Universidade de Aveiro Departamento de Biologia
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Respostas de biomarcadores a disruptores endócrinos em *P. microps*

Biomarker responses to endocrine disruptors in *Pomatoschistus microps*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada - Ramo Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos, Investigadora de Pós-doutoramento do Departamento de Biologia e CESAM (Centro de estudos do Ambiente e do Mar) da Universidade de Aveiro e co-orientação do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia e CESAM da Universidade de Aveiro

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Palavras-chave

AChE, Biomarcadores, Compostos Disruptores Endócrinos, PCB-77, *Pomatoschistus microps*, p,p'-DDE, Vitelogenina, 17β-estradiol

Resumo

A presença de compostos químicos de origem antropogénica, nos mais variados ecossistemas aquáticos, já não é um assunto novo. No entanto, apesar da proibição da utilização de muitos destes compostos, a sua presença continua a ser detectada mesmo a concentrações baixas. As zonas costeiras, mais propriamente os estuários, são objecto de grande preocupação. Devido ao seu elevado valor ecológico e económico e, também, o facto de serem um destino final de uma grande quantidade de compostos maioritariamente de origem antropogénica, torna necessário a sua monitorização e o desenvolvimento de métodos com espécies autóctones que permitam uma melhor avaliação do impacto desses compostos.

Assim, o objectivo principal deste trabalho consistiu na determinação e avaliação das respostas de biomarcadores a determinados contaminantes disruptores endócrinos (EDCs), utilizando o peixe estuarino *Pomatoschistus microps* como organismo-teste e na avaliação da viabilidade de utilizar a quantificação da vitelogenina (vtg) nesta espécie como biomarcador de exposição a esses compostos. Esta avaliação foi realizada após 21 dias de exposição, tanto em juvenis (em corpo inteiro) como em fêmeas (fígado e gónadas). Em conjunto com a vtg foram analisados os índices hepato e gonadossomáticos (HSI e GSI, respectivamente) para a disrupção endócrina e a avaliação da acetilcolinesterase (AChE) para exposição a neurotóxicos. Os EDCs testados, a nível sub-letal, foram o 17β-estradiol (E2), o pesticida p,p'-DDE e o PCB-77, todos considerados compostos disruptores endócrinos de acção estrogénica e/ou antiestrogénica. Os resultados mostraram, em juvenis, um aumento na vtg por acção do 17β-estradiol e uma diminuição nos seus valores por acção do PCB-77. Nas fêmeas, foram encontrados resultados significativos com o aumento da vtg no fígado depois da exposição ao PCB-77 e nenhuns resultados significativos nos outros parâmetros. A exposição ao p,p'-DDE não induziu alterações significativas nos parâmetros endócrinos analisados. Relativamente à AChE, o PCB-77 parece aumentar a sua actividade nos juvenis e observa-se o resultado oposto nas fêmeas. Por sua vez, o p,p'-DDE parece não afectar a actividade da AChE nas fêmeas. Em conclusão, os juvenis de *P. microps* parecem responder à contaminação por EDCs a concentrações ambientais relevantes de E2 e PCB-77 e a utilização da vtg neste estágio de vida parece apropriado para identificar a contaminação por EDCs em estudos de monitorização ambiental. As fêmeas deste peixe, no geral, parecem não ser suficientemente afectadas pelas concentrações dos EDCs testados.

keywords

AChE, Biomarkers, Endocrine disruptors compounds, PCB-77*, Pomatoschistus microps*, p,p'-DDE, Vitellogenin, 17β-estradiol

abstract

The presence of chemical compounds, in the most diverse aquatic ecosystems it was not a recent subject. However, in spite of the prohibition of the use of many of these compounds, their presence in the environment keeps being detected even at low concentrations. The coastal areas, namely the estuaries, are object of great concern. Due to their great ecologic and economic value and, also, the fact of being the final destination of a lot of compounds mainly from anthropogenic sources, become necessary its monitoring and the development of methods with autochthonous species that allow a better evaluation of the impact of those compounds. Thus, the main objective of this work consisted in the determination and evaluation of the biomarker responses to selected endocrine disruptor compounds (EDCs) using the estuarine fish *Pomatoschistus microps* as organism test and in the assessment of the viability to use vitellogenin quantification (vtg) in this species as a biomarker of exposure to these compounds. This evaluation was realized after 21-days exposure, both in juveniles (whole body) and females (liver and gonads). In addition to vtg were analysed the hepato and gonadossomatic indexes (HSI and GSI, respectively) for endocrine disruption assessment and acetylcholinesterase (AChE) for exposure to neurotoxicants. The EDCs tested, at sub-lethal level, were the 17β-estradiol (E2) and the pesticides p,p'-DDE and PCB-77, all considered endocrine disruptors with estrogenic and/or antiestrogenic activity. The results showed, in juveniles, an increase in vtg-like proteins by action of 17β-estradiol and a decrease in its values by action of PCB-77. In females, it was found significant results with an increase in liver vtg-like proteins after exposure to PCB-77 and no significant results in the other endpoints. The p,p'-DDE exposure did not induce any significant alterations in the endocrine endpoints analyzed. Relatively to AChE, the compound PCB-77 seems to increase its activity in juveniles and the opposite result was observed in females. In turn, p,p'-DDE seems to not affect the AChE activity in females. In conclusion, the juveniles of *P. microps* seem to respond to EDC contamination at environmental relevant concentrations of E2 and PCB-77 and the use of vtg in this life stage seems appropriate to track EDC contamination in field biomonitoring studies. The female fish, in general, do not seem to be clearly affected by the exposure to these EDCs at the concentrations tested.

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CHAPTER 1.

General Introduction

1.General Introduction

1.1.Endocrine disrupting compounds

Pollution is defined by the Group of Experts on the Scientific Aspects of Marine Pollution from the United Nations as 'the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of the sea water and reduction of amenities' (GESAM, 2000).

In the case of estuaries and other coastal areas, since they are located in high populated areas, their exposition to a great quantity of contaminants from human, industrial and agricultural sources is even higher (Dolbeth et al., 2007; Paerl, 2006). This ecosystem has great importance at various levels since it is considered one of the most productive natural habitats, where large phytoplankton populations support a variety of organisms, including many commercially and recreationally important marine fish and crustacean species that use it as nursery grounds (Fulton and Key, 2001). For these reasons it is important to protect these ecosystems from the input of environmental contaminants such as endocrine disruptors compounds (EDCs) and try to find tools that can help to evaluate the degree of contamination and exposure in the natural populations that inhabit, use these places for reproduction and/or in an early-life stages (Boudreau et al., 2004; Monteiro et al., 2007).

Endocrine disruptors compounds are a diverse group of substances that have been detected in wastewater effluents and surface waters around the world, namely in estuaries. This contamination is due to industrial wastes, agricultural runoffs, and municipal wastewater effluents where elimination through sewage treatment plants is incomplete (Benotti et al., 2009; Boudreau et al., 2004).

In definition, an EDC is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, its progeny, or (sub) populations (World Health Organization, 2002). Their

effects are mainly in aquatic ecosystems which are visible at population levels, namely in fish, where EDCs can lead to altered sex steroid levels (e.g. 17β-estradiol), masculinization, vitellogenin induction or feminization (Boudreau et al., 2004; Ferreira et al., 2009).

The action, main sources (natural or synthetic) and compounds included in the group of EDCs were described years ago by Cheek and McLachlan (1998). The industrial/synthetic chemicals mostly used in the past, and still detected in the environment, include organochlorine pesticides (OCPs), herbicides and polychlorinated biphenyls (PCBs) (Porte et al., 2006). The EDCs in use more recently include plasticizers, surfactant breakdown products, pharmaceuticals (oral contraceptives) and metals (e.g. cadmium) (Benotti et al., 2009; Campbell et al., 2006; Goksoyr, 2006). The natural sources, in turn, include human and animal hormones (estradiol, estriol, and estrone), phyto and mycoestrogens found in sewage/animal husbandry runoff and intentionally or accidentally as food and feed ingredients (Goksoyr, 2006; Matozzo et al., 2008).

To evaluate the effects of EDCs in fish oogenesis and reproduction, it is important to consider all possible mechanisms of action, including the role of both hormone receptors, the estrogen (ER) and androgen receptor (AR) or membrane-bound receptors, as well as receptor-independent mechanisms. So, these compounds are divided according their mode of action: interaction of EDCs with the hormone-receptors; alteration of processes (production, transport and secretion) involved in steroid synthesis or alteration of processes involved in sex steroid metabolism (Garcia-Reyero et al., 2006; Goksoyr, 2006). In interaction with sex hormone receptors, they can mimic the endogenous estrogens binding to the ER or stimulate abnormal hormonal responses binding to the AR, by acting as agonists or antagonists (Garcia-Reyero et al., 2006). For instance, certain endocrine disrupters may act as antiestrogenic agents and antagonize the normal ER pathway, inhibiting the expression of target genes (Vaccaro et al., 2005).

This work will focus in three compounds in particular: the steroidal estrogen 17β-estradiol (E2); the pesticide 1,1-dichloro-2,2-bis (4-chlorophenyl)ethane (p,p'-DDE) derivative from 1,1,1-trichloro-bis-2,20-(4chlorophenyl) ethane (DDT); and one congener of the polychlorinated biphenyls (PCB-77) (Matozzo et al., 2008). These compounds, due

to their ability to bind to ER/AR, are considered EDCs because they obey to one of the three characteristics as reviewed by Mills and Chichester (2005): presence in the environment at high concentrations, persistence and bioaccumulation or constantly entering in the environment.

1.1.1. 17β-estradiol (E2)

The estrogenic hormone E2 is one of the compounds detected in effluents coming from sewage treatment plants and is considered an estrogenic EDC, since it induces an estrogen-like response due to their ability to bind to ERs and induce the production of vitellogenin (vtg) (Campbell et al., 2006; Denslow and Sepúlveda, 2007)

Despite being the major estrogen in females, which main role is promoting gonadal growth and development (Goksoyr, 2006; Nagahama and Yamashita, 2008), it has been reported as an environmental contaminant by its constant input in aquatic ecosystems mainly from domestic sewage, animal residues and agriculture runoff (Ahmad et al., 2009; Ying et al., 2002). It has been observed a significant increase in concentrations of E2 in the aquatic environment, especially in urban areas, and their levels in water could reach up to 200 ng/l (Bowman et al., 2000). Due to this and its ability to cause endocrine alterations it is necessary an effective evaluation of the toxicity of this aquatic contaminant (Kramer et al., 1998). In addition, E2 is recommended for use as reference compound in EDC testing, e.g, according to Organization for Economic Co-operation and Development (OECD) guidelines (2009). It was verified by Teles et al. (2005) that this compound has a time-related reduction in water and microorganisms were capable of transforming E2. This compound has an half-live of 0.2 to 9 days (Jürgens et al., 2002) and as a natural steroid E2 has a low octanol:water partition coefficient (log K_{ow}=3.94) (Ying et al., 2002).

1.1.2. 3,3',4,4'- tetrachlorobiphenyl (PCB-77)

This compound is a congener of polychlorinated biphenyls (in a total of 209) differing, among them, in the number and position of chlorine atoms on the biphenyl molecule and is considered one of the most toxic (Corsolini et al., 2005; McFarland and Clarke, 1989). These compounds were widely used in the past in industrial and

agricultural applications and, due to its lipophilic, metabolization rate and persistency in the environment have been biomagnified and bioaccumulated and are still detected in the environment and biota, even after so long since its banning in the 1970's (Axmon and Rignell-Hydbom, 2006; Fouial-Djebbar et al., 2010; Ross, 2004).

PCBs, due to its structure resembling dioxins with two benzene rings are able to rotate around a carbon–carbon bond (Calo et al., 2010; Corsolini et al., 2005), can interact with the estrogen receptor or alter estrogen metabolism, mimicking the action of the natural steroid E2, due to their bind to the ER proteins, stimulating ER signals with a contemporary increase of vtg expression (Calo et al., 2010; Ferreira et al., 2009). In addition, besides causing endocrine disruption, they are responsible for a number of adverse health effects including teratogenesis, neurotoxicity, immunotoxicity, reproductive toxicity and carcinogenesis (Calo et al., 2010).

PCBs have half-lives in water of about 3 to 9 years as reviewed in Hillery et al. (1997) and the partition coefficient octanol:water of congener 77 in particular is 68.4 (Rantalainen et al., 2000).

