Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2010, 22(10) 1613–1622
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Assessing the estrogenic potency in a Portuguese wastewater treatment plant using an integrated approach

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Received 26 November 2009; revised 11 December 2009; accepted 15 January 2010

Abstract

The estrogenic potency of a wastewater treatment plant (WWTP) was evaluated using chemical and biological analyses, which showed that after the station treatment processes some of the selected endocrine disruptor compounds (EDCs) were still present in the treated effluent (e.g., bisphenol A, alkylphenols, estrone). Thus, the most common endocrine EDCs were identified and quantified and the overall estrogenicity of the treated effluent assessed by integrating the results. Male goldfish (*Carassius auratus*) were used as biological indicators in a 28-day experiment. Vitellogenin (Vtg), gonadosomatic and hepatosomatic indices, steroids (17β-estradiol and 11-ketotestosterone) and histopathology were biomarkers used in fish to evaluate WWTP treated effluent estrogenicity, in combination with instrumental analyses. The results showed a significant increase ($P < 0.01$) in plasma and liver Vtg, which were significantly correlated ($r = 0.66$; $P < 0.01$). The gonadosmatic index was significantly ($P < 0.01$) reduced in exposed fish. The steroid analyses revealed significant elevations in 17β-estradiol and depressed 11-ketotestosterone concentrations. The histological examinations show changes in exposed fish gonads, such as regressed testes and in some cases (43% to 75%) the development of ovo-testis in fish exposed to 50% and 100% treated effluent.

Key words: *Carassius auratus*; endocrine disruptors; vitellogenin; steroids; intersex
DOI: 10.1016/S1001-0742(09)60297-7

Introduction

There has been increasing awareness that a group of substances, usually termed endocrine disruptor compounds (EDCs), may negatively affect the endocrine system of wildlife and humans (Vos et al., 2009). These chemicals are discharged to the aquatic environment through point (sewage treatment, pulp mill and industrial effluent) and non-point (urban and agricultural runoff) sources (Folmar et al., 2002).

These compounds, which are both natural and man-made, are able to mimic the action of natural steroids. The most intensively studied group of EDCs are environmental estrogens such as 17β-estradiol (E2) and alkylphenols (APEs), which are common in sewage effluents (Bjerselius et al., 2001). However, many other natural and synthetic chemicals are estrogenic and may cause endocrine disruption, e.g., bisphenol A, 17β-ethynylestradiol, organochlorine pesticides, polychlorinated biphenyls, polynuclear aromatic hydrocarbons and phytoestrogens (Folmar et al., 2001a). The alkylphenols are the final breakdown products of alkylphenol polyethoxylates (APEOs) and are widely used as detergent emulsifiers and wetting and emulsion agents in various domestic and industrial applications. Nonylphenol and octylphenol are the most representative breakdown metabolites of APEO degradation, showing higher toxicity to wildlife than the parent compounds (Coldham et al., 1998).

The compound bisphenol A is used in the production of epoxy resins and polycarbonate plastic for household and industrial applications and, therefore, can be expected to be present in wastewater (Furhacker et al., 2000). It was recently shown that BPA also has estrogenic potency, causing adverse changes in the reproductive health and fecundity of animals and humans (Belfroid et al., 2002). Natural and synthetic steroids, such as 17β-estradiol and 17α-ethynylestradiol, are also found in wastewater effluents originating from human waste. They are very potent estrogens that can cause adverse effects in living organisms (Tyler et al., 1998). These compounds have been identified and quantified in many WWTPs, principally in developed countries (Petrovic et al., 2004; Maurício et al., 2006; Liu et al., 2009). Several studies of fish,

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both in the field and laboratory, have now clearly shown that reproductive disturbances can occur following their exposure to environmental estrogens such as those already mentioned (Routledge et al., 1998; Diniz et al., 2005; Filby et al., 2007).

Vitellogenin (Vtg) is the main precursor of yolk protein in oviparous vertebrates and is synthesised by the liver in response to endogenous oestrogens (Heppel et al., 1995). Vtg is normally synthesized in mature females, where the level of estrogen is above the threshold required to induce it, and male and juvenile fish only produce background levels of this protein. However, upon exposure to estrogen or an estrogen mimic, they will be induced to synthesize Vtg, since they have the gene for it in their livers (Folmar et al., 2001b). The potential of Vtg as a biomarker has already been demonstrated in various fish species (Jobling et al., 1998; Thompson et al., 2000). Thus, Vtg has been used as an ideal biomarker for measuring the exposure of oviparous animals to estrogen or to estrogen-mimicking compounds (Tyler et al., 1996). Some studies have suggested that exposure to EDCs can alter the steroid concentrations in fish, e.g., the plasma 11-ketotestosterone and 17β-estradiol concentrations, and deregulate their endocrine systems (Noaksson et al., 2003). Thus, the sex hormone balance has also been advocated as a reliable and complementary biomarker of potential reproductive disruption (Solé et al., 2003; Diniz et al., 2009).

