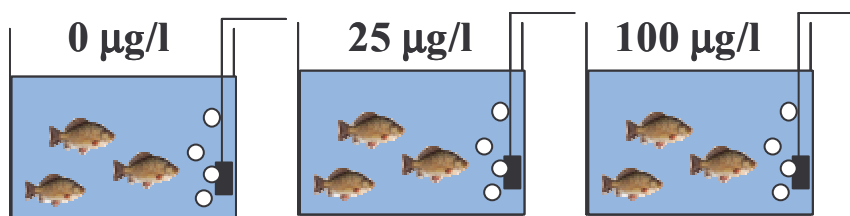


Figure VII.1 - Experiment design showing nominal concentrations of NP added to each aquarium.

C. carpio (Fig. VII.2) were exposed to nominal concentrations of NP (0, 1, 25 and 100 µg/L) during a period of 28 days. At the end all fish were sampled, weighed and measured. The organs (liver, kidney, gonad and muscle) were quickly excised, weighed and frozen in liquid nitrogen at -80°C . The concentrations of NP were determined in the aquaria water and organs by HPLC acopped to a fluorescence detector, after extraction. Sub-samples of the organs were taken and placed in Bouin fixative for 48 h before being processed using conventional histological techniques.



Figure VII.2 - Common carp (*Cyprinus carpio*).

Results and discussion

The highest levels of NP were detected in the liver, suggesting an important role in the bioaccumulation and possibly detoxifying processes. The GSI reduction in exposed fish may indicate a negative effect of NP on gonad development. The Sertoli cell layer is altered in fish exposed to 100 NP µg/L. The data analyses suggests that exposure to high doses of the weak estrogen agonist, such as NP, can compromise reproductive fitness.

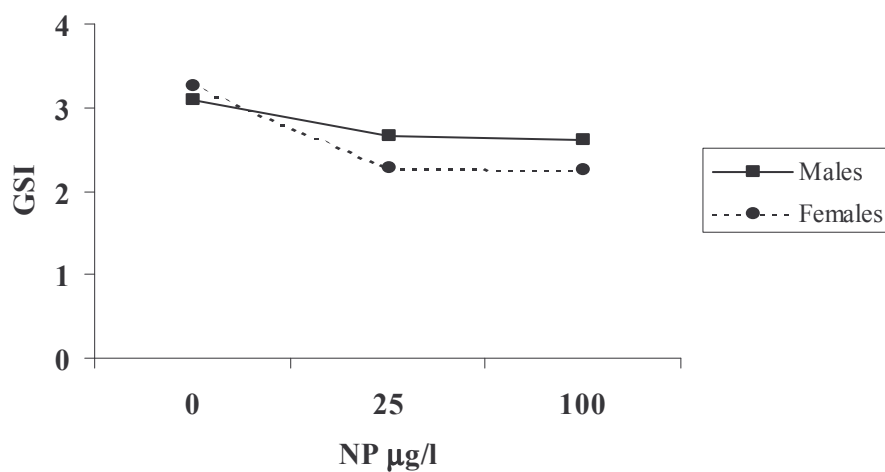


Figure VII.3 - Gonadosomatic index (GSI).