1.1.3. 1,1- dichloro-2,2-bis(4-chlorophenyl)ethane (p,p'-DDE)

The p,p´-DDE is the main metabolite of the insecticide DDT (one of the OCPs) and, despite have been prohibited some decades ago (also in the 70's) in many countries (as other OCPs), remain in the environment and it is considered one the most widespread and abundant environmental contaminant (Donohoe and Curtis, 1996; Garcia-Reyero et al., 2006; Kwong et al., 2008). This is, mainly, due to its persistent and highly lipophilic characteristics that allows their strongly adsorption to the sediments and its bioaccumulation in adipose tissue leading to its biomagnification along food chains (Bayley et al., 2002; Gillis et al., 1995; Makita, 2008). The partition coefficient octanol:water of this compound has the value of log kow=5.8 (Finizio et al., 1997). Its half-life time in water is about 50000 hours (±6.3 years) (Beyer et al., 2000).

Its widespread, uncontrolled and intensive use for years to control agricultural pests and vectors of human disease, even actually in developed countries, is still a problem of great relevance to human health due to its predominance in environment (mainly in the

sediments) and in living organisms such as fish consumed by humans (de la Cal et al., 2008; Denslow and Sepúlveda, 2007; Kristensen et al., 2006).

Relatively to its mode of action, p,p'-DDE has been found, *in vitro* screening assays using ARs, to be primarily antiandrogenic (Wells and Van Der Kraak, 2000) but is also reported to act weakly, between others, as a fish ER agonist (Kristensen et al., 2006).

The ability of OCPs to induce antiestrogenic effects in female teleost fish have great ecological significance, since competitive binding of some of these weak E2 agonists to the ER could reduce E2 function, leading to impaired gonadal development, a decrease in the vtg production and ending in the production of poor quality eggs and a reproduction decrease (Denslow and Sepúlveda, 2007).

1.2.Biomarkers

It is not possible to monitor all the contaminants (from natural or anthropogenic source) that can be considered threats to the environment. The use of biomarkers is becoming an useful tool for pollution monitoring since they can be used as early warning signals of possible damage, in this case, in aquatic ecosystems (van der Oost et al., 2003).

Application of these tools in laboratory and fieldwork can give an important linkage between toxicity observed in the experimental conditions and the evaluation of the effects in the field (van der Oost et al., 2003). In definition, they represent any change (effect), through a biological response in the organisms, induced by any environmental chemical, at the hierarchical level of a whole organism or below (biochemical, physiological, or histological level), indicating a deviation from the normal status that cannot be detected in the intact organism and used for monitoring purposes (Hallgreen, 2009).

According to van der Oost et al. (2003), biomarkers can be subdivided into three classes:

-biomarkers of exposure: covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;

-biomarkers of effect: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease;

-biomarkers of susceptibility: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.

The screening of multiple biomarker responses (biochemical parameters) can help obtaining important information about organism toxicant exposure and stress. In fish it has been tested parameters at various levels, including endocrine and neurological biomarkers (van der Oost et al., 2003). However, their use in biomonitoring programs needs validation as ecological relevant biomarkers with studies that improve their sensitivity and selectivity (van der Oost et al., 2003).

In this work, were chosen several biomarkers including vtg, gonadossomatic and hepatossomatic indexes (GSI and HSI, respectively) as reproductive/endocrine biomarkers and acetylcholinesterase as neurological biomarker, indicator of exposure to neurotoxins.

1.2.1.Acetylcholinesterases

Cholinesterases are, usually, divided in two main classes: the acetylcholinesterases (AChEs) with more affinity for acetylcholine and the butyrylcholinesterases (BChEs) with more affinity for butyrylcholine, also known as non-specific esterases or pseudocholinesterases (van der Oost et al., 2003). In fish, AChEs are found in brain and muscle tissue while BChEs in liver and plasma (Fulton and Key, 2001). AChE is a key enzyme of the nervous system and its inhibition is considered a biomarker of fish exposure to neurotoxins (mainly organophosphorus and carbamates pesticides) but can serve, however, as a bioindicator of fish stress (Pavlov, 1994) in a variety of aquatic and terrestrial animals, including fish (Chuiko et al., 2007; Corsi et al., 2005; Fulton and Key, 2001). For this reason, the inhibition of the activity in this enzyme, detected in organisms exposed to any anticholinesterase agents, means that the compound reached to the target site and produced a physiological effect (Fulton and Key, 2001).

PCBs are generally accepted as developmental neurotoxicants (Muthuvel et al., 2006) with a decrease in this biomarker observed in different animals, such fish and rats, exposed to these compounds (Barra et al., 2001; Muthuvel et al., 2006; Venkataraman et al., 2008).

In case of p,p'-DDE, since it belongs to the organochlorine insecticides, can produce toxicity by inhibiting cholinesterase enzymes in both vertebrate and invertebrate organisms (Fulton and Key, 2001).

1.2.2.Vitellogenin (vtg)

This phospolipoprotein, egg yolk protein precursor in females, is produced in liver, through binding/activation of the ER by the presence of elevated levels of 17β-estradiol, then is transported by blood from liver until the ovary to be incorporated in the oocyte and serve as nutrient reserve (Mommsen and Walsh, 1988; Tyler and Sumpter, 1996). Female fish invest a lot of energy in all these processes and, as they are under hormonal control which can be challenged by EDCs, the vtg production is affected as well as other vital functions (Hallgreen, 2009). Normally, it results in induction of vtg levels in females or appearance in males or juveniles and is widely accepted as an evidence of exposure to estrogenic chemicals in environment or laboratory studies (de Vlaming et al., 2007; Porte et al., 2006) more specifically, as a good biomarker of endocrine disruption in male fish (de Vlaming et al., 2007). Despite this, some researchers have also examined females and have seen depression or total suppression of vitellogenesis by exposure to EDCs which can lead to alterations in egg quality and, consequently, the future success of progeny cannot be assured (Denslow and Sepúlveda, 2007). Changes in vtg, sex steroid hormones and GSI are used as biomarkers to assess the possibility of contaminants to cause alterations on fish endocrine/reproductive systems (Bosker et al., 2010).

1.2.3.Hepatossomatic index (HSI)

Hepatossomatic index is the ratio between the weight of the liver and the total body weight of the fish: (liver weight / total body weight) x 100. It is a quite general and non-specific parameter but, it's low cost, ease and rapidity still make it a valuable tool

and can serve as an initial screening biomarker to indicate exposure and effects to environmental contaminants. The disadvantage of HSI is it sensitivity for non-pollutant factors (e.g. season, disease, nutritional level)(van der Oost et al., 2003).

1.2.4.Gonadassomatic index (GSI)

Gonadassomatic index is commonly used as a biomarker to assess the potential of contaminants that cause adverse effects on fish reproductive systems (Bosker et al., 2010) and is measured with the formula (gonad weight / total body weight) ×100. The weight of the gonads was subtracted from the body weight to minimize the effect of the reproductive cycle on this index.

1.3.The *Pomatoschistus microps* Krøyer (1838) as fish model

The use of fish as indicator species is due to their paper(s) in the food chain, the capacity of bioaccumulate toxic compounds, responds at very low concentrations of a certain compound and their large abundance and distribution within various habitats (van der Oost et al., 2003). So, their utilization in ecotoxicology it is from great importance since they allow the evaluation of the effects from various contaminants under different exposition conditions.

The euryhaline common goby, *Pomatoschistus microps*, is one of the most abundant fish species present in the coastal waters of northwestern Europe such as estuaries, lagoons and shores (Leitão et al., 2006; Pampoulie, 2001). This epibenthic species occurs abundantly in shallow soft-bottom areas (Magnhagen and Wiederholm, 1982). This kind of habitats, as said above, can act as recipients for sewage, and can be potentially polluted by EDCs and are constantly changing their conditions (e.g. salinity and temperature). However, this species shows a high level of plasticity to deal with those changes and, in addition to their abundance, high fecundity and its role in the food chain as predators of macro and meio-fauna and as a prey for big fish and seabirds (Leitão et al., 2006; Pampoulie, 2001; Pihl, 1985) makes it a species with a very important role in those kind of environments. Besides this, it is a small fish, making it an ideal species to work in laboratory as mentioned in Denslow and Sepúlveda (2007) about other model

species. It was chosen for this work, due to all the above reasons, but also because it was used in other studies as test organism and have already shown to be a good model species in different ecotoxicological and monitoring studies (Christiansen et al., 1998; Fonseca et al., 2011; Monteiro et al., 2005; Monteiro et al., 2007; Vieira et al., 2008).

In this work, the fact of not using males as the natural choice for monitoring EDCs, was due only to the difficulty of collecting enough organisms (they are in minority) in the field to perform the tests in the laboratory. However, the relevance of using juveniles and adult females is also important in the study of endocrine disruption as seen in other works. The juveniles were used because developmental processes in early life stages also depend on hormones, including sex steroids, and may be disrupted by EDCs (Boudreau et al., 2004). The adult females, in turn, due their importance to reproduction and maintenance of the populations could be used to determine the action of EDCs by the quantification of vtg or its incorporation into the oocyte, which may result in alterations in the format/number of eggs, bioaccumulation of compounds that could be transferred to the progeny, affecting the rate of natural hormones and leading to problems in development (Kime and Nash, 1999).

1.4.Objectives and thesis organization

The main objective of this work was to evaluate the effects of selected EDCs in the estuarine fish, the common goby *Pomatoschistus microps.* To reach this, the following specific objectives were performed:

- Assess the effects of 21-day exposure of *P.microps* to 17β-estradiol, PCB-77 or p,p'-DDE on vtg-like protein levels;
- Assess the effects of PCB-77 and p,p'-DDE on AChE activity;
- Assess the effects on GSI and HSI in females exposed to PCB-77 and p,p'-DDE;
- Investigate if juveniles and females of *P.microps* are suitable for EDC testing.

This thesis is organized in four chapters:

The Chapter 1 corresponds to general introduction where are described the main subjects of the work, Chapters 2 and 3 to two articles to be submitted in indexed journals and Chapter 4 to general discussion/conclusion.

In Chapter 2, the article presents the effects in vtg and AChE in juveniles of *P.microps* exposed for 21-days to 17β-estradiol and PCB-77 while in Chapter 3 it is analyzed those same biomarkers in addition to GSI and HSI in *P. microps* females after PCB-77 and p,p'-DDE exposure.

In the last chapter of this work is presented the general discussion/conclusions about the principal aspects of this work.

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Chapter 2.

Biomarker responses to EDCs exposure in juveniles of the estuarine fish *Pomatoschistus microps*

Biomarker responses to EDCs exposure in juveniles of the estuarine fish *Pomatoschistus microps*

Keywords: acethylcholinesterase, common goby, vitellogenin, polychlorinated biphenyls, 17-β-estradiol.

Summary:

Endocrine disruptors are a diverse group of compounds, with natural or synthetic origin, that have been detected in wastewater effluents and surface waters around the world. They have different modes of action and one of their main targets is the endocrine system. The main objective of this work was to evaluate the response of biomarkers in *Pomatoschistus microps* juveniles after exposure to selected EDCs, the model compound 17β-estradiol and the polychlorinated biphenyl PCB-77. The experimental work consisted in the quantification of vtg-like proteins and acetylcholinesterase (AChE) activity in the fish *Pomatoschistus microps* after 17β-estradiol and PCB-77 21-days exposure, in independent experiments. The results showed an increase and a decrease in the vtg of fish exposed to 17β-estradiol and PCB-77, respectively. In case of AChE activity it was observed an increase in the highest concentration tested of PCB-77. The quantification of vtg in juveniles of *P.microps* can be considered a valuable tool to track the presence and action of estrogenic compounds in estuaries.

1.Introduction

An endocrine disrupting compound (EDC) is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, or its progeny, or (sub) populations (World Health Organization, 2002). Endocrine disruptors are a diverse group of compounds, with natural or synthetic origin, that have been detected in wastewater effluents and surface waters around the world (Benotti et al., 2009; Boudreau et al., 2004). They are divided according to their mode of action: interaction with hormone-receptors; alteration of processes involved in steroid synthesis or alteration of processes involved in sex steroid

metabolism (Garcia-Reyero et al., 2006). In the interaction with sex hormone receptors, EDCs can mimic the endogenous estrogens binding to the estrogen receptor (ER) or stimulate abnormal hormonal responses binding to the androgen receptor (AR), by acting as agonists or antagonists. For instance, certain EDCs may act as antiestrogenic agents which antagonize the normal hormone receptor pathway, inhibiting the expression of target genes (Vaccaro et al., 2005).