The main objective of this study was to assess the sewage discharge-related estrogenicity that could be observed at various levels of the biological organization of fish. Several endpoints (e.g., somatic indices, steroids, Vtg and histology) were therefore evaluated to determine the effects at the organism level. In addition, quantification of the target endocrine chemicals in the WWTP effluent provided information on the persistence of these substances and is complementary to the bioassay data. Thus, integration of the results from different levels of organization provides a better understanding of the negative effects of treated sewage effluents on the aquatic environment.

1 Materials and methods

1.1 Selection of the wastewater treatment plant

The Chelas Waste Water Treatment Plant (WWTP) is located in the east of Lisbon (Portugal) and discharges its treated effluent into the River Tagus estuary (Fig. 1). It consists of an interceptor system, which includes five pumping stations to collect downtown wastewater. The WWTP was selected as a model to study as it receives great volumes of urban wastewater and variable quantities of industrial effluents produced by companies in the municipal district. The WWTP was designed to collect and treat 52,500 m^3 of urban wastewater per day, corresponding to approximately 211,000 equivalent inhabitants. The WWTP provides secondary and tertiary treatment, with activated sludge treatment (including nitrogen and phosphorous removal) and final disinfection with an ultra-violet system before the effluent is discharged into the River Tagus estuary. It is, therefore, a typical, large-scale municipal WWTP. Table 1 summarizes the WWPT technical data.

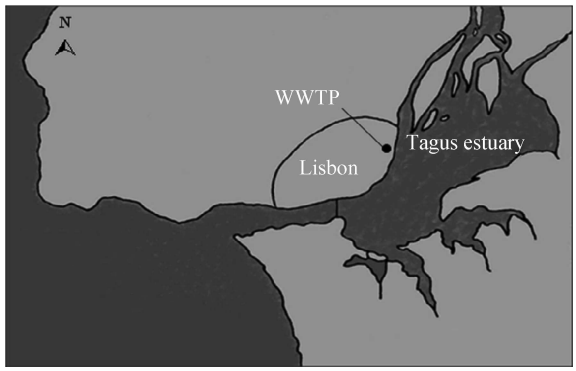

Fig. 1 Location of the wastewater treatment plant (WWTP) in the River Tagus estuary.

1.2 Sampling and sample preparation

At 4-hour intervals over a 24-hour period, composite samples were collected in amber glass bottles from different sampling points of the WWTP. The aim was to evaluate the efficiency of unit operations in removing the previously selected compounds and to characterize the physicochemical parameters of the WWTP wastewater (pH, temperature, dissolved oxygen, TSS, COD and BOD_5) so as to gain a better understanding of the effluent quality during the sampling period and, therefore, the plant performance.

The procedure for sample pre-treatment and extraction was similar for ELISA and LC-MS-MS analysis and was carried out according to previous reports (Petrovic and Barceló, 2002, Petrovic et al., 2002). Briefly, wastewater samples (300 mL) were filtered in a glass-fibre filters (0.45 μm; Macherey-Nagel, Germany) and acidified with 1 mol/L acetic acid buffer (pH 5) to eliminate particulate matter and other suspended solid matter and then stored at 4°C in the dark. EDCs were always extracted, within 24 hours of collection, using OASIS HLB 3 cm^3 solid phase extraction (SPE) columns (Waters, USA). Prior to extraction, the columns were conditioned with methanol and water. EDCs were eluted with 2 × 5 mL of dichloromethane (BPA and 17β-estradiol) or methanol (APEs), depending on the EDCs for analysis, and then evaporated to dryness under a gentle stream of nitrogen and reconstituted with 10% methanol to a final volume of 2 mL.

Table 1 Technical data from Chelas WWTP

Physico-chemical parameters	Mean of Min. and Max. concentrations	Treated effluent
BOD_5 (mg O_2/L)	270/290	25
COD (mg O_2/L)	440/595	125
TSS (mg/L)	180/435	35
Total N (mg/L)	45/55	7.5
Total P (mg/L)	10/15	10.0
Mean flow (m^3/day)	49	

BOD_5: biochemical oxygen demand; COD: chemical oxygen demand; TSS: total suspended solids; total N: total nitrogen; total P: total phosphorus.

1.3 LC-MS-MS analysis conditions

Alkylphenols (nonylphenol and octylphenol), alkylphenol ethoxylates (APn_{EO}, n_{EO}: 1–15), bisphenol A (BPA) and estrogens (E1-estrone, E2-estradiol, E3-estriol, EE2-ethynyl estradiol, DES-diethylstilbestrol, E2-gluconate, E1-sulfate) were analysed using the LC-MS-MS methods (Petrovic et al., 2003; Rodriguez-Mozaz et al., 2004). LC-MS-MS analyses were performed on a Waters 2690 series Alliance HPLC (Waters, USA) with a quaternary pump equipped with a 120-vial capacity sample management system. The analytes were separated on a 5-μm, 125 × 2 mm i.d. C18 reversed phase column Purospher STAR RP-18 endcapped (Merck, Germany). The sample injection volume was set at 10 μL to APEs, APEOs and BPA, and to 25 μL to estrogens. A binary mobile phase gradient with methanol and water was used for analyte separation at a flow rate of 200 μL/min. The elution gradient was linearly increased from 30% methanol to 85% methanol in 10 min, then increased to 95% methanol in 10 min and kept isocratic for 5 min.