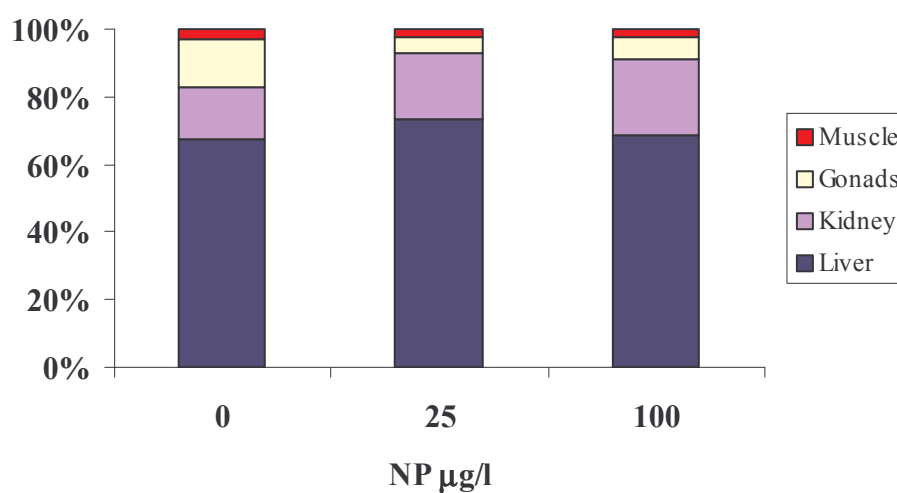


Figure VII.4 - Percentages of NP (calculated from µg/g wet weight) in muscle, kidney, gonads and liver of carp exposed to different concentrations of NP in water (0, 25 and 100 µg/L).

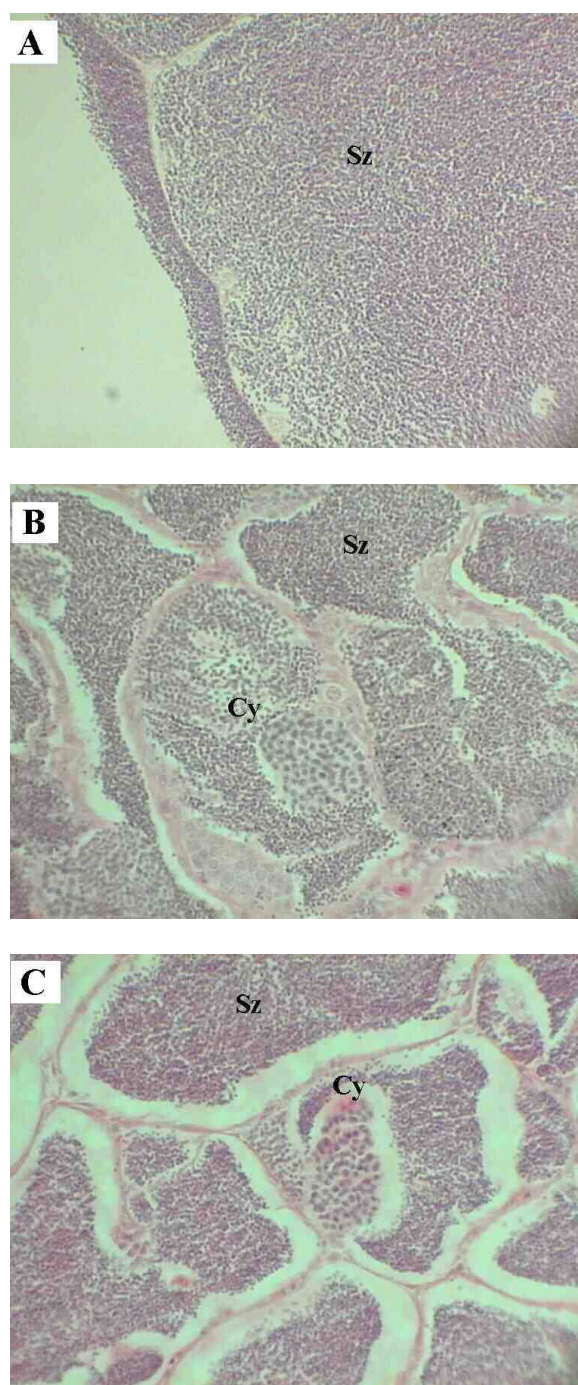


Figure VII.5 - *Cyprinus carpio* gonad (male), showing the seminiferous lobules in control fish (A) and in fish exposed to 25 and 100 NP $\mu\text{g/L}$ (B and C, respectively). Spermatogenesis was active in exposed fish but the number of Sertoli cells decreased in 100 $\mu\text{g/L}$ treatment. Note peripheral lacuna (Fig. 5C; Lp - arrowhead) caused by decrease of Sertoli cells. Legend: Sg (seminiferous lobules); Cy (spermatogenic cysts); Sz (spermatozoa). Arrowhead – Lp (Peripheral lacuna). Magnification 400X.. H&E stain. Sections cut at 5 μm .

Acknowledgements

To the Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project “*Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater*”, (POCT/36303/MGS/2000) and also the author PhD grant (SFRH/BD/3093/2000). We also thank to “Aquário Vasco da Gama” and E. Mateus.