Among EDCs are persistent organic compounds such as the polychlorinated biphenyls (PCBs) or steroidal hormones such as the 17β-estradiol (Benotti et al., 2009; Campbell et al., 2006; Goksoyr, 2006). The estrogenic hormone 17β-estradiol is recommended for use as reference compound in EDC testing, e.g. according to OECD guidelines (2009). Despite being the major estrogen in females, it has been reported as an environmental contaminant by its input in aquatic ecosystems mainly from domestic sewage (Ahmad et al., 2009; Ying et al., 2002).

PCBs were widely used in the past and, due to its lipophilic and persistency characteristics, they are bioaccumulated and are still detected in the environment and biota, even after so long since its banning (Ross, 2004). PCBs can present, due to their dioxin-like characteristics and depending on their affinity to the hydrocarbon receptor (AhR), both estrogenic and antiestrogenic activity (Calo et al., 2010). The AhR mediates production and regulation of sex steroid hormone-related, in both normal physiology and in dioxin toxicity (Bock and Kohle, 2009). They can, indeed, activate one of ERs in fish (Mortensen and Arukwe, 2008), acting as agonists or antagonists of hormone receptors (Miller-Perez et al., 2009). The 3,3',4,4'- tetrachlorobiphenyl (PCB-77) is considered one of the most toxic congeners of polychlorinated biphenyls (Corsolini et al., 2005) and is well documented as an AhR agonist with anti-estrogenic activity (Mortensen et al., 2006).

Since estuaries are located in high populated areas and exposed to a great quantity of contaminants from human, industrial and agricultural sources it is important to protect these ecosystems from these activities and to find tools that help to evaluate the degree of contamination and exposure in the natural populations that inhabit or use these places for reproduction.

The use and assessment of biomarkers is considered of great importance since they can be used as early warning signals of possible damage in aquatic ecosystems (van der Oost et al., 2003). This is possible due to their capacity to predict effects at low levels of biological organization and responses at cellular and molecular level have great relevance, because they detect exactly how the specific exposure changed biochemical pathways (Denslow and Sepúlveda, 2007).

Brain acethylcholinesterase is a key enzyme of the nervous system known as a biomarker of fish exposure to neurotoxins (mainly organophosphorus and carbamates pesticides) but beside that, can also be used as a bioindicator of fish stress (Pavlov, 1994). It seems that the developing nervous system is sensitive to PCBs and these compounds are generally accepted as developmental neurotoxicants (Muthuvel et al., 2006). Despite the lack of studies in fish, it was shown that chub (*Leuciscus cephalus*) living in Lambro River (Italy) had a decreased brain AChE activity when the total PCB content in fish was increased (Barra et al., 2001). In case of other animals such rats it was observed, after exposure to a mixture of PCBs, a decrease of AChE activity in some zones of the brain (Muthuvel et al., 2006; Venkataraman et al., 2008).

The induction of vitellogenin (vtg) synthesis is already accepted as a good biomarker of endocrine disruption in fish (de Vlaming et al., 2007). This phospolipoprotein, the egg yolk protein precursor in females, is produced through activation of the estrogen receptor by 17β-estradiol. Vitellogenin is then transported by blood to the ovary where it is incorporated into the oocyte to serve as nutrient reserve (Mommsen and Walsh, 1988; Tyler and Sumpter, 1996). However, its induction in females or appearance in males or juveniles of fish is widely accepted as an evidence of exposure to estrogenic chemicals in environmental or laboratorial studies (de Vlaming et al., 2007; Porte et al., 2006). In case of PCB-77 it seems to have the ability to reduce the E2-induced production of Vtg in rainbow trout hepatocytes in a concentration-dependent manner (Petersen and Tollefsen, 2012).

The model species used in this work, the common goby, *Pomatoschistus microps* (Krøyer, 1838) is one of the most abundant fish species present in the general coastal waters as estuaries, lagoons and shores (Leitão et al., 2006; Pampoulie, 2001). This

widespread and highly fecund fish has an important role in the food chain as intermediate predator and a high capacity to adapt to the constant changes that occur in coastal areas (Dolbeth et al., 2007; Pampoulie, 2001). *P. microps* has already been used in other studies as test organism and have shown to be a good indicator species (Christiansen et al., 1998; Fonseca et al., 2011; Monteiro et al., 2005; Monteiro et al., 2007; Vieira et al., 2008).

The main objectives of this work were (i) to evaluate the responses of biomarkers (vtg and AChE) after a sub-lethal exposure of *Pomatoschistus microps* to 17β-estradiol and PCB-77 and (ii) to determine if vtg is an eligible tool to track EDCs in juveniles of this estuarine species.

2.Material and Methods

2.1.Chemicals

All chemicals used in these experiments were obtained from Sigma-Aldrich (Germany), except the Bradford reagent which was purchased from Bio-Rad (Germany).

2.2.Fish sampling and laboratory maintenance

The sampling site selected for this study (41^{o}53'27.28''N; 8^{o}49'30.81''W) is located in the Minho river estuary (NW coast of Portugal). This site presents low levels of environmental contamination (Santos et al., 2012) and has been used as a reference site in several studies (Guimarães et al., 2009; Monteiro et al., 2007; Quintaneiro et al., 2006).

Two fish samplings were performed, one for each test compound, and local water phisico-chemical parameters (temperature (T), pH, dissolved oxygen (DO), salinity (Sal) and conductivity (Cond) were measured using a multiparameter VWR mod SympHony SP90M5. The measures showed the following values: T=16^{o}C; pH=7.85; DO=92.9%; Sal=3 mg/l; Cond=24.13mS/cm for the first sampling (17β-estradiol test) and T=14.6^{o}C; pH=8.79; DO=103.4%; Sal=6mg/l; Cond=10.65mS/cm for the second sampling (PCB-77 test).

Juvenile fish were collected using a landing net at low tide. Fish were then transported to the laboratory (travel duration of about 4h) in three separated containers filled with local water at which Ocean Fish Prodac marine salt was added to gradually

increase salinity. The organisms were acclimated to laboratorial conditions for two weeks before the beginning of the toxicity tests. They were placed in three separate aquariums filled with well-aerated, filtered artificial seawater in a controlled room with photoperiod 8h dark: 16h light at 20±1^{o}C temperature. The water medium for both acclimation and experiments was prepared dissolving marine salt Ocean Fish Prodac to simulate seawater (salinity=35‰), partially renewed every 2/3 days. Fish were fed daily, twice a day, with the dry food TetraMin®.

All procedures involving fish handling were conducted according to the Guide for the Care and Use of Laboratory Animals of the European Union - in Portugal represented by Decreto de Lei nº 129/92 de 06 de Julho, Portaria nº1005/92 de 23 de Outubro de 1992.

2.3.Test conditions

Fish were exposed to 17β-estradiol and PCB-77 under the same conditions of temperature and photoperiod of the acclimation period. The water medium parameters were measured after every medium change. At the end of each test, the number of dead fish was recorded and the live fish were anesthetized with MS-222 (except in animals exposed to PCB-77, since it can interfere with AChE activity, one of the endpoints assessed), weighted, measured and sacrificed by decapitation upon ice. Fish were then frozen in liquid nitrogen and stored at -80^{o}C until Vtg (entire fish in 17β-estradiol experiment/ decapitated fish in PCB-77 experiment) and AChE analysis (fish head).

2.3.1.Juvenile test exposure to 17β-estradiol (E2)

Before the test exposure, the appropriate amount of E2 was previously dissolved in dimethyl sulfoxide (DMSO). It was added, daily, to the experimental recipients in order to ensure the maintenaice of nominal concentrations since it has high loss rate in water and a fast uptake by fish in the first 4h (Teles et al., 2005). Also, for these reasons, it was not possible to perform chemical analysis to this test medium.

The juvenile fish were exposed in groups of 4/5 animals with 12-15 fish per treatment, during 21 days, to E2 at the nominal concentrations of 6.25; 12.5; 25; 50 and 100 ng/l and, also, to a negative control and a solvent control (0.0000333% v/v DMSO).

The water physico-chemical parameters measured before every water change ranged between: T=19.3±0.04^{o}C; pH=7.82±0.02; DO=61.9±1.01%; Sal=35.5±0.03 mg/l; Cond=130.94±0.15 nS/cm. The values are presented in mean±SE.

2.3.2.Juvenile test exposure to PCB-77

Three groups of four *P. microps,* per treatment, were exposed in individual 1-L glass recipients to seven concentrations of PCB-77, a negative control and a solvent control (0.041% DMSO). This was performed during 21 days with medium renewal every 2/3 days. The values measured before every water change ranged between: T=18.2±0.3^{o}C; pH=8.22±0.04; DO=81.9±3.5%; Sal=35.5±0.1 mg/l; Cond=50.25±2.3 nS/cm. The values are presented in mean±SE. Samples from the highest concentration of PCB-77 tested, were collected after 2-3 days exposure, before the medium change. Samples were kept at 4^{o}C and subjected to a posterior chemical analysis. The real concentrations of PCB-77 tested were 171.69; 42.92; 10.73; 2.68; 0.67; 0.17 and 0.04 ng/l.

2.4.Biomarker analysis

2.4.1.Protein quantification for biomarkers

Protein content of the samples was determined, in quadriplicate, by the Bradford method (Bradford, 1976) adapted to microplate, using γ-globulins as standard and a wavelength of 595 nm. A Labsystem Multiskan EX microplate reader was used for all protein and enzimatic determinations.

2.4.2.Acetylcholinesterase activity

One fish head per sample (n=7-12 per treatment) was homogenized, using a sonicator Branson S-250A, in 1 ml of phosphate buffer (0.1 M, pH 7.2). The supernatants obtained after centrifugation (4^{o}C, 6000 rpm, 3 min) were diluted and used for further protein and AChE activity quantification. The method used to determine AChE activity was performed according to Ellman et al. (1961) adapted to microplate by Guilhermino et al. (1996). The enzymatic activity is expressed in nmol/ml/mg de protein.

2.4.3.Vtg like-proteins

Fish samples, (n=7-12 in treatment with 17β-estradiol and n=4-12 in treatment with PCB-77) were homogenized through sonication, in 1 ml of homogenization buffer (containing 125 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA and 1 mM dithiothreitol at pH 8) for each 200 g of weight and then centrifuged at 12000 g for 20 min at 4^{o}C. Vitellogenin was then determined by the indirect alkali-labile phosphate method following the protocol presented in Gagné et al. (2003) with some alterations (Hallgren et al., 2009). Briefly, 100 µl of the supernatant were mixed with 54 µl of acetone (35% of final volume) for 5-10 min at room temperature and then mixed with a vortex agitator at least three times and then centrifuged at 10000 g for 5 min. After acetone removal, 50-100 ul (depending of pellet size) of 1 M NaOH were added to samples that were then maintained for 90 min at 70^{o}C (Hallgren et al., 2009), to allow hydrolysis of bound phosphates (Gagné et al., 2003). The levels of free phosphates were determined in the aqueous phase according to the phosphomolybdenium method (Stanton, 1968). Results are expressed as mg PO4/mg protein.

2.5.Statistical analysis

For each biomarker, data from different treatments were transformed (if necessary) to achieve normal distribution and then were analysed using one-way analysis of variance (ANOVA). To identify significant differences between control and treatments the Dunnett's test was used. Data outliers have been removed considering mean ± 3x standard deviation or mean ± 2x standard deviation.

The mortality rate was calculated considering the accumulated mortality in all treatments vs. control, and values of LC50 were determined using the Minitab version 14.0 with Probit Analysis. All other statistical analysis was performed using SigmaStat for Windows, version 11.0.

3.Results

3.1.Test exposure to 17β-estradiol (E2)

At the end of the experiment, the fish were measured and weighted obtaining values for length ranging from 2.0 to 3.2 cm and for weight from 87.9 to 362.1 mg. At the end of the test, the mortality rate was null in the controls and the highest mortality rate was observed in the highest concentration (73.3%) with an estimated LC50 at a concentration of 68.13 ng/l with an 95% confidence interval (CI) between 52.4175 and 95,5457 ng/l.