A bench-top triple quadrupole mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with a pneumatically assisted electrospray probe and a Z-spray interface was used. The capillary voltage was set at 2.8 kV, extractor lens at 7 V and RF lens at 0.6 V. The source and desolvation temperatures were 150 and 350°C, respectively. The nitrogen (99.999% purity) flows were optimised at 50 L/hr for the cone gas and 540 L/hr for the desolvation gas. For MS-MS experiments the argon collision gas was maintained at a pressure of 0.58 Pa. To measure estrogens, the MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150°C; desolvation temperature, 350°C; extractor voltage, 4 V; and radio-frequency (rf) lens, 0.2 V. Nitrogen was used as both nebulizing and desolvation gas. The flow rate of the nebulizing gas was set at 60 L/hr and that of the desolvation gas at 550 L/hr. Argon was used as the collision gas with a pressure of 0.25 Pa.

Quantitative LC-MS-MS analysis of compounds detected under negative ionisation (NI) conditions (NP, OP, BPA and estrogens) was carried out in multiple reactions monitoring (MRM) mode, while compounds detected under positive ionisation (PI) conditions (NPEOs and OPEOs) gave only molecular adduct ions $(M^+Na)^+$ and produced no fragmentation. As a result these compounds were analysed using a single stage MS in selected ion monitoring (SIM) mode. Table 2 shows the MRM and SIM ions, and the limits of detection (LODs) obtained.

1.4 Biological assays

A group of four fiberglass tanks (1 m^3) were set up at the WWTP, supplied with different concentrations of the treated wastewater effluent (25%, 50% and 100%) produced by mixing different percentages of effluent with tap water (mains water previously de-chlorinated) as shown in Fig. 2. One tank was supplied exclusively with tap water (the reference tank). All tanks received a continuous flow of water (8 L/min) and aeration using an air pump.

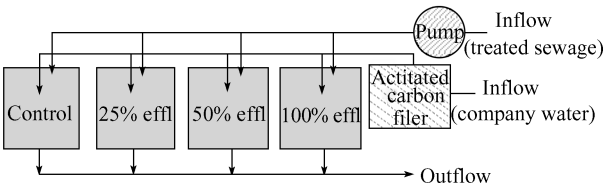

Fig. 2 Diagram of experimental tanks at the WWTP.

Table 2 LC-MS-MS conditions and limits of detection

Negative ionisation mode							
Compound	MRM 1	Cone (V)	Collision (eV)	MRM 2	Cone (V)	Collision (eV)	LOD (ng/L)
NP	219 → 133	30	30	219 → 147	30	30	2
NP1EC	277 → 219	10	30	219 → 133	30	30	2
NP2EC	219 → 133	30	30	321 → 219	10	30	1
OP	205 → 133	30	30	205 → 147	30	30	2
OP1EC	263 → 205	10	30	205 → 133	30	30	2
OP2EC	307 → 133	30	30	307 → 205	10	30	1
BPA	227 → 133	30	30	227 → 211	30	30	2
E2–estradiol	271 → 183	50	45	271 → 145	50	45	1
E3–estriol	287 → 171	50	40	287 → 145	50	40	1
E1–estrone	269 → 145	50	40	269 → 143	50	45	1
EE2–ethynyl-estradiol	295 → 145	50	40	295 → 159	50	40	2
DES–diethyl-stilbestrol	267 → 222	30	35	267 → 237	30	50	0.5
E2–gluconate	447 → 113	40	30	447 → 271	40	50	5
E1–sulfate	349 → 269	40	40	349 → 145	40	40	0.1

Positive ionisation mode		
Compound	*m/z*	LOD (ng/L)
NPEO (n_{EO}: 1–15)	287 + 44*n*	50 ($NPEO_1$) 20 ($NPEO_2$) 10 (NPEO, n_{EO}: 3–15)
OPEO (n_{EO}: 1–15)	273 + 44*n*	50 ($OPEO_1$) 20 ($OPEO_2$) 10 (OPEO, n_{EO}: 3–15)

To carry out the exposure experiments, sexually mature male goldfish (*Carassius auratus*), obtained from local producers (Aquário Vasco da Gama) and weighing an average of (15 ± 2) g were used as a test organism in a semi-field assay.

Before the tests, the fish were acclimated in a 300-L glass aquarium at the laboratory for two weeks and fed with commercially available pellets (Tetra fish food, UK). To perform the exposure tests, they were randomly distributed in each tank (n = 44) and fed daily with commercial pellets. Some physicochemical parameters (temperature, dissolved oxygen, pH and conductivity) were checked weekly. Cumulative mortality was registered as well.