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**Annex VIII - Bioaccumulation and effects of 4-n – Nonylphenol in *Cyprinus carpio*.
Preliminary Results**

Bioaccumulation and effects of 4-n-Nonylphenol in *Cyprinus carpio*. Preliminary Results

M.S. Diniz and I. Peres (2001)

Presented as Poster at Iberian Conference on Environmental Protection. Instituto Piaget – Viseu, 12 a 14 de Julho.

Abstract

The non-ionic surfactants alkylphenol polyethoxylates (APEOs) are usually used as emulsifiers in house and industrial cleaning agents, chemical and agriculture products and as plasticizers. Nonylphenol is a potent APEO with a structure similar to the endogenous estrogens and, therefore, can affect the biota causing endocrine disruption. Thus, it is very important the study of the bio-accumulation effects on living organisms to allow a better comprehension and understanding of the endocrine deregulation processes. In this study, groups of four carp (*Cyprinus carpio*) were randomly distributed by three glass tanks, in a semi-static exposure experiment, during 28 days. The carp were exposed to concentrations of 0, 25 and 100 µg/L of 4-n-nonylphenol under a constant temperature ($15\pm1^{\circ}\text{C}$). At the end of the experiment period nonylphenol concentrations in gonad, liver, kidney and muscle were quantified by HPLC using a fluorescence detector. Sub-samples of the organs were taken for histological analysis and tissue changes caused by nonylphenol exposure were evaluated. The results show that fish bioaccumulated increasing concentrations of nonylphenol in a dose-dependent manner. Histological analysis revealed that nonylphenol caused histopathological changes in *C. carpio* tissues.

Key words: nonylphenol, bioacummulation, endocrine disruption, *Cyprinus carpio*

Introduction

The APEOs have been widely used in the last 50 years in a vast diversity of commercial and domestic applications (Tsuda, et al., 2000), being used as emulsifiers in industrial cleaning, and domestic products (Nichols, et al., 2001). The APEOs are degraded successively to forms less biodegradable, such as the NP (nonylphenol), by the treatment processes of the station treatment plants (STPs) before being discharged into the aquatic environment.

Recent studies showed that the NP is one of the most potent APEOs metabolites of degradation (Arukwe, et al., 2000; Nichols, et al., 2001), having a similar structure to the endogenous estrogens and, consequently, can act by deregulating the endocrine system of fish.

Thus, studies performed in various fish species exposed to NP showed several anomalies, such as morphological and histological changes in gonads (Arukwe, et al., 2000; Schwaiger, et al., 2000). In addition, it was shown that the alkylphenols, as the NP, have the capacity to induce vitellogenin synthesis in male and juveniles of bony fish, such as the rainbow-trout (*Oncorhynchus mykiss*) (Jobling et al., 1996) or the carp (*Cyprinus carpio*) (Gimeno et al., 1998).

Thus, since NP possesses estrogenic activity, is of the greatest importance to study the adverse effects and bioaccumulation in living organisms, particularly at the reproductive level. The main objective of the present study is to contribute for a better comprehension concerning the bioaccumulation and adverse effects of the NP in fish.

Materials and Methods Sexually mature carp (*Cyprinus carpio*) (Fig. VIII.1), (N=12, mean weight: $14,59 \pm 2,36$ g), were randomly distributed in three groups and maintained in 50 liters aquaria, containing tap water with prior removal of chlorine. The fish were kept under a constant temperature of $15 \pm 1^\circ\text{C}$ and to a photoperiod regimen of 16-hour light: 8 hour dark. The animals were fed daily with pellet food (Alpis).

The water in aquarium was contaminated with nominal concentrations of 0, 25 and 100 $\mu\text{g/l}$ of 4-n-Nonilfenol (Fluka, Chemika 74430). During the experiment period (28 days) the water in the aquarium was renewed and contaminated with the different concentrations of NP.