In Figure 2.1 is presented the results obtained in vtg-like proteins quantification. There were no significant differences between the control and control solvent (CS). Comparing each treatment with control, there are significant differences in all the concentrations tested, 6.25; 12.5; 25 and 50 ng/L of 17β-estradiol with control ($P<0.001$), except for the highest concentration 100 ng/l ($P>0.05$). The lowest observed effect concentration (LOEC) is verified at the lowest concentration tested, 6.25 ng/L.

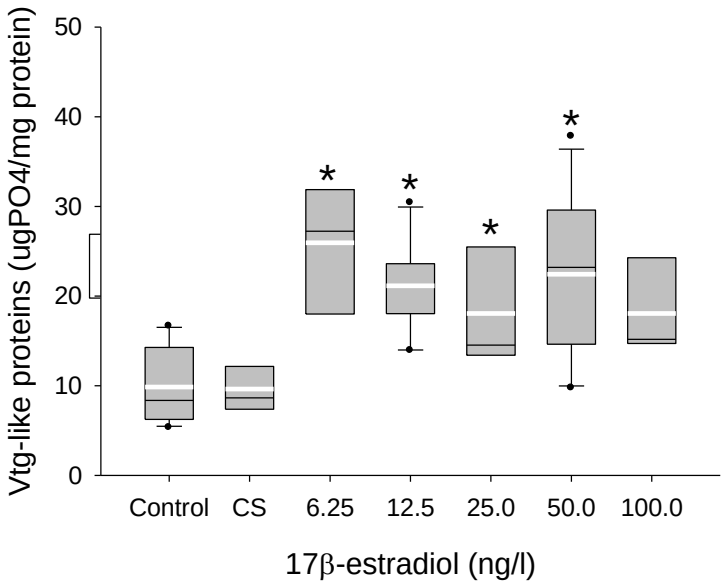

Figure 2.1: Effects of 17β-estradiol in vitellogenin levels of the juvenile fish *P. microps* after 21 days of exposure. Results are expressed as mean±SE; *significantly different from control ($P<0.05$, Dunnett's test) and •represents all the outliers; CS- Control of solvent. The white line represents the mean of the results.

3.2.Test exposure to PCB-77

In this experiment, the final measures of fish were between 2.1 and 3.2 cm to length and between 97.9 and 334.5 mg to weight in the exposed fish. The mortality rate was variable among treatments and the estimated LC_{50} was the value of 47.52± 22.87 ng/l (mean ± SE) with CI not calculated.

The effects of PCB-77 in the vtg levels are presented in Figure 2.2. There are no significant differences between treatments with the lowest concentrations tested relatively to control but, in the highest concentrations it was observed a tendency of vtg-like proteins to decrease. The lowest concentration where is observed a significant decrease of vtg was at 10.73 ng/l ($p<0.05$).

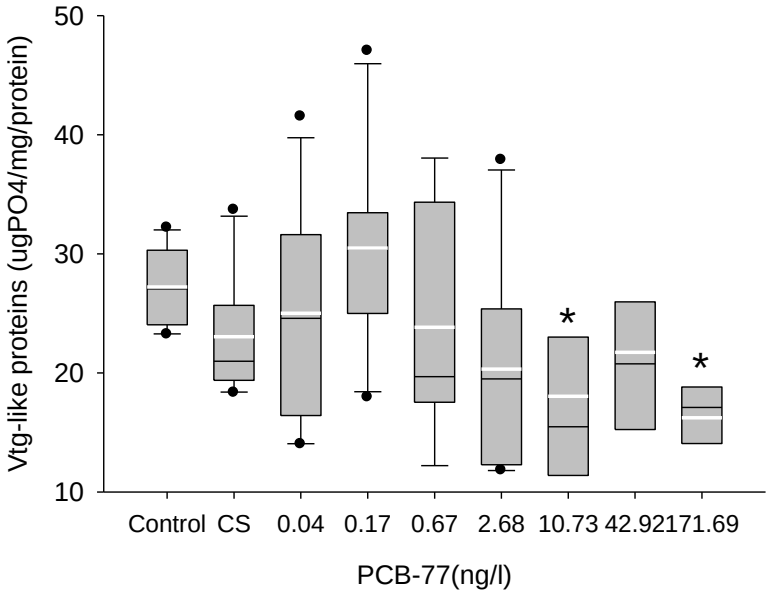

Figure 2.2: Effects of PCB-77 in vitellogenin levels in the fish *P.microps*. Results are expressed as the mean±SE; *significantly different from control (P<0.05, Dunnett's test) and •represents all the outliers.; CS-control of solvent. The white line represents the mean of the results.

In the quantification of AChE activity (Figure 2.3) it was observed a significant difference between the negative control and solvent control (P<0.05). In this case, the statistical analysis of PCB-77 treatments was performed in relation to solvent control. Therefore, it was observed a significant difference between the highest concentration of PCB-77 (171.69 ng/L) and the solvent control (P<0.05, Dunnett's test).

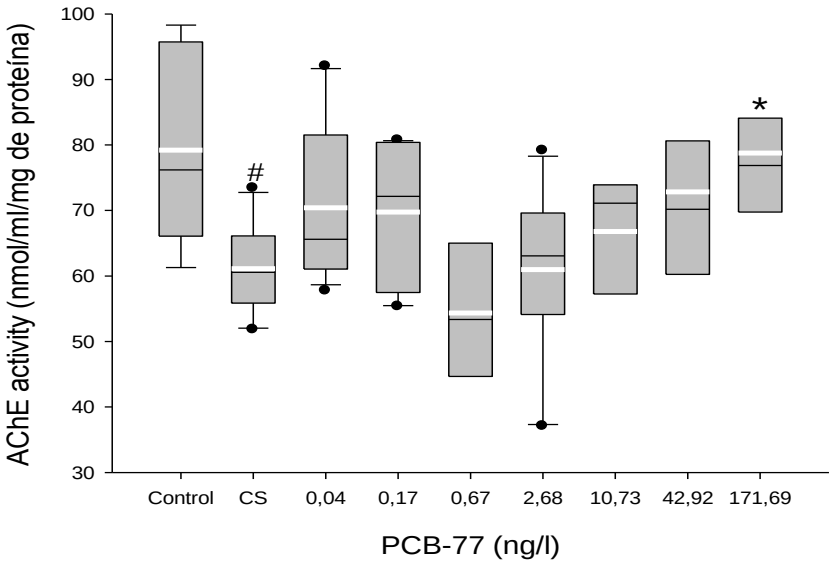

Figure 2.3: Determination of AChE activity in head fish of *P. microps* after 21 days of PCB-77 exposure. Results are expressed as the mean±SE; #significantly different from control; * significantly different from solvent control (P<0.05, Dunnett's test) and •represents all the outliers; CS- Control of solvent. The white line represents the mean of the results.

4.General Discussion

In the present work it was analysed the responses of the biomarkers vtg and AChE to verify if the compounds tested, 17β-estradiol and PCB-77, were able to cause endocrine disruption and/or neurotoxicity in juveniles of the fish *Pomatoschistus microps*. The measurement of endpoints in whole organisms allows the quantification of real effects of EDCs in the target species (Campbell et al., 2006) and to use the species as a representative bioindicator of their habitat which, in this case, are the estuaries and other coastal areas (Chang et al., 2009). However, the disadvantage is that the results just can be associated to the deficiency of a specific organism response to certain EDCs and, in the field, the specific cause or the exact location of the source could not be known with certain (Chang et al., 2009).

The Alkali-Labile Phosphates method (ALP), used in this experiment, is based on the determination of labile phosphates released by vtg after hydrolysis in alkali and cannot provide quantitative measures of egg-yolk protein concentrations but is considered an effective and simple way to quantify this biomarker of response to EDCs (Gagnaire et al.,

2009). This is possible because vtg is stated to be the only phosphorous protein in the blood of oviparous vertebrates and together with the high degree of phosphorylation enables the indirect quantification of vtg via ALP (Hallgren et al., 2009). This method is, also, considered simple and cheaper than other methods (e.g. ELISA) and should facilitate large scale environmental monitoring at many locations and in many fish species (Hallgren et al., 2009).

4.1.Test exposure to 17β-estradiol (E2)

In this study, after 21 days exposure to E2, it was verified an expected increase in the values of the vtg-like proteins measured in the whole fish, relatively to the control organisms, explained due to the estrogenicity of this compound. As said before, this compound is recommended as reference in many EDCs tests and therefore it is suitable for comparison of inter-test sensitivity. In addition, due the fact of its natural presence is necessary for the production of vitellogenin (observed mainly in mature females) (Ferreira et al., 2009), it appearance in juveniles (as well in males) could be accepted as evidence for estrogenic chemical exposure (Porte et al., 2006).

The lowest concentrations of E2 used in the present work are environmentally relevant, since similar values are measured in surface waters with median and mean concentrations below 1 and 5ng/L, respectively (de Vlaming et al., 2007; Labadie and Budzinski, 2005). The observation of vtg induction at the lowest concentration tested, 6.25 ng/l E2, demonstrates that *P. microps* juveniles are sensitive to low levels of E2 and might be useful to track estrogenic contamination in coastal areas. Indeed, the lowest concentration of E2 that causes an induction of vtg in *P. microps* juveniles is below the lowest observed effect concentration (LOEC) values found in other studies for other fish species, namely in male zebrafish and juvenile rainbow trout, that are around 20 ng/l (van den Belt et al., 2003) and are very close to the LOEC value determined by Holbech et al. (2006) in juveniles of medaka (≤8.66 ng/l). In turn, the no observed induction of vtg in the highest treatment (100ng/l) could be explained by the high mortality recorded in this treatment. The mortality rate occurred in this work at the concentration of 100 ng/l E2 was not observed in other studies with fish, for instance with *Danio rerio* (Brion et al., 2004; van den Belt et al., 2003), this can be due to the species sensitivity to the

compound. Therefore, to use E2 as reference compound in future studies with *P. microps* juveniles, e.g. as a positive control, could be recommend the use of concentrations above the LC_{10} (8.50036 ng/l of E2 with an 95% CI between 20.4418 and 24.5800 ng/l), instead of 100 ng/l E2 as recommended for other fish species (OECD, 1984).

The concentrations used in this work were previously tested in *Danio rerio* by Brion et al. (2004) who verified that the exposure to these concentrations of E2 resulted in vtg induction whatever the life stage exposed was (embryo-larvae, juvenile and adult life stages) at least at the highest concentration tested (100 ng/l). Similar results were seen in other studies with other fish species, in agreement with the results obtained in this work, even when the vtg quantification was done at different exposure times (Hahlbeck et al., 2004; Holbech et al., 2006). So, it can be said that the ability of E2 to induce vtg in fish is well documented in several species of fish (Kramer et al., 1998; Panter et al., 1998; Routledge et al., 1998; Thorpe et al., 2000) and, more specifically, in other small-size laboratory species as juveniles of fathead minnows (Tyler et al., 1999), rainbow trout (*Oncorhynchus mykiss*) (de Vlaming et al., 2007) and summer flounder (Mills et al., 2001). In fact, the presence and changes in vtg concentrations in plasma of juvenile fish, has been used as a biomarker to evaluate the effects of xenoestrogen exposure (Calo et al., 2010) and proved to be a very sensitive and a generally consistent endpoint to detect an estrogenic or an antiestrogenic effect (Panter et al., 2002). In addition, juveniles, in contrast to adult females, accumulate vtg in the plasma due to the lack of or the immature state of the ovaries (Donohoe and Curtis, 1996) and this life stage is recommended for use in OECD test guidelines for endocrine disrupter assessment purposes (Huet, 2000).

It is known that exposure to low concentrations of contaminants could lead to estrogenic responses and this is very concerning given the levels that become detected in the various water environments (Chang et al., 2009). During early life stages, this exposure can lead to an altered pattern of egg production in the subsequent adults as concluded by Brion et al. (2004).

4.2.Test exposure to PCB-77

4.2.1.Vtg-like proteins

Relatively to the PCB-77 exposure, this compound is considered a dioxin-like and aryl hydrocarbon receptor (AhR) agonist with antiestrogenic activity (Mortensen and Arukwe, 2008; Mortensen et al., 2006). It was described in other teleost fish and both in vivo and in vitro studies, that exposure to AhR agonists could be associated with reduced vtg synthesis (Arukwe et al., 2001; Mortensen et al., 2006).