Fish were exposed to various concentrations of wastewater effluent for four weeks and at the end of the experiment they were sampled to collect blood from the caudal vein, using heparinized syringes (2 mL) treated with aprotinin (10 Trypsin Inhibitory Units (TIU)/ mL; Sigma-Aldrich, USA). After centrifugation at 4000 ×g (15 min at 4°C), the blood plasma was immediately stored at –80°C in Eppendorf tubes, awaiting later analysis.

The vitellogenin analyses were carried out by the enzyme-linked immunoassay (ELISA) method in accordance with a protocol described elsewhere (Denslow et al., 1999). Briefly, microtiter (Nunc-Roskilde, Denmark) wells were coated with diluted (1:200) male carp plasma samples (50 μL), in phosphate buffered saline (PBS) solution. The micro plate was incubated overnight at 4°C, washed and blocking buffer (10% BSA in PBS with 0.02% sodium azide) was added to each well. After 2 hr incubation at room temperature microplates were washed again. The carp monoclonal antibody (Biosense, Norway) diluted to an appropriate concentration (0.1–5 μL/mL) was added to each micro titer well and incubated for 60 min at 38°C. Subsequently to new plate wash, a secondary antibody (1:1000) was added (goat-antimouse immunoglobulin-IgG conjugated to alkaline phosphatase, Sigma-Aldrich, USA) to plate wells and incubated (60 min at 38°C). After washing, the substrate (*p*-nitro-phenylphosphate–PNPP, Sigma-Aldrich, USA) was added (100 μL) to each microplate well, incubated at room temperature for 10–30 min and stop solution (50 μL; 3 mol/L NaOH) added to stop the development process. The microplates were read at 405 nmol/L in a microplate reader (BioRad-Benchmark, USA). Vtg was quantified by constructing a calibration curve, preparing standards by serial dilutions of the carp Vtg standard (Biosense, Norway) to give a range from 10 to 1000 ng/mL. The coefficient of variation was calculated for each triplicate sample and, if it exceeded 15%, samples were run again. Standard curves fitted by log-linear regression were used to quantify Vtg concentration, with R^2 values of 0.96 to 0.99.

Plasma concentrations of 11-ketotestosterone (11-KT) and 17β-estradiol (E2) were measured using EIA kits (Estradiol EIA kit and 11-KT EIA kit, Cayman Chemicals, USA), in accordance with the kit protocols provided by Cayman Chemicals (2002, 2003). Both the assays are based on the competition between free estradiol or 11-KT and a tracer (estradiol linked to an acetylcholinesterase or 11-KT-acetylcholinesterase (AchE) molecule) for a limited number of estradiol or 11-KT-specific rabbit antiserum binding sites. Because the concentration of the estradiol tracer (or 11-KT tracer) is held constant while the concentration of free estradiol or 11-KT (standard or sample) varies, the amount of free estradiol (or 11-KT) tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free estradiol (or 11-KT) in the well. This rabbit antiserum estradiol (or 11-KT) (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AchE) is added to the well. Plasma steroid concentrations were measured by constructing standard curves, using standard estradiol and 11-KT and standard serial dilutions to a range between 7.8 and 1000 pg/mL according to information furnished by Cayman Chemical (2002, 2003). A computer spreadsheet provided by Cayman Chemical for data analysis was used to calculate the assay results.

After blood sampling, fish were euthanized and dissected to remove the organs (gonads and liver) and then weighed. The gondosomatic index (GSI) and the hepatosomatic index (HSI) for males and females were determined to assess exposure effects at organism level. The indices were calculated as follows: GSI = (gonad weight/total body weight) × 100 and HSI = (weight/total body weight) × 100.

After sub-samples (from caudal and mid portions) of the removed gonads had been taken and placed in Bouin-Hollande fixative for 48 hr, they were washed and placed in a series of graded ethanol (30% to 70%) and subsequently embedded in paraffin wax blocks using conventional techniques, as described in Martoja and Martoja (1967). Sections were cut (5–7 μm) and stained with haematoxylin and eosin (H&E). Male gonads were evaluated by histological observation, using a microscope (Leica-ATC 2000, Germany), and classified at one of four maturation stages, according to the scale proposed by Gupta (1975).

1.5 Statistical analysis

Statistical analysis of the results was carried out by one-way ANOVA, after the data had been checked for assumptions of normality and homogeneity (Leven's test) and, if necessary, appropriately transformed. The post-hoc Tukey test was used to compare pairs of means and detect significant differences ($P < 0.05$). Correlation analysis (Pearson) was carried out to examine the significance of the relationships between vitellogenin concentrations and GSI, HSI and steroid (11-KT and E2) concentrations. The software Statistica 5.0 (Statsoft Inc., USA) was used for the data analysis. Microplate Manager 4.0 (BioRad Software, USA) was used to construct a standard curve and determine vitellogenin concentrations, by extrapolating observances to the standard curve.

2 Results

2.1 Biological assays

At the end of the experiment cumulative male mortality was less than 10% for all treatments. The highest levels of plasma vitellogenin (262 ± 19.7 μg/mL) were registered in male fish exposed to 100% of treated sewage effluent. The Vtg measurements for fish exposed to different concentrations of treated sewage effluent were significantly different ($P < 0.01$) from controls and also among treatments (Fig. 3).