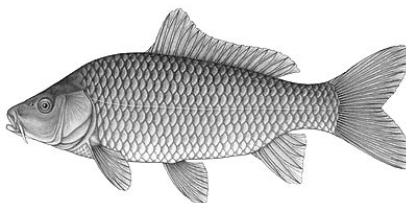


Figura VIII.1 - Carp (*Cyprinus carpio*)

After 28 days fish were sampled, weighed, measured and the organs removed (liver, gonad, kidney and muscle). The size of the gonad was express as total percentage of the body mass (Gonadosomatic Index – GSI). Sub-samples of the organs were taken and placed in Bouin's fixative for 48 hours. Then, the organs were processed using the usual histological techniques for light microscopy examination. Another sub-samples were stored at -80°C until further analysis of the NP concentrations in fish tissues.

Results

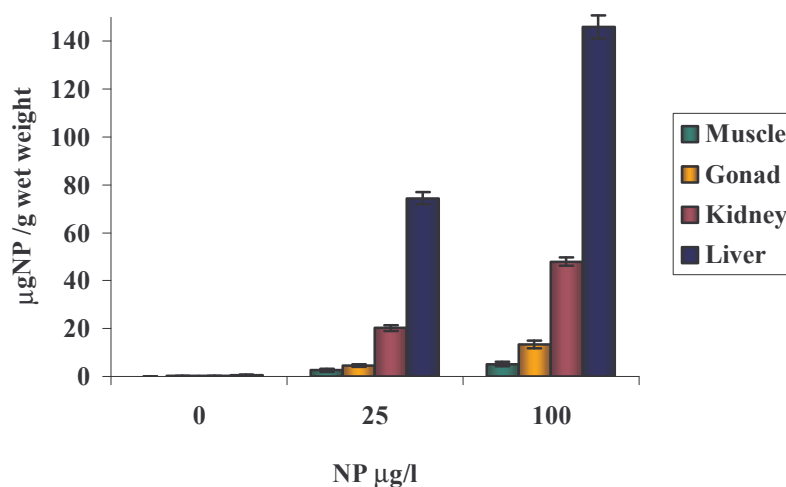


Figure VIII.2 - NP levels ($\mu\text{g/g wet weight}$) in muscle, kidney, gonads and liver of carp, exposed to different concentrations of NP (0, 25 and 100 $\mu\text{g/l}$).

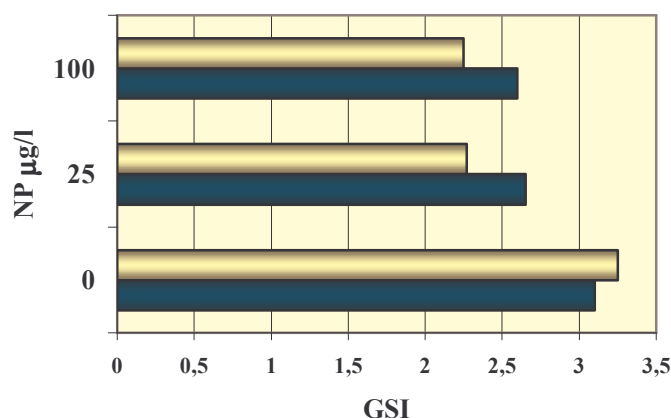


Figure VIII.3 - Gonadosomatic Index in carps exposed to 0, 25 and 100 $\mu\text{g/l}$ of NP.

The liver shows the highest concentrations, which suggests the possibility of the liver have a

major role in the bioaccumulation and metabolizing process of NP in *Cyprinus carpio*.