As seen in the results, it was verified a slightly inhibition of vtg-like proteins with significant differences at the concentration of 10.73 ng/L and in the highest concentration (171.69 ng/l). The work realized by Calo et al. (2010) indicates a similar result with a decrease in levels of vtg below the control (anti-estrogenic response) after an exposure to PCB-26 (10^{-8} M), a congener with the same characteristics that PCB-77. This antiestrogenic activity was reported, also, by other works in fish hepatocytes (Bemanian et al., 2004; Mortensen and Arukwe, 2007). These opposite effects could be explained by the different toxicological and biological actions of AhR through different pathways explained and revised by Calo et al. (2010) with the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context or on their cross-talk with sex steroid receptor through promotion of proteolysis.

However, it is known that the development and execution of vtg assays represents some difficulties (once this is one protein inherently unstable due to its design to degrade after incorporation in the oocyte) and, for this reason, it is need a proper sample handling (Goksoyr, 2006).

4.2.2.AChE activity

In the quantification of AChE activity, after PCB-77 exposure, it was observed significant decrease of the control solvent (DMSO) relatively to control. The use of a solvent is because PCBs are very hydrophobic and it is needed a carrier solvent to facilitate their introduction into aqueous solution (Dillon and Burton, 1991). The authors Barbosa et al. (2003) reported a toxic effect of DMSO, although not as much as other

solvents and with no observed effects in *Daphnia magna*. Due to this result, the statistical analysis was performed relatively to the solvent control and it was verified an unexpected and significant increase in the highest concentration tested. A similar enhancement of AChE was observed by Isoda et al. (2002) in other class of EDCs, the phytoestrogens (genistein and daidzin). These phytoestrogens had the capacity to bind to the ER and enhanced the AChE activity in a rat cell line. However, it was possible identify this effect in other works realized in brain homogenates of rat after exposure to aluminum (Zatta et al., 2002) and, more recently, in the Atlantic salmon after exposure to an anti-parasitic drug (Ucán-Marín et al., 2012). More than a biological effect to a neurotoxic exposure, this enhancement of AChE could be due the acclimation to the laboratory conditions confirmed by the work performed by Quintaneiro et al. (2008).

4.3.Conclusions and future perspectives

In general, the fish *P. microps*, at juvenile stage, seem to respond to EDC contamination as it were registered alterations in vtg-like proteins by exposure to environmental relevant concentrations of E2 and PCB-77. Therefore, the determination of vtg in the juvenile life stage of this estuarine species seems appropriate to track EDC contamination in field biomonitoring studies. In case of PCB-77, vtg-like proteins were found to decrease, in accordance to its anti-estrogenic mode of action in other fish species described in the literature. Furthermore, PCB-77 induced AChE, which may indicate only a situation of stress due to the acclimation to the laboratory conditions.

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Chapter 3.

Evaluation of biomarkers responses to different EDCs in *Pomatoschistus microps* females

Evaluation of biomarkers responses to different EDCs in *Pomatoschistus microps* females

Summary

The presence of endocrine disruptor compounds (EDCs) has been detected in many aquatic environments, including estuaries. The main objectives of this work were: (i) to assess the responses in different biomarkers after a sub-lethal exposure to the EDCs, PCB-77 (0.37 – 2872 ng/l) and p,p'DDE (0.1205 – 241 ng/l), in females of the estuarine species *Pomatoschistus microps* and (ii) to determine if the different biomarkers of endocrine disruption measured (e.g. vitellogenin-like proteins, gonadossomatic index) are suitable to be used in the monitoring of EDCs in this estuarine species. After the two independent exposures of 21 days, the hepato and gonadossomatic indexes (HSI and GSI), the levels of vtg-like proteins and acetylcholinesterase (AChE) activity were measured. The main results were that, for the concentrations tested, the responses of the several biomarkers were not significantly altered in vtg of the gonads as well in GSI and HSI after exposure to PCB-77 and in all of the endpoints after exposure to p,p'-DDE.

Keywords: Acetylcholinesterase, common goby, GSI, HSI, PCB-77, p,p'-DDE, vitellogenin.

1.Introduction

The presence of endocrine disruptor compounds (EDCs) in aquatic environments, more specifically in wastewaters, was suspected for several decades and their effects are being studied from some decades until now. These type of compounds are found in many environmental compartments around the world (surface water, ground water supplies, wastewater effluents, sea water, and sediments) and provide from natural sources (including phyto and mycoestrogens) (Goksoyr, 2006; Matozzo et al., 2008) or anthropogenic sources (include estrogens released into the environment via sewage effluents, pesticides, chemical industry contaminants, organotin compounds, polychlorinated biphenyls (PCBs), phthalate plasticizers, detergent breakdown products, pharmaceuticals and personal care products, combustion byproducts and surfactants) that are released through agriculture and industry wastes, accidental spills and, indirectly,

through diffuse sources such as storm water runoff (Chang et al., 2009; Falconer et al., 2006).

The EDCs are exogenous substances that can alter function(s) of the endocrine system (the biochemical messengers or communication systems of glands, hormones and cellular receptors that control the body's internal functions) and consequently cause adverse health effects in an intact organism, its progeny, or (sub)-populations (World Health Organization, 2002). The principle reason of being called EDCs is due to their capacity to either mimic the molecular effects of endogenous hormones or alter hormonal homeostasis, interfering with the synthesis, secretion, transport, binding metabolism and/or excretion of endogenous hormones such as estrogen, testosterone and/or thyroid (Chang et al., 2009; Denslow and Sepúlveda, 2007) that are essentials to maintain the homeostasis, reproduction, metabolism, development, and/or behavior of living species (Chang et al., 2009).

Depending on the endocrine endpoints, they can be estrogenic, androgenic, or thyroidal compounds (Chang et al., 2009) with the ability to work as agonists or antagonists to the various endogenous hormones (Falconer et al., 2006).

The importance of estuaries is recognized worldwide for providing essential ecological functions, services and recreational activities (Kennish, 2002; Paerl, 2006). However, due their localization, they are subjected to a variety of anthropogenic stresses, mainly for being a final destination of a large quantity of nutrients and pollutants derived from urban, agricultural and industrial effluents (Paerl, 2006), including EDCs (Shahidul Islam and Tanaka, 2004). Among the different EDCs that might be found in estuaries, there are persistent contaminants such as polychlorinated biphenyls (PCBs) and pesticides such as dichloro-diphenyl-trichloroethane (DDT) and its metabolites, which are considered endocrine-disrupting compounds because they present one of the characteristics that defines them; they are persistence and bioaccumulate in the environment (Mills and Chichester, 2005).

The coplanar polychlorinated biphenyl 3,3',4,4'- tetrachlorobiphenyl (PCB-77) have been shown *in vivo* to have both estrogenic and antiestrogenic activities, being an example of an antiestrogenic chemical which may alter the estrogenic response through

binding to the androgenic aryl hydrocarbon receptor (AhR) (Calo et al., 2010; Geyer et al., 2000). This compound is considered one of the most toxic PCB congeners (Corsolini et al., 2005) and, as well as DDT and its metabolites, belongs to a larger group called persistent organic pollutants (POPs) which possess a very high persistency and bioaccumulation potential in aquatic and terrestrial organisms including humans (Geyer et al., 2000).

The dichloro-2,2-bis(4-chlorophenyl)ethane (p,p'-DDE) is one of DDT metabolites, a compound that belongs to the organochlorine pesticides (OCPs), a large class of compounds used to control agricultural pests and vectors of human diseases and, due to their early widespread use and high chemical stability and lipophilicity, they continue to bioaccumulate in animal tissues, and are currently one of the most common and widely distributed types of pollutants worldwide (Denslow and Sepúlveda, 2007). Relatively to its mode of action it directly inhibits steroid synthesis (Jorge Chedrese and Feyles, 2001) and has been found, through *in vitro* screening assays using androgen receptors (ARs), to be primarily antiandrogenic (Kelce et al., 1995) but is also been reported that p,p'-DDE can act weakly as a fish estrogen receptor (ER) agonist (Garcia-Reyero et al., 2006).

Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants or of host response (van der Oost et al., 2003). Their use and assessment is considered of several importance since they can be used as early warning signals of possible damage in aquatic ecosystems (van der Oost et al., 2003) with the advantage that the information obtained, often, can be easier and less costly to collect compared with longer-term responses at higher levels of biological organization such as growth and/or reproduction (Miller et al., 2007).

The amount of the phospholipoglycoprotein vitellogenin (vtg), activated by ERs as 17β-estradiol, controlled through the hypothalamic–pituitary–gonadal (HPG) axis (Ankley et al., 2005), produced by the liver cells and required for normal oocyte maturation (Arukwe and Goksoyr, 2003), is a critical step for successful reproduction in the developing female fish (Mills and Chichester, 2005). It was used as an endpoint to evaluate the estrogenic effect of certain compounds and its quantification in whole organism assays and its increasing levels in immature fish and females has been widely

accepted as a biomarker for estrogenic endocrine disruption, as reviewed by Matozzo et al. (2008).

The acethylcholinesterase enzyme (AChE) is widely distributed among animals, both in vertebrates and invertebrates (de la Torre et al., 2002) and is responsible for the removal of the neurotransmitter acetylcholine (ACh) from the synaptic cleft through hydrolysis (Fulton and Key, 2001) at the nerve endings to prevent continuous nerve firings, which is vital for normal functioning of sensory and neuromuscular systems (van der Oost et al., 2003). This enzyme is not a biomarker of endocrine disruption, but can be indicative of stress in the general status of the fish (Pavlov, 1994) and other animals as are known to be altered by PCB and DDT (Schmidt et al., 2004; Zala and Penn, 2004). The inhibition of acetylcholinesterase is well documented as a specific biomarker target for assessing the exposure of non-target aquatic organisms to organophosphate and carbamate insecticides unlike to other groups of compounds. However, in the work realized by Bocquené et al. (1995) was observed that DDT, do not inhibit AChE in four species of fish at the maximum concentrations tested. Relatively to the PCBs, they are generally accepted as developmental neurotoxicants since the developing nervous system is sensitive to these compounds (Muthuvel et al., 2006), but it is known that PCBs are not ChE inhibitors and very few works try to demonstrate the relation between AChE and PCBs (Chuiko et al., 2007). However, it is known that PCB-77, DDT and its metabolite p,p'-DDE, are considered potent neurotoxicants that, at least after neonatal exposure, can lead to permanent disturbances in the cholinergic system and behavior in adults of mouse inducing persistent effects in the brain (Eriksson et al., 1992; Eriksson et al., 2002).

The common goby (*Pomatoschistus microps*) used in this work as a model species is an epibenthic and euryhaline fish that inhabits breeds in unpredictable environment such as estuaries in northern Europe and coastal lagoons in the Mediterranean Sea (Pampoulie, 2001) and plays an important ecological role because of its high abundance, high fecundity and trophic role as a predator on meio- and macrofauna and as prey for larger fish species and seabirds (Monteiro et al., 2006; Quintaneiro et al., 2008). The body size (maximum length, approx. 5 cm) and sluggish behavior of *P. microps* make it suitable for rearing and handling in the laboratory (Christiansen et al., 1998). *P. microps* has

already been used in other studies as test organism and have shown to be a good indicator species (Christiansen et al., 1998; Fonseca et al., 2011; Monteiro et al., 2005; Monteiro et al., 2007; Vieira et al., 2008).

Therefore the main objectives of this work were: (i) to assess the responses in different biomarkers (vtg, GSI, HSI and AChE), after a sub-lethal exposure of 21 days to PCB-77 and p,p'DDE in *Pomatoschistus microps* females; (ii) to determine if the various biomarkers of endocrine disruption (vtg, GSI and HSI), measured in *Pomatoschistus microps* females, are suitable to be used in the monitorization of EDC contamination in estuaries.

2.Material and Methods

2.1.Chemicals

All chemicals used in these experiments were obtained from Sigma-Aldrich (Germany), except the Bradford reagent which was purchased from Bio-Rad (Germany).

2.2.Fish sampling and laboratory maintenance

The fish sampling site (41^{o}53'27.28''N; 8^{o}49'30.81''W), located in the Minho river estuary (NW coast of Portugal) presents low levels of environmental contamination (Santos et al., 2012) and has been used as a reference site in different studies (Guimarães et al., 2009; Monteiro et al., 2007; Quintaneiro et al., 2006).