Regarding Vtg measurements in liver and gonad homogenates, results show that the liver registers the greatest concentrations of this protein (Fig. 4). However, no significant differences are observed among treatments as far as liver Vtg and gonad Vtg is concerned. Statistical analysis revealed that plasma Vtg and Vtg in the liver homogenate were significantly correlated ($r = 0.66$; $P < 0.01$). A significant correlation was also observed between Vtg plasma and Vtg in the gonad homogenate ($r = 0.37$; $P < 0.01$).

The steroid determination revealed a significant increase ($P < 0.01$) in E2 concentrations in fish exposed to the different concentrations of sewage effluent. The highest levels of E2 (4410 ± 564 pg/mL) were recorded in fish exposed to 100% treated sewage effluent. Because the concentrations of plasma 11-KT diminished significantly in comparison with controls (Fig. 5). A significant negative correlation was found between 11-KT and E2 ($r = –0.62$; $P < 0.01$).

GSI showed a significant ($P < 0.01$) decrease in all treatments in comparison with controls, while the lowest values (GSI = 1.70 ± 0.21) were identified in fish exposed to 100% treated sewage effluent. A significant negative correlation was detected among plasma Vtg ($r = –0.90$; $P < 0.01$), liver Vtg ($r = –0.63$; $P < 0.01$) and the GSI. The hepatosomatic index (HSI) analysis revealed that there were no significant differences between controls and exposed males (Fig. 6).

After the experimental period the testes of control fish had a normal appearance and the spermatogenesis was active. When the testes were examined, they showed cells at all spermatogenic stages and were classified as maturing (Stage II). The lumina were filled with spermatozoa and the lobules contained spermatogenic cysts. No ovotestis condition was observed in male control fish.

The histopathological analysis of the treated testes showed that treated sewage effluent has a negative effect, causing changes at tissue and cell level. Fish exposed to 25% treated effluent appeared similar to the controls, except for the fact that some of the sperm cells seemed to be hypertrophied.

Fish exposed to 50% and 100% sewage effluent exhibited spermatozoa in the seminiferous lobules but the seminiferous lobules seemed to have a more diminished diameter than in previous treatments. The tissue seems to have degenerated and hypertrophied cells were observed around seminiferous lobules (Fig. 7a). Some fish exposed to 100% of the effluent presented regressed testes and spermatogenesis seemed to be inhibited. Additionally, histological observation showed a few oocytes (Fig. 7) scattered within the testis tissue in fish exposed to different

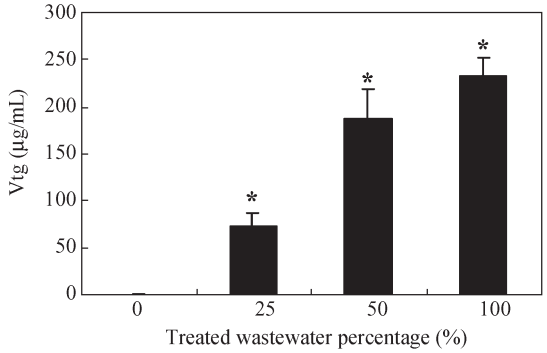

Fig. 3 Vitellogenin (Vtg) concentrations in exposed fish. * Significant differences from controls.

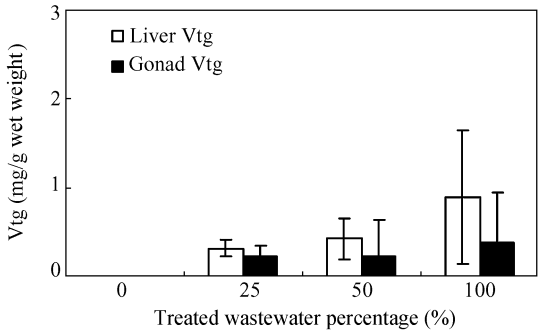

Fig. 4 Liver and gonad Vtg concentrations.

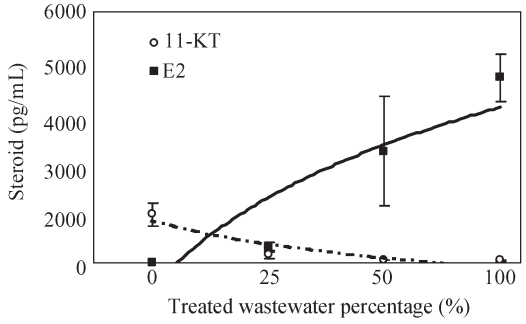

Fig. 5 Steroid concentrations in fish exposed to the different concentrations of treated effluent.