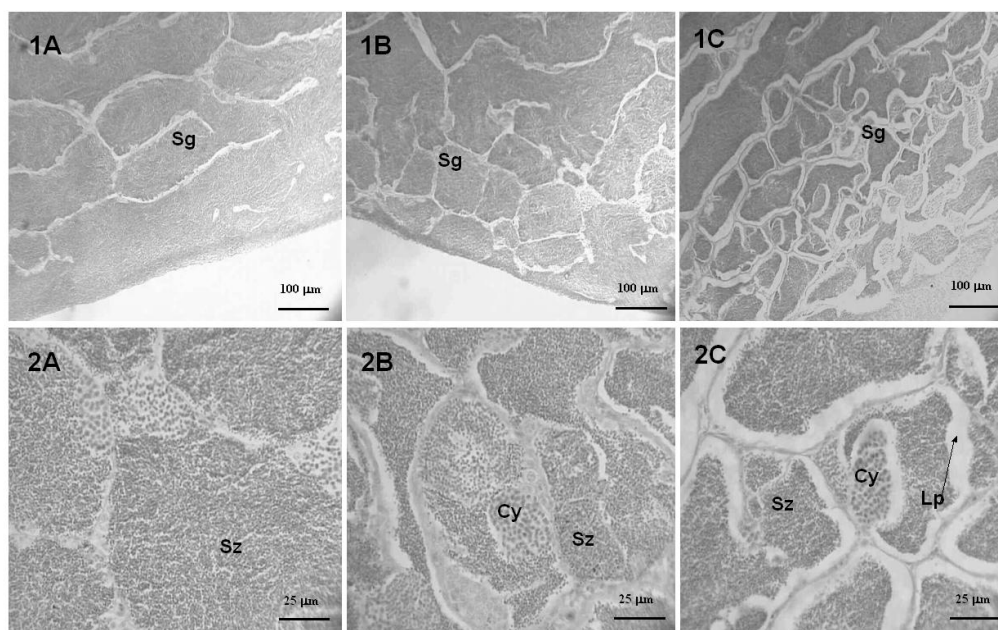


Figure VIII.4 - *Cyprinus carpio* gonad (male), showing the seminiferous lobules in controls (1A-2B) and in fish exposed to 25 e 100 $\mu\text{g/l}$ (1B-2B e 1C-2C, respectively). Notice a reduction in the seminiferous lobules diameter in exposed fish. Legend: Sg (seminiferous lobules); Cy (spermatogenic cysts) Sz (spermatozoa). Bar: 100 μm (1A, 1B, 1C) and 25 μm (2A, 2B, 2C). Coloration H&E. (cut 5 μm).

The Fig. VIII.3 show a slight decrease in the gonadosomatic index (GSI), of fish exposed to the different treatments. The reduction in the gonad weight seems to be dose-dependent.

The light microscopy examination revealed that testes of the fish exposed to the nominal concentrations of 100 NP $\mu\text{g/l}$ show morphological alterations in the gonads (Fig. VIII.4).

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Annex IX - European Legislation and Policy actions on Endocrine Disruptors

Table IX.1 - Main sources and uses of some environmentally persistent chemicals

Abbreviation	Type of chemical	Applications/sources
ACB	Alkylated chlorobiphenyls	PCB substitutes
CP	Chlorinated paraffins	C10-C30 alkanes with 30–70 % chlorine, plasticisers for use in polymer manufacture, metal working fluids, flame retardants, paint additives
Cyclodienes	Aldrin, endrin, dieldrin, endosulfan, chlordane, heptachlor	Pesticides
DDE	4-dichloro-diphenyl-dichloroethene	4 Degradation product of DDT
DDT	4, 4-dichloro-diphenyl-trichloroethane	Insecticide (still used in tropical developing countries)
HAC	Halogenated aliphatic compounds	Volatile halogenated solvents such as tri- and tetrachloroethylene and ethylene dichloride tar
HCB	Hexachlorobenzene	Formerly used as a fungicide; also a combustion by-product
HCH	Hexachlorohexanes	Used as insecticide. Several persistent isomers including lindane (gamma isomer)
HMs	Heavy metals	Large numbers of potential sources e.g. combustion by-products, industrial processes, water treatment sludges, batteries, paints, antifouling coatings, zinc and cadmium from car tyres, mercury in dental amalgam, nickel from diesel, cadmium from phosphate fertilisers, arsenic, copper and chromium from wood preservatives
NPN	Nonylphenol	Stable degradation intermediate of nonylphenol ethoxylates used as detergents and additives in latex and plastic goods
Oms	Organo-metallic compounds	Mainly mercury, lead and tin compounds; mercury in paints; seed disinfectants; anti-sliming agents; lead in petrol; tin in marine anti-fouling agents
PAC	Polycyclic aromatic compounds	Heterocyclic aromatic compounds, derivatives of PAHs (such as nitro-, chloro- and bromo-PAHs)
PAE paint	Phthalic acid esters (phthalates)	Plasticisers (e.g. in PVC — polyvinyl chloride); additives, varnishes; cosmetics; lubricants
PAH	Polycyclic aromatic hydrocarbons	Crude oil; by-products of incomplete combustion by-products of fuel and wood; creosote wood preservative; coal tar
PBB/PBDE	Polybrominated biphenyls/diphenyl ethers	Intermediates for chemical industry; brominated flame retardants
PCB	Polychlorinated biphenyls (and their degradation products)	More than 200 substances (but not all congeners are found in technical product or in the environment); insulating fluid in transformers; cables; plasticisers; oil and paint additives; hydraulic fluids; combustion by-products
PCC	Polychlorinated camphenes	Pesticides e.g. toxaphene, campechlor
PCDD/F	Polychlorinated dibenzo-p-dioxins/dibenzofurans, collectively referred to here for simplicity as 'dioxins'	More than 200 substances; mainly by-products from combustion and other chemical processes, such as incineration; paper pulp bleaching and metal refining; as contaminants impurities in PCBs, PCP, transformer oils; and chlorinated phenolic herbicides; contaminants; incinerators; paper pulp bleaching
PCDE	Polychlorinated diphenyl ethers	By-products of PCP manufacture; PCB substitutes; pesticide additives
PCN	Polychlorinated naphthalenes	Insulating fluids in capacitors; flame retardants; oil additives; wood preservatives, pesticides; combustion by-products
PCP	Pentachlorophenol	Fungicides; bactericides; wood preservatives
PCS	Polychlorinated styrenes	By-products of chemical processes
PCT	Polychlorinated terphenyls	PCB substitutes