Two fish samplings of *P. microps* females were performed using a landing net at low tide. The first sampling was performed for PCB-77 test on January/2012 and the second for p,p'-DDE test on March/2012. The physico-chemical parameters of local water (temperature (T), pH, dissolved oxygen (DO), salinity (Sal) and conductivity (Cond)) were measured using a multiparameter VWR mod SympHony SP90M5. The values registered were: first sampling - T=9.4^{o}C; pH=7.5; DO=99%; Sal and Cond were not possible to be measured; second sampling (p,p'-DDE test) - T=14.6^{o}C; pH=8.79; DO=103.4%; Sal=6 mg/l; Cond=10.65 mS/cm. The transport to the laboratory (travel duration of about 4h) consisted in distribution of the fish through three separated containers filled with local water at which Ocean Fish Prodac marine salt was added to gradually increase salinity. In

the laboratory, the organisms were acclimated for two weeks before the beginning of the toxicity tests. The acclimation consisted in distribution for three aquariums filled with well-aerated, filtered artificial seawater (salinity=35‰) collocated in a controlled room with photoperiod 8h dark: 16h light at 20±1^{o}C temperature. The water medium for both acclimation and experiments was prepared dissolving marine salt Ocean Fish Prodac to simulate seawater, partially and was renewed every 2/3 days. Fish were fed twice a day with the dry food TetraMin®.

All procedures involving fish handling were conducted according to the Guide for the Care and Use of Laboratory Animals of the European Union - in Portugal represented by Decreto de Lei nº 129/92 de 06 de Julho, Portaria nº 1005/92 de 23 de Outubro de 1992.

2.3.Test conditions

Fish were exposed to PCB-77 and p,p'-DDE under the same conditions of temperature and photoperiod and feeding of the acclimation period. The water medium parameters were measured after every medium change. At the end of each test, the number of dead fish was recorded and the survivors were sacrificed by decapitation upon ice, weighted and measured. All fish were dissected and their gonads and livers were excised and weighted to posterior GSI and HSI calculation, respectively. These organs and also the head were placed in individual tubes and frozen in liquid nitrogen. The tubes were then stored at -80^{o}C until vtg (gonads and liver) and AChE analysis (fish head). Samples of the water medium from the highest concentration of both the compounds tested were collected and kept at 4ºC until chemical analysis.

2.3.1.Test exposure to PCB-77

Three groups of four *P. microps* per treatment were exposed in individual 1-L glass recipients to six concentrations of PCB-77, a negative control and a solvent control (0.078% DMSO). The test was conducted during 21 days according to Organization for Economic Cooperation and Development guidelines (OECD, 2006) and the medium renewal was performed, partially, every 2/3 days. The water medium parameters measured after every water change were: T=19.12±0.09^{o}C; pH=8.01±0.01;

DO=59.5±1.39%; Sal=35.23±0.08mg/l; Cond=54.40±0.43nS/cm (mean ±SE). Based on the results of chemical analysis the concentrations of PCB-77 tested were 2872.85; 478.8; 79.8; 13.3; 2.22 and 0.37ng/l.

2.3.2.Test exposure to p,p'-DDE

The female fish were exposed in groups of 3 animals, 12 fish per treatment, during 21 days, to five concentrations of p,p'-DDE and, also, to a negative control and a solvent control (0,008% DMSO). The measured concentrations of p,p'-DDE tested were 0.1205; 0.241; 2.41; 24.1 and 241 ng/l. The water medium parameters measured during the test were: T=17.9±0.06^{o}C; pH=8.13±0.04; DO=83.47±2.18%; Sal=35.66±0.23 mg/l; Cond=130.94±0.15 nS/cm (mean ±SE).

2.4.Biomarker analysis

2.4.1.Protein quantification for biomarkers

Protein content of the samples was determined, in quadriplicate, by the Bradford method (Bradford, 1976) adapted to microplate, using γ-globulins as standard and a wavelength of 595nm. A Labsystem Multiskan EX microplate reader was used for all protein and enzimatic determinations.

2.4.2.Acetylcholinesterase activity

One fish head per sample (n=7-12 per treatment) was homogenized, using a sonicator Branson S-250A, in 1 ml of phosphate buffer (0.1 M, pH 7.2). The supernatants obtained after centrifugation (4^{o}C, 6000 rpm, 3 min) were diluted and used for further protein and AChE activity quantification. The method used to determine AChE activity was performed according to Ellman et al. (1961) adapted to microplate by Guilhermino et al. (1996). The enzymatic activity is expressed in nmol/ml/mg de protein.

2.4.3.Vtg like-proteins

Liver and gonad samples (n=8-12 per treatment in the test with PCB-77 and n=9-12 per treatment with p,p'-DDE) were homogenized through sonication, in 1 ml of homogenization buffer (containing 125 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA and 1 mM dithiothreitol at pH 8) for each 200 g of weight and then centrifuged at 12000 g for 20 min at 4°C. Vitellogenin was then determined by the indirect alkali-labile phosphate method as described by Gagné et al. (2003) with some alterations introduced by Hallgren et al. (2009). Briefly, 100 µl of the supernatant were mixed with 54 µl of acetone (35% of final volume) for 5-10 min at room temperature and then mixed with a vortex agitator at least three times and then centrifuged at 10000 g for 5 min. After acetone removal, 50-100 µl (depending of pellet size) of 1 M NaOH were added to samples that were then maintained for 90 min at 70°C (Hallgren et al., 2009), to allow hydrolysis of bound phosphates (Hallgren et al., 2009). The levels of free phosphates were determined in the aqueous phase according to the phosphomolybdenium method (Stanton, 1968). Results concerning Vtg-like proteins are expressed as µgPO_4/mg protein.

2.5.Statistical analysis

For each biomarker, data from different treatments were transformed (if necessary) to achieve normal distribution and homogeneity of variances and were then analysed using one-way analysis of variance (ANOVA). To identify significant differences between control and treatments the Dunnett's test was used. When normality and/or homogeneity of variances were not achieved, a non-parametric test (test Kruskal-Wallis One Way Analysis of Variance on Ranks) was performed. Data outliers have been removed considering mean ± 3x standard deviation or mean ± 2x standard deviation.

Relatively to the variability associated with AChE in the PCB-77 experiment it is important to refer that all data was included in the statistical analysis. The low AChE registered (present both in controls and treatments) were not considered as outliers by the statistical analysis performed, contrarily to the higher values registered that are closer the mean AChE values registered in previous works with this species collected in the same estuary (Monteiro et al., 2005; Vieira et al., 2008).

The mortality rate, calculated considering the accumulated mortality in all treatments vs. control, and values of LC_{50} were determined, if the mortality rate was low, using the Minitab version 14.0 with Probit Analysis. All other statistical analysis was performed using SigmaStat for Windows, version 11.0.

3.Results

3.1.Weight, length and mortality rates

At the end of the experiments, the fish were measured and weighted obtaining values for length ranging from 3 to 5.3 cm and for weight from 272.7 to 1253.2 mg in the PCB-77 exposure and 2.5 to 4 cm for length and 179.4 to 535.2 mg for weight after p,p'-DDE exposure.

Although these experiments were not designed to examine lethality, the value for LC_{50} in this test was extrapolated and was above the highest concentration used (7743.36 ± 7381.63 ng/l; LC_{50} ± SE). The mortality rates at the end of test were 0; 17; 25; 8; 8; 25; 0; 25% in the control, solvent control and in the treatments 0.37; 2.22; 13.3; 79.8; 478.8; and 2872 ng/l respectively.

There was not possible calculate the value of LC_{50} for the p,p'-DDE test but the the mortality rates at the end of the test were 0; 33; 17; 17; 17; 8; and 8% corresponding to control, control solvent and the treatments 0.1205; 0.241; 2.41; 24.1 and 241ng/l, respectively.

3.2. AChE activity

In the Figure 3.1 is presented the data obtained in AChE activity after PCB-77 exposure (1) and after p,p'-DDE exposure (2). As seen in the Figure 3.1-1, in the PCB-77 experiment, it was verified a significant difference between control and one of the treatments (Kruskal-Wallis One Way Analysis of Variance on Ranks, P=0.002), namely the concentration of 2.22 ng/l that present a significant reduction in AChE levels ($P<0.05$, Dunnetts's test). Relatively to p,p'-DDE exposure, there was no observed differences between the different treatments and the control ($P<0.05$, Dunnet's test).

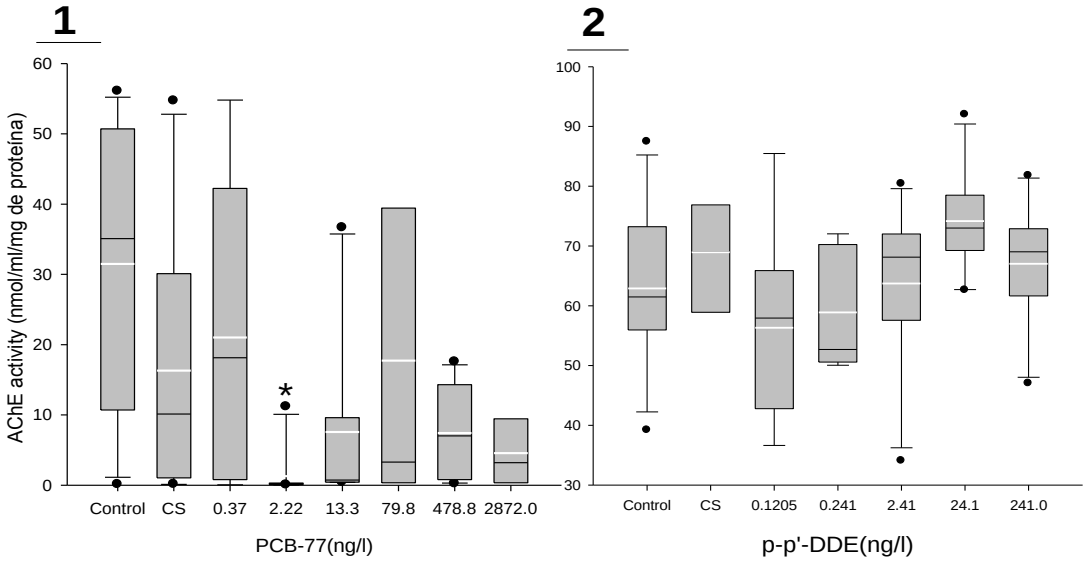

Figure 3.1: Determination of AChE activity in head fish of *P. microps* after 21 days of PCB-77 exposure (1) and p,p'-DDE (2). Results are expressed as mean±SE; * significantly different from control (P<0.05) and •represents the outliers; CS- solvent control. The white line represents the mean of the results.

3.3. Vtg-like proteins

The results obtained in the vtg-like proteins levels in the gonads of females exposed to PCB-77 and p,p'-DDE are presented in Figure 3.2. As observed in the Fig. 3.2-1 there was no significant differences observed between control and treatments (P=0.118; ANOVA). For comparison, the results referred to the effects of PCB-77 in the amount of vtg-like proteins in liver are displayed in Figure 3.2-1 and in this organ it was observed significant differences between the control and the different treatments (p<0.05; Kruskal-Wallis One Way Analysis of Variance on Ranks) with an increase of the levels of vtg at the concentration of 478.80ng/l PCB-77 (p<0.05, Dunn's Method).

The effects in p,p'-DDE (Fig. 3.2-2) in the vtg values in gonads and liver and there was no significant differences observed (p=0.423 and 0.679, respectively) between control and all the treatments.