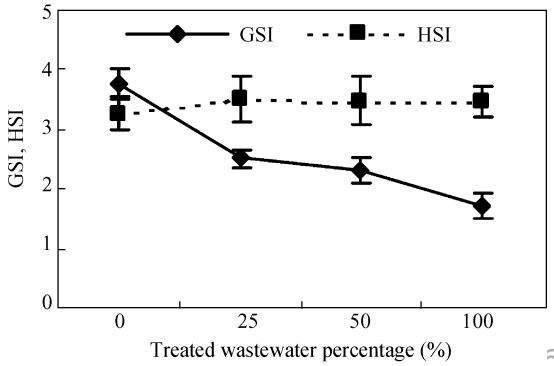

Fig. 6 Gonadosomatic and hepatosomatic indices.

concentrations of treated sewage effluent (Table 3). The development of gonadal ducts was not detected.

2.2 Analytical chemistry

The results for the estrogenic compounds selected are shown in Tables 4 and 5. They report the concentrations after the operations listed.

3 Discussion

The induction of Vtg in male and juvenile fish is used as a biomarker of estrogenic exposure (Sumpter and Jobling, 1995; Kime et al., 1999). High plasma Vtg concentrations have been reported in wild male carp, *Cyprinus carpio* (Folmar et al., 1996; Solé et al., 2002), in rainbow trout (*Oncorhynchus mykiss*) (Harries et al., 1996, 1999) and in walleye (*Stizostedion vitreum*) (Folmar et al., 2001a) that are exposed to or live near sewage treatment plants. They have also been reported in laboratory studies with chemicals known to be present in sewage, in fish such as salmon (*Salmo salar*) (Arukwe et al.,1998, 2000) and flounder (*Platichthys flesus*) (Christensen et al., 1999) exposed to NP, and carp (*Cyprinus carpio*) (Casini et al., 2002), adult medaka (*Oryzias latipes*) (Kang et al., 2002) and rainbow trout (*Oncorhynchus mykiss*) exposed to E2 (Bjoernsson and Haux, 1985; Christiansen et al. 1998) and bisphenol A (Lindholst, 2000, 2003).

The significant increases in plasma Vtg in male fish exposed to the different treated effluent concentrations indicate the estrogenic activity of the WWTP. In addition, the Vtg was detected in male livers and gonad homogenates, which in turn were significantly correlated to the plasma Vtg. If the presence of Vtg in male livers reflects the synthesis of Vtg in this organ, the existence of Vtg in the testes suggests that this protein reaches the testes via the circulatory system and may cause adverse effects. Folmar et al. (2001b) indicated that Vtg accumulation in organs possibly resulted in hepatocyte hypertrophy, the disruption of spermatogenesis and the obstruction or rupture of renal glomeruli. In addition, they also suggested that Vtg

Table 3 Presence of gonadal ducts and oocytes in testes of fish exposed to different percentages of effluent

Treated wastewater percentage (%)	Ocytes (%)	Gonadal ducts (%)
0	0	nd
25	0	nd
50	43	nd
100	75	nd

n = 40; nd: not detected.

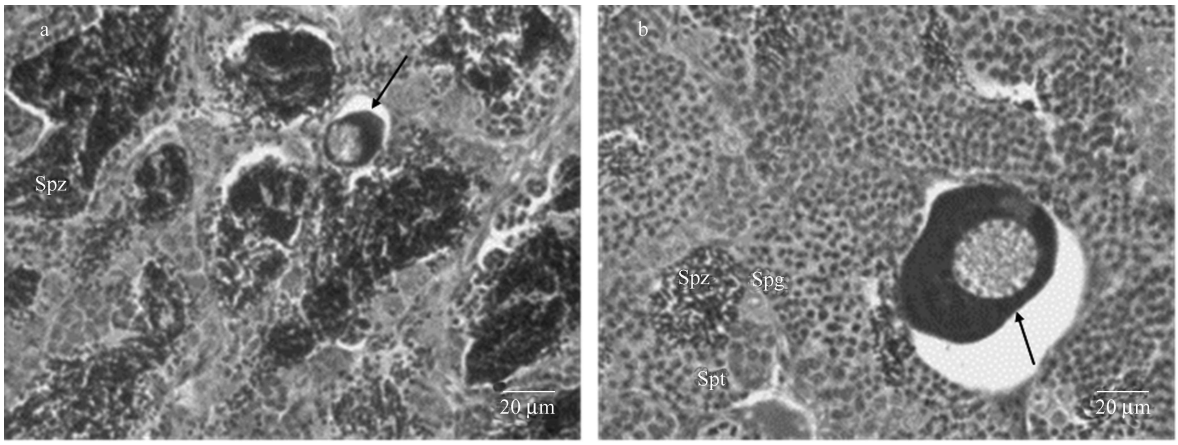

Fig. 7 Ovo-testis in fish exposed to 100% treated effluent (a), Spz (spermatozoa), Spg (spermatogonia), Spt (spermatocytes); Ocyte (arrowhead) (b).