Source: Based on Swedish EPA, 1993

Table IX.2 - Some Legislation and Regulations in EU that can be amended to control EDCs.

Legislation and Regulations that can be amended to control EDCs	
Type of Control	Legislation and Regulations
Water Quality	Habitats Directive (92/43/EEC)
	Surface Water Abstraction Directive (75/440/EEC)
	Groundwater Directive (80/68/EEC)
	Drinking Water Directive (80/778/EEC)
Industrial Sector	Sludge Directive (86/278/EEC)
	Urban Wastewater Treatment Directive (91/271/EEC)
	Integrated Pollution Prevention and Control (96/61/EC)
	Sludge (Use in Agriculture) Regulations 1989 (SI 1263)
Substances	Marketing and Use Directive (76/769/EEC)
	Detergent Directive (76/769/EEC)
	Environmental Protection (Prescribed Processes and Substances) Regulations 1991 (SI 472)
	Dangerous Substances Directive (76/464/EEC)
	Biocidal Products Directive (98/8/EC)
	Control of Pesticides Regulations 1986 (SI 1510)

Table IX.3 - Some ecological impacts and possible associations with chemicals

Observation/impact	Species	Substance	Association
Large-scale effects			
Eggshell thinning	Guillemot, eagle, osprey, peregrine falcon	DDT/DDE	5
Reproduction	Seal, otter	PCB	4
Skeletal malformation	Grey seal	DDT, PCB	4
Pathological changes	Seal	PCB, DDT, metabolites	3
Reproduction	Mink	PCB	5
Reproductive disturbances	Osprey	DDT, PCB	5
Reproductive disturbances	Eagle	DDT, PCB	2-3
Reproduction (M74 syndrome)	Salmon	Chlorinated substances	2
Imposex Molluscs	e.g. dogwhelk	TBT	5
Impairments in wildlife in relation to endocrine disrupting chemicals (EDCs)			
Sperm quality, cryptorchidism	Panther		2-3 (effects observed in inbred population)
Population decrease	Mink, otter		2-3
Female reproductive disorders	Seal		4-5
adrenocortical hyperplasia			4-5
Eggshell thinning Birds			4-5
Embryotoxicity and malformations			4-5
Malformation of reproductive tract			2-3
Reproductive behaviour			2-3
Microphalli and lowered testosterone levels	Alligators		3-4 (effects seen in connection with accidental contamination)
Vitellogenin	Fish		4-5
Masculinisation			3-4
Lowered testosterone levels			2-3
Reduced testis size			2-3
M74 and early mortality syndromes			1-2
Imposex Molluscs			5

Notes: The strength of the association is assessed on the scale: 1 = no observed; association, 2 = suspected; association, 3 = weak; association, 4 = clear; association, 5 = significant; association.; Sources: EEA, 1998 (largescale; effects); Swedish EPA, 1998b (impairments in; wildlife in relation to EDCs)