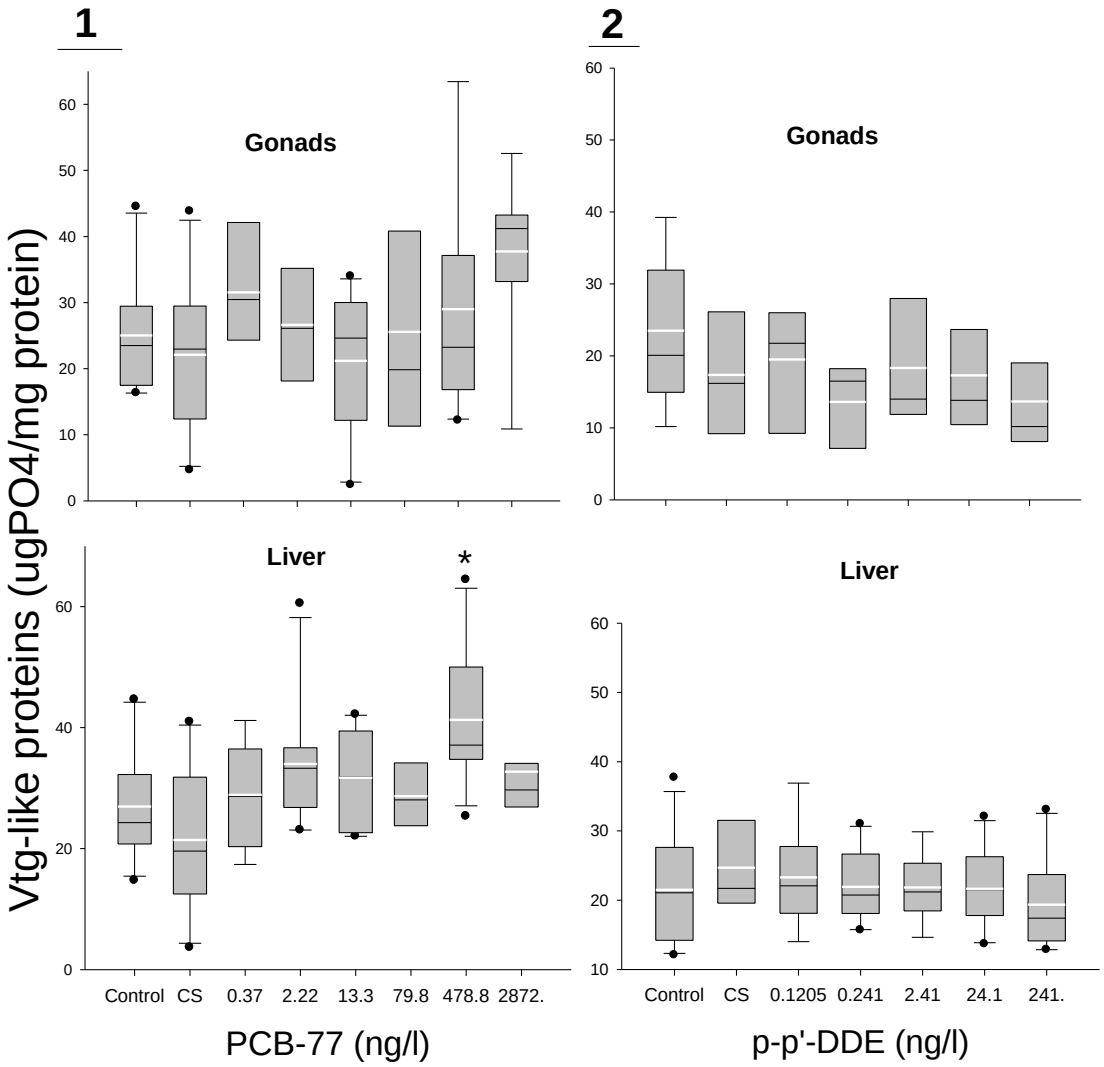

Figure 3.2 - Vitellogenin-like protein levels in gonads and liver of the female fish *P. microps* after 21 days of exposure to PCB-77(1) and p,p'-DDE (2). Results are expressed as mean±SE; *significantly different from control (P<0.05) and •represents the outliers; CS- solvent control. The white line represents the mean of the results.

3.4.GSI and HSI

The determination of GSI and HSI at the end of exposure to PCB-77 (1) and p,p'-DDE (2) is presented in Figure 3.3. No significant differences were verified in both indexes (p=0.095 and 0.053, respectively) relatively to the control. Relatively to GSI and HSI, after p,p'-DDE exposure (Fig. 3.3-2) and it was verified that there was no significant differences in both indexes between the control and the other treatments (p=0.178 and 0.126, respectively).

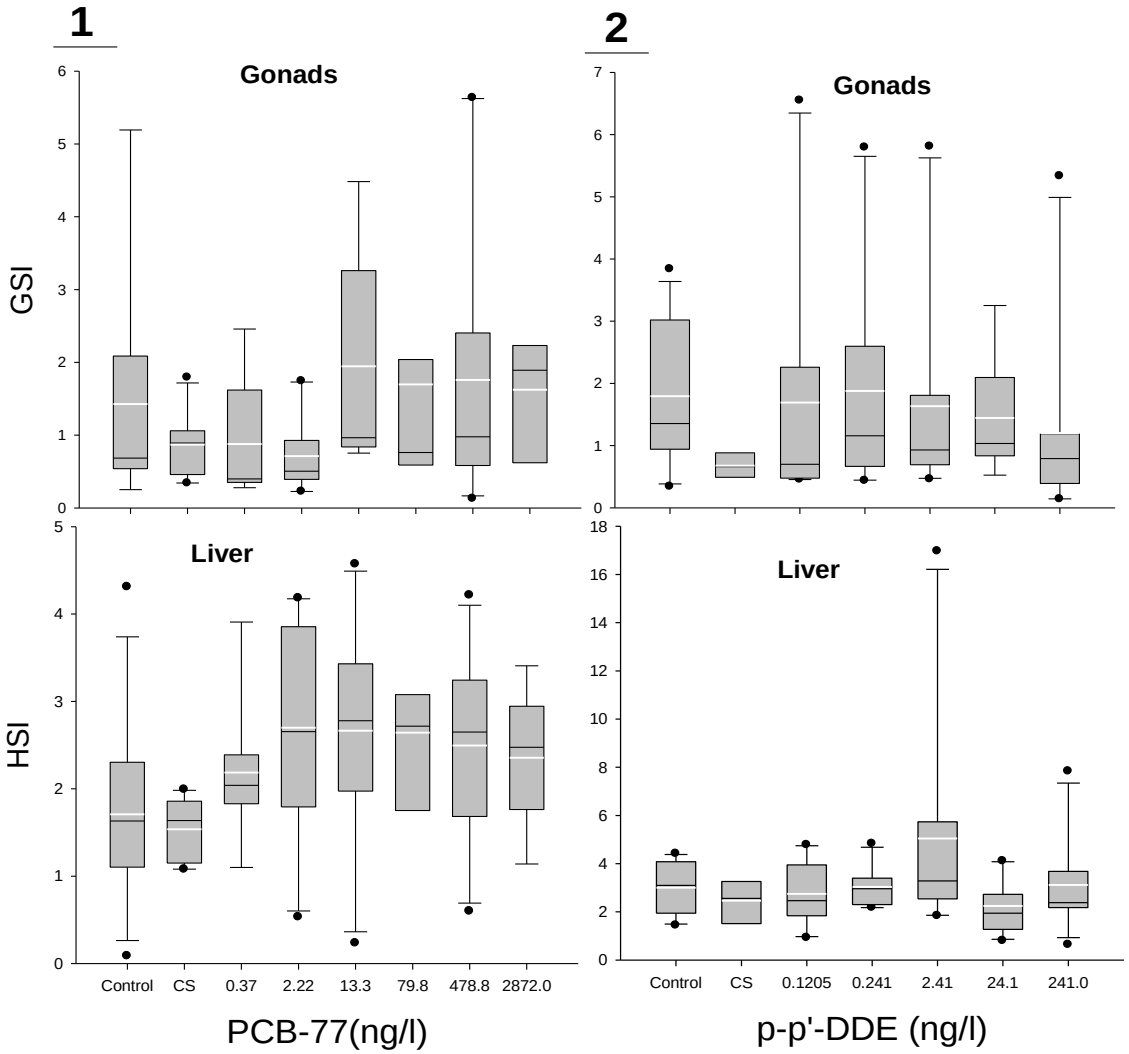

Figure 3.3: Gonadossomatic (A) and hepatossomatic (B) indexes of *P. microps* females after 21 days exposure to PCB-77(1) and p,p'-DDE (2). Results are expressed as mean±SE and •represents the outliers. The white line represents the mean of the results.

4.Discussion

In this work, the main objectives were to evaluate the effects that two endocrine disruptor compounds (PCB-77 and p,p'-DDE) can cause in biomarkers of endocrine disruption such as the levels of vtg-like proteins in gonads and liver and in the GSI and HSI of females of the fish *P. microps*. In addition, the head of the fish were utilized to assess the neurotoxicity by the measurement of AChE activity.

In this context, the fish are often used as sentinel organisms since they accumulate and respond to low concentrations of toxic compounds (Çavaş and Ergene-Gözükara, 2005) but, namely, coastal species such as *P. microps* may accumulate high levels of

organochlorine compounds (PCBs, DDT and its metabolites) due to the proximity to discharge points (Shahidul Islam and Tanaka, 2004). This has great ecological concern since xenobiotics can impair reproductive functions and subsequently threaten survival of the species (Shahidul Islam and Tanaka, 2004)

Normally, the sex of the specimens chosen to realize experiments to test endocrine disruption using vtg as endpoint is the male due to the lack or low level of vtg. The fact of using females of *P. microps* in the present experimental setup is related mainly to the difficulty in field, to collect enough male organisms to perform the tests. However, the use of adult females as bioindicators through vtg quantification, in endocrine disruption, is of great importance due to their role in the reproduction and maintenance of the populations (Kime and Nash, 1999) and both inhibition and stimulation of vitellogenesis can have direct repercussions on the reproductive capacity of fish (Kime et al., 1999). Miller et al. (2007) illustrates how vitellogenin concentrations, in reproductively active female fish (effects in individuals), appear to reflect both important mechanisms of action of EDCs and provide a direct linkage to egg production (population level).

The female biomarkers, as vtg and GSI, had a great potential to be used as signposts and it was demonstrated that there are significant quantitative relationships between them and reproductive output, regardless of mode of action of the compound and the species used (Bosker et al., 2010).

EDCs are more likely to cause long-term sublethal effects (Ankley et al. 2004) and, so, the value of LC_{50} in both exposures was calculated only if the mortality rate registered was high. It was observed, in both tests that did not happen and only in the case of PCB-77 test the LC_{50} value was calculated by extrapolation with an associated high standard error.

4.1.AChE activity

The inhibition of AChE activity is a specific biological effect of exposure to agricultural pesticides including OPs and CBs but can be applied in field biomonitoring programmes on fish species since, although less specific, it was considered a general marker of exposure to neurotoxic contaminants including metals and organochlorines (Corsi et al., 2003a)

The choice of the assessment of the AChE activity in the entire head of this fish was decided due to a previous work by Monteiro et al. (2005) that characterized the class of cholinesterases present in the different head tissues of this fish and concluded that AChE was the predominant type.

The differences observed in the AChE activity between the controls of the two tests 31.48 ± 5.66 (mean ± SE) in the experiment with PCB-77 and 60.23 ± 4.43 (media ± SE) in the experiment with p,p'-DDE) might be explained by the existing variability associated with field sampling of animals, that in this case was performed in different seasons of the year (January and March of 2012, respectively). Besides the seasonality that might affect AChE in *P. microps* (Monteiro et al. 2007), animals were collected in a reference site, the Minho river estuary, but can still be subjected to several environmental uncontrolled factors and some degree of chemical contamination (Monteiro et al., 2005; Vieira et al., 2008).

The exposure to PCB-77 demonstrates an inhibition in the AChE activity in the treatment 2.22 ng/l relatively to the control. This inhibition is usual in works with PCBs both in laboratory and field studies with other species. For instance, in the field, Durou et al. (2007) demonstrated that in worms, the PCB concentrations and AChE activity, were inversely related. As well, in the study performed by Venkataraman et al. (2008), it was verified a decrease in activity of AChE in selected brain regions in treated rats after exposure to a mixture of PCBs.

Relatively to studies that use fish, Khan and Thomas (1996) demonstrated disruption of neuroendocrine functions in Atlantic croaker *Micropogonias undulates* after exposure to a mixture of PCBs and concluded that it is important to consider the potential adverse effects of PCBs exposure that could interfere with the neuroendocrine function during investigations of the reproductive toxicity of these compounds. Moreover, it was found that chub (*Leuciscus cephalus*) living in Lambro River (Italy) had a decreased brain AChE activity when the total PCB content in the fish was increased (Barra *et* al., 2001).

At the concentrations tested, no effects were observed relatively to the action of p,p'-DDE in this enzyme. This is in accordance with the findings of Bocquené et al. (1995)

that found that DDT did not inhibit AChE in the four species of fish used at the maximum concentrations tested (in case of DDT, up to 10µg/l).