Table 4 Alkylphenolic compounds and BPA concentrations in samples measured by LC-MS-MS (unit: µg/L)

Treatment steps	BPA	OP	OP1EC	OP2EC	NP	NP1EC	NP2EC	NPEO (n: 3–15)
Screening	1.55	0.46	< LD	0.31	1.17	0.58	2.40	76.50
Grit removal	0.15	< LD	< LD	0.28	0.52	0.62	1.96	54.50
Filtration	0.31	< LD	< LD	1.80	0.13	1.82	12.00	15.40
UV desinfection	< LD	< LD	< L D	3.11	0.70	2.91	94.20	4.50

BPA: bisphenol A; OP: octylphenol; OP1EC: octylphenol carboxylate; OP2EC: octylphenol ethoxy carboxylate; NP: nonylphenol; NP1EC: nonylphenol carboxylate; NP2EC: nonylphenol ethoxy carboxylate; NPEO: nonylphenol ethoxylates (sum of oligomers with 3 to 15 ethoxy groups). LD: limit of detection.

Table 5 Steroids concentrations in samples measured by LC-MS-MS (unit: ng/L)

Treatment steps	E2-gluc	E1-sulf	E3	E2	EE2	E1 estrone	DES
Screening	nd	8.4	52.8	nd	nd	39.6	nd
Grit removal	nd	nd	nd	nd	nd	nd	nd
Filtration	nd	3.5	nd	nd	nd	nd	nd
UV desinfection	nd	3.4	nd	nd	nd	nd	nd

E2-gluc: b-estradiol 17glucuronide; E1-sulf: estrone 3-sulfate; E3: estriol; E2: 17β-estradiol; EE2: ethynyl estradiol; DES: diethylstilbestrol; n = 3 (mean value; sd < 7%). nd: not detectable.

accumulates in the testis vasculature, through obstructive blockage, rather than in the gonadal tissue itself, which was further supported by the apparent lack of specific Vtg receptors in the testes of fish.

Sex steroid hormones play important roles at all stages of the reproductive cycle in vertebrates and most teleost fish. The role of the main androgen is played by 11-KT, while testosterone is usually present in the blood at lower concentrations (Kime, 1993). Experimental studies show that 11-KT can induce one or more of the male reproductive traits, including spermatogenesis and spermiation, secondary male sexual characteristics and male reproductive behaviour (Godwin and Thomas, 1993). Taking this into account, it is not surprising that there is increasing interest in measuring and monitoring the effects of endocrine disrupting chemicals on their reproductive function (Nash et al., 2000). Our results show a decrease in 11-KT concentrations in fish exposed to the different dilutions of wastewater effluent and at the same time a significant increase in the plasma E2 concentrations. However, plasma steroids showed some individual variability. In addition, the statistical analysis revealed a significant negative correlation between 11-KT and E2. Studies from other authors have also demonstrated depressed levels of 11-KT or Testosterone (T) and increasing concentrations of E2 in male fish plasma (Folmar et al., 2001a; Bjerselius et al., 2001). However, in various species of fish, plasma 11-KT levels have been found to be low, even during the peak period of sexual development, showing no positive correlation with the development of male reproductive traits (Pankhurst, 1999). Additionally, Solé et al. (2003) have suggested that fish show lower T levels than those of normal males as a consequence of the reduced amount of testicular tissue rather than a diminished amount of T production per unit mass of testicular tissue.

Estrogenic substances present in wastewater are taken up by fish and thereby stimulate biosynthesis of Vtg. The increased Vtg levels stimulate the synthesis of endogenous E2 that again induces Vtg production. Therefore, the high levels of Vtg observed in exposed male can be related to the increasing levels of circulating E2. In some field studies, Vtg presence, or a change in the normal pattern, has been concomitant with altered plasma levels of sex steroids such as testosterone and estradiol in male carp (Harries et al., 1999).

In this study, the GSI decreased, which is consistent with other studies that reported severe effects in gonad weight. For example, in fathead minnows (*Pimephales promelas*) exposed in a treatment wetland, besides significant Vtg induction, the GSI was significantly reduced at the inflow site, however, no differences were observed in the HSI (Hemming et al., 2001). Wild carp, *C. carpio*, collected near an STP showed a reduction in GSI (30). Lavado et al. (2004) showed a decrease in GSI and spermatogenesis in carp (*C. carpio*) collected from five sites along the lower course of the River Ebro (Spain) and suggested that the proximity of STP effluent was a major cause of the endocrine disruption observed. Ashfield et al. (1998) found a significant reduction in the GSI of fish (*O. mykiss*) exposed to NP and NP1EC and suggested that an appropriate GSI is a crucial factor in successful reproduction.

Several studies carried out in the laboratory as well in the field have shown that EDCs can cause histological changes in testes and other organs such as the liver and kidney, for instance, the testes of male eelpout (*Zoarces viviparus*) exposed to NP and E2 showed severe effects, such as degenerated lobules (Christiansen et al., 1998) and in male fathead minnows (*P. promelas*) exposed to E2 and E1 the increase in plasma vitellogenin levels was accompanied by the inhibition of testicular growth (Panter et al., 1998). Bjerselius et al. (2001) exposed male goldfish (*C. auratus*) to E2 and observed a reduction in the GSI. Juvenile common carp (*C. scarpio*) exposed to NP and EE2 showed histological alterations in the kidney, the liver and the spleen after treatment with EE2, whereas NP-exposed fish did not show any tissue lesions (Schwaiger et al., 2000).