Due to the fact, before referred, that AChE is usually used as bioindicator to carbamates and organophosphorous compounds, there is not much literature were the objective is determine the alteration in AChE activity in organochlorine compounds. Relatively to other organochlorines compounds, one the main conclusions were that no effects were observed on *in vitro* AChE activity after incubation in chironomids with one pesticide and one metal (lindane and zinc), the mean of AChE activity remained within 0.1% of controls at all concentrations for both compounds (Ibrahim et al., 1998). The inhibition of AChE activity is a specific biological effect of exposure to agricultural pesticides including OPs and CBs but can be applied in field biomonitoring programmes on fish species since, although be less specific, it was considered a general marker of exposure to neurotoxic contaminants including metals and organochlorines (Corsi et al., 2003b). The choice of the assessment of the AChE activity in the entire head of this fish was decided due to a previous work by Monteiro et al. (2005) that characterized the class of cholinesterases present in the different head tissues of this fish and concluded that AChE was the predominant type.

4.2.Vtg-like proteins (gonads and liver)

In this work, the vtg-like proteins quantification presented only significant differences when measured in the liver after exposure to PCB-77, where it was verified an induction of its production. Since this compound is known to be an endocrine disruptor with a coplanar structure and dioxin-like behavior, they can present weak estrogenecity and ability to increase the values of vtg, despite the fact of being documented as an AhR agonist with anti-estrogenic activity (Mortensen et al., 2006).

This situation can happen, as explained by (Robertson and Hansen, 2001) because the estrogenic effects may not be only explained by binding and activation of the ER and but also due to a mechanism whereby some PCBs, through inhibition of estrogen sulfotransferase, can inactivate and increase the amount of E2 in target tissues. This is verified with 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126) that present also estrogenic and

antiestrogenic activity in rat reflecting the possible estrogenicity of its metabolites (Robertson and Hansen, 2001)

As see in the results, the induction of vtg occurred in the liver but it was not observed in the gonads and this could be explained due the fact that the liver, as the place where vtg is synthetized and particularly in female fish, contains high concentrations of estrogen receptors that leads to the production of large amounts of vitellogenin when stimulated by estrogens (Sumpter and Jobling, 1995). A study, where a dietary exposure with a mixture of PCBs performed in the laboratory have shown a decrease in ER binding capacity in livers of the Atlantic croaker was mentioned by Garcia et al. (1997)

It is important to take in account that the measure of high levels of vitellogenin alone may not necessarily be indicative of exposure to an environmental estrogen (Kime et al., 1999) since it can be affected by other variables (e.g., nutritional status) (Miller et al., 2007) and it is necessary relate these values with other characteristics of the reproductive endocrine system (Kime et al., 1999).

Relatively to the no observation of significant effects in vtg in the gonads after PCB-77 exposure, it was observed the same result in the work with the same compound and realized by (Monosson et al., 1994) that concluded that this exposure impairs both maturation of adult females, and survival of their offspring. In addition, the same authors concluded that low concentrations of PCB-77 needed to decrease ovarian growth, oocyte maturation, circulating sex steroid hormone could be more than sufficient to decrease larval survival in the adult female fish.

As said before, the p,p'-DDE is mainly antiandrogenic (Kelce et al., 1995) but in fish their mode of action is considered a weakly ER agonist (Garcia-Reyero et al., 2006). The concentrations of p,p'-DDE tested in this experiment did not cause any significant alteration in the levels of vtg-like proteins in *P.microps* females. This is in agreement with the study realized by Donohoe and Curtis (1996), where the p,p'-DDE was not significantly estrogenic to trout and the authors considered that this result was consistent with other *in vivo* studies that reported lack of effect of p,p'-DDT (other metabolite of DDT) on uterine or oviduct weights. This tendency was also observed in other study by Carlson et

al. (2000) which concluded that there was no evidence that this chemicals act as endocrine disruptor in fish. They microinjected embryos of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) with several contaminants, including p,p´-DDE, and after 6 months it was not observed changes in sex ratio, gonadal histology or steroid production. In addition, there are several reports about the complete absence of endocrine-disrupting activity of this compound, despite its well-documented uptake in fish (Carlson et al., 2000; Mills et al., 2001; Zaroogian et al., 2001).

Some studies found that vtg is up-regulated by p,p'-DDE in largemouth bass (Larkin et al., 2002), whereas several papers reported that vtg it was not induced by p,p'-DDE treatment in flounder *Paralichthys dentatus* (Mills et al., 2001; Zaroogian et al., 2001).

In females, a relatively constant induction rate of vtg could be related with a steady uptake by oocytes (Vega-López et al., 2006) and a low plasma concentration is indicative of pollution induced dysfunction at the pituitary or ovarian level but it measurement alone must therefore be used with careful since the absence of an effect on exposure to a potential xenoestrogen does not necessarily imply no effects (Kime et al., 1999). Other factors that may also contribute to a variable vitellogenic response include water temperature, migratory behavior, previous and type of EDC exposure (Mills and Chichester, 2005).

Variability in fish size, within and between experiments, is likely to have little impact on the vitellogenin response, due to the low background and the magnitude of the effect (Panter et al., 1998).

4.3.GSI and HSI

Both of these indexes, in both exposures to the two compounds, did not present significant differences when the values were compared between the several treatments and the control. This means that the exposure to both organochlorine compounds did not interfere with these parameters in the female fish of *P. microps* at the concentrations tested. Despite the fact of these chemicals are known for their estrogenic and/or anti-androgenic activity (Toppari et al., 1996)it was verified a similar result by (Daouk et al.,

2011) that observed, in a laboratory exposure of females of *Danio rerio* to a PCB mixture that both parameters did not present significant differences.

In the work cited above realized by (Versonnen et al., 2004), in addition to the no observed effects in vtg, it was not observed effects in GSI and HSI after the exposure to methoxychlor, a compound structurally analogue to DDT.

Due to the no observed effects in this endpoint, it can be inferred that vitellogenin quantification is a more sensitive biomarker than GSI for determining estrogenic exposure as suggested by Panter et al. (1998).

4.4.Conclusions

One of the significant results were observed in the vtg of the liver after PCB-77 exposure and, take into account that this organ synthetized vtg, the measure of this endpoint in this organ need to be considered in future works to detect alterations after exposure to this or other EDCs.

The other endpoints assessed (vtg in gonads, GSI and HIS) do not present significant responses to low concentrations of PCB-77 and p,p'-DDE tested in the females of *P. microps* and, for that reason, it was not possible infer about their relevance. The AChE results were only significant to the PCB-77 and, due to the unexpected response, it was difficult to infer about the mean of this result.

However, the obtained responses were sufficient to understand and/or considered their utilization in monitoring endocrine disruption and/or neurotoxicity in this species of estuarine waters.

Therefore, it can be recommended testing both compounds with higher concentrations (even if the environmental relevance was low) and the assessment of more biomarkers (e. g. histology of gonads) could be taken in account.

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Chapter 4

General Discussion

1. General Discussion

The presence of a great quantity of compounds in the estuarine waters is the main reason of this work. The need to assess how these compounds affect the organisms (mainly at endocrine level) that live in this type of environments, through the use of biomarkers, is essential to their application in biomonitoring programs.

In the beginning of this work, the main objective was to evaluate the effects of selected EDCs in one estuarine fish species, the common goby *Pomatoschistus microps*.

The first and more usual choice was working with males of *P.microps* but, as said before, their collection was not very easy in the field due to their low number in comparison with females and juveniles. Thus, and as the most part of the life-cycle bioassays developed for EDC testing takes in account reproductive endpoints, there is a need for estuarine bioassays that could assess the possible impacts of EDCs during early life stages (Boudreau et al., 2004) and this become a viable reason to work with juveniles. Their utility, in a whole body evaluation, was confirmed in Chapter 2. The results obtained in vtg demonstrate that *P. microps* juveniles are sensitive to environmental relevant concentrations of E2 and PCB-77 and might be useful to track contamination by EDCs in estuaries. The measurement of AChE, in the all head of the fish and after PCB-77 exposure, revealed an induction of this endpoint which is difficult to be explained due to the lack of similar results in the literature.

The Chapter 3, where *P. microps* females were used as model species, revealed some results that were not significant in the endpoints measured at the concentrations of PCB-77 and p,p'-DDE tested but, however, the significance of the results with statistical meaning need to be taken in consideration such as the increase of the levels of vtg in liver. In addition, in the future, the work with *P. microps* females could be complemented with the assessment of more endpoints, such as the measurement of sex ratio, histology (from both tissues of liver and gonads) and/or differences in length and weight. Vitellogenin, in combination with sex ratio as endpoint, provides a test that is very sensitive to estrogenic as well as androgenic chemicals (Holbech et al., 2006).

The measurement of AChE activity was performed to detect if the tested compounds, PCB-77 and p,p'-DDE, were neurotoxicants and to take advantage of the head of the fish, obtaining therefore more information from the same number of animals. It was observed opposite effects of PCB-77 in females and juveniles with an inhibition and induction of activity, respectively. This may have to do with the obvious stage of life, difference in sensitivities or different pathways of interaction of this compound with the enzyme. However, the enhancement of AChE activity after PCB-77 exposure could be only a signal of stress. In p,p'-DDE, due to the lack of significant results, its neurotoxic potential cannot be inferred. It is important to denote that the values observed in controls for juveniles are in range with others measured by other authors (Monteiro et al., 2005; Quintaneiro et al., 2008). In females, in both exposures, the values are below those found in the literature; one of the obvious reasons could be due to the two different life stages that could have different AChE levels and no data concerning AChE activity in *P. microps* females is available in the literature to compare the values.

In the particular case of the AChE activity measured after PCB-77 exposure, it were denoted some differences in controls relatively to the p,p'-DDE exposure. This could be explained by the existing variability associated with field sampling of animals. These differences denote low values (some closest to zero) that, beyond the previous explanation, the probability of technical errors could be considered and a possible repetition of the test was a viable option. In addition, it should be taken in account some technical/sampling/acclimation cautions that could prevent the variability observed for instance in the AChE activity in the PCB-exposure with females. In the field, the handling of the fish needs to be the less possible. The acclimation begins in the transport to the laboratory and the exposure to gradual concentration of salts was revealed of great importance along the three samplings. This species had a great capacity of adaptation verified by Quintaneiro et al. (2008) that tested the effects of transportation and acclimation in AChE measurement and do not find any interference but, in the laboratory, is essential the correctly monitoring and maintenance of the physico-chemical parameters.

Since the pollution in the environment is generally characterized by a mixture of compounds, rather than by a single chemical, that may result in additive or antagonistic effects of some biological responses (Calabrese, 1995), future research in this area should include the testing of mixtures of EDCs commonly found together in estuaries.

Based in the results obtained in this work, some considerations should be taken in account in future investigations that include evaluation of EDCs in *P. microps:*

The use of juveniles, due to the sensitivity showed to low concentrations of E2 and when comparing their response to PCB-77 exposure with females, could be considered the best choice in terms of life stage to monitor and/or study EDCs.

In case of using females, the organ that seems to respond better to the presence of EDCs is the liver, since the levels vtg-like proteins are elevated in relation to the control in the PCB-77 exposure. However, the values associated to this result need to be analysed with precaution since this is the local of synthesis of vtg and it was a process that could depend from other variables than the presence of endocrine disruptors.

As concluded in the Chapter 2, the E2 can be considered a positive control to use in EDCs tracking with juveniles of this species, with the recommend concentrations above the LC_{10} obtained in this work (8.50036 ng/l of E2 with an 95% CI between 20.4418 and 24.5800 ng/l).

The lack of results in the GSI and HSI indexes in the exposure to both compounds can be related with the sensitivity of these parameters since the high variation between individual fish and their level of maturation can interfere with the results as seen in the study realized by Bogers et al. (2007) for HSI and by Panter et al. (2000) for GSI.

In general, at the concentrations tested and after the evaluation of the several endpoints analyzed, both PCB-77 and p,p'-DDE seem to interfere with the organisms. However, the response and due to the effects observed after exposure to PCB-77 in the juveniles, this compound seems to present characteristics of endocrine disruptor contrarily to p,p'-DDE which results do not allow to infer about its capacity to deregulate the endocrine system of this species, at least at the concentrations tested.

2.Bibliography

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