In this study, fish exposed to 50% and 100% treated effluent show regressed testes, spermatogenesis inhibition, and oocyte development within testis tissue, suggesting that EDCs are affecting the endocrine system of the fish. In fish exposed to EDCs, besides the observed increase in plasma Vtg, the most pronounced effects are the high incidence of ovo-testis in wild populations, especially those near WWTP effluent (Solé et al., 2001).

The results show several disturbances at the various levels of organization, from the biochemical to organism level, as shown for instance by the Vtg or GSI, respectively. In addition, combined with the biological data, the chemical analysis provides information on estrogenic potency at many organization levels.

The effective operation of wastewater treatment plants plays an important role in minimizing the release of xenobiotic compounds into the aquatic environment (Byrns, 2001). Once in the environment, the fate of these compounds is determined by microbial transformation and physicochemical processes such as autoxidation (Johnson and Sumpter, 2001).

In general, the biodegradation of APEOs is limited by the formation of relatively stable metabolites, nonylphenol and octylphenol, their mono- and diethoxylates, and mono carboxylic acids, in particular (Zhang et al., 2008; Liu et al., 2008). In the present study the nonylphenolic compounds were in the form of persistent metabolites, the most abundant being nonylphenoxy carboxylic acids (Table 4). Studies carried out in the Glatt River (Ahel et al., 2000) also showed that the total concentration of the nonylphenoxy carboxylic acids was significantly higher than that of the lipophilic metabolites and the metabolites often exceeded the predicted no-effect concentration (PNEC) of 0.33 g/L proposed in a risk assessment report to the European Union. Spengler et al. (2001) suggested that the relatively high concentration may result from its semipolar character, which leads to a less pronounced tendency to adsorb onto suspended particles than that of the hydrophobic NP.

Elevated levels of APE metabolites were found in the influent of the WWTP and, although the station treatment processes were able to remove substantial amounts of

the compound, it was still possible to find metabolites in the treated effluent (Table 4). In this study the NPEC concentrations were also above the EU risk assessment proposals.

The mean value for BPA in the WWTP treated effluent was below the detection limit (Table 4). However, after sand filtration the effluent showed a mean value of 310 ng/L, which is in the same range as the concentrations found in other WWTP effluents (Belfroid et al., 2002; Ding and Wu, 2000). The present study showed that an average elimination rate of > 95% was observed and most of the bisphenol A was possibly removed from the wastewater in another way than through effluent discharge. Nevertheless, the BPA levels measured in the present study are not likely to produce estrogenic effects in the aquatic ecosystem if we take the estuarine water dilution effects into consideration (Belfroid et al., 2002).

According to Spengler et al. (2001), steroids (except for mestranol) are found in almost 80% of WWTP effluents, showing median values ranging between 0.4 ng/L (EE2) and 1.6 ng/L (E2). Although, the steroids investigated could not be detected, with the exception of E1 (Table 5), that does not mean that they are not present in the WWTP effluent. In fact, a previous study on E2 in WWTP effluent showed the presence of this compound (Maurício et al., 2006). A possible explanation for the non-detection of E2 was the low sample volume collected (500 mL) which may not have been enough to detect the low levels of E2 usually present in domestic WWTP effluents.

However, the concentrations of the selected EDC are considered weak and by itself they do not explain the biological results. Therefore, the results may suggest the presence of other EDCs that can cause the observed results. Another possibility is that EDCs, although weak, can act additively.

As a concluding remark, the chemical identification and quantification of certain EDCs, combined with the biological data, in the treated effluent provide an integrated measure of the total estrogenic potency of the effluent and result in a more comprehensive characterization of the WWTP effluent.

4 Conclusions

Instrumental analyses are usually used to identify and quantify EDCs in WWTPs effluent. *In vivo* bioassays can be used to provide useful information that can complement instrumental analyses. Therefore, this study adopted an evaluation of the estrogenic effects by measuring several biological indicators at different levels of organization and also combining it with results from chemical analysis. The main results can be concluded as:

(1) The altered mean plasma steroids (11-KT and E2), plasma, liver and gonad Vtg, somatic indices and histopathological changes were observed in exposed carp and make available a multi-level approach of the results which supports the estrogenic assessment of the STP effluent.

(2) Chemical identification and quantification of some EDCs at the treated effluent combined with biological data provide an integrated measure of the total estrogenic potency of the effluent and result in a more comprehensive characterization of the WWTP effluent.

Acknowledgments

This work was supported by the Portuguese Foundation for Science and Technology (FCT/MC) to its financial support to the project "Comprehensive Assessment of Impacts of Endocrine Disruptors Compounds from Urban Wastewater" (No. POCT/36303/MGS/2000) and also the authors Ph.D grant (No. SFRH/BD/3098/2000, SFRH/BD/3093/2000). We also thank to Eng. J. Martins from SIMTEJO (Chelas-Waste Water Treatment Plant) for WWTP facilities in the sampling procedure.

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