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RESEARCH ARTICLE

# The endocrine-disrupting effect and other physiological responses of municipal effluent on the clam *Ruditapes decussatus*

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**Abstract** In order to document the potential endocrine disrupting and toxic effect of the municipal wastewater effluents discharged into the Sfax coastal area (South of Tunisia), specimens of clam *R. decussatus* were collected from a reference site and were in vivo exposed to treated sewage effluent for 30 days. To this end, estrogenic and androgenic activities were measured in the gills to assess potential accumulation and regulation of active compounds. After effluent exposure androgenic activity in organic extracts increased up to fivefold compared to controls and remained elevated, while estrogenic activity was not significantly affected by exposure. As a consequence, remarkable disruptions in the gametogenesis activity, glycogen content, and Vitellogenin-like protein levels in male clams were observed. A parallel analysis of heavy metals in clam tissues was determined. A significant uptake of Ni, Zn, and Pb in soft tissues of exposed clams was observed. The significant increase of malondialdehyde (MDA) concentrations as a function of exposure time implies that clams have been exposed to an oxidative stress probably due to the presence of high metal concentrations in sewage effluent. Correlation analysis has revealed a statistically significant and positive relationship between MDA levels and metal concentrations in clams' tissues. The acetylcholinesterase activity was not significantly affected by exposure. Altogether, these results showed that a short-term exposure to a mixture of chemical compounds released by the Sfax wastewater treatment plant induce adverse physiological and reproductive effects in *R. decussatus*. Further studies are underway in order to evaluate its long-term impacts on aquatic wildlife in the gulf of Gabes area.

**Keywords** *R. decussatus* · Municipal wastewater effluents · Androgenic and estrogenic activities · Biomarkers · Reproduction · Endocrine disruption

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## Introduction

The presence of toxic agents in the ecosystem has increased in recent years, especially in aquatic environments. Some chemical products that are released into the environment have the potential to disturb the normal physiology and endocrinology of humans and wildlife (Colborn et al. 1993; Sumpter 1998; Tyler et al. 1998; Guillette and Craine 2000; Gravato and Santos 2003; Salo et al. 2007; Gagné et al. 2011). Substantial scientific evidence indicates that a multitude of environmental contaminants have steroid hormone mimicking properties and in some cases, produce biological responses qualitatively similar to those produced by endogenous hormones (Sumpter and Jobling 1995; Katsiadaki et al. 2002; Falconer et al. 2006). These endocrine-disrupting chemicals (EDCs) enter aquatic environments through diverse human sources including medical (Thornton et al. 1996), industrial (Parrot et al. 2006), agricultural (Horrigan et al. 2002), and

especially wastewater treatment activities (Aerni et al. 2004; Johnson et al. 2005; Barber et al. 2007; Vajda et al. 2008; Schultz et al. 2010).

Among EDCs, estrogenic steroid hormones have attracted considerable attention, as they are a potential cause for enhanced feminization of fish exposed to treated wastewater; feminization of fish populations has been observed in the UK near sewage treatment plants (Jobling et al. 1998). Bateman et al. (2004) revealed the appearance of oocytes in the testes (ovotestis; an intersex condition) of male fish. Xenoestrogens are also able to induce the female yolk precursor protein vitellogenin and to provoke abnormal gonadal development in male fish (Purdom et al. 1994; Harries et al. 1997; Tyler et al. 1998). Invertebrates exposed to sewage treatment plant (STP) effluents have been shown to exhibit morphological abnormalities consistent with exposure to estrogenic chemicals as well. Mussels display significantly higher hemolymph and gonadal vitellogenin (Vtg) after exposure to STP effluents in the laboratory, and mussels caged downstream of an STP discharge in Quebec had significantly higher Vtg than those at the upstream site (Gagné et al. 2002). Gross et al. (2001) shown that a significant number of *Gammarus pulex* females captured downstream of an STP in the UK displayed an abnormal structure of oocytes in vitellogenesis and intersexuality in harpacticoid copepods was correlated with exposure to STP effluents (Moore and Stevenson 1994).

More recently, many studies have indicated the occurrence of other steroid hormones that mimic biological activities of the male sex hormone androgen called xenoandrogens in surface waters and sediments (Tilton et al. 2002). Activities of such chemicals have been described in association with paper and pulp mill effluents (Svenson and Allard 2004) and effluents from wastewater treatment plants (Blankvoort et al. 2005). Studies have documented the masculinization and impair immune function, reproduction, and development of aquatic organisms exposed to androgens (Bettin et al. 1996; Parks et al. 2001; Milla et al. 2011).

Besides sexual hormones, a variety of synthetic chemical compounds including pesticides, plasticizers, surfactants and heavy metals are frequently associated with treated municipal discharges and reach, in this way, aquatic environment (Hemming et al. 2004). These compounds may also interfere with hormonal systems (Burkhardt-Holm 2010) and cause physiological alterations, immunotoxicity, and genotoxicity in aquatic organisms (Gravato and Santos 2003; Salo et al. 2007).

In developing countries like Tunisia, studies on endocrine disruptors are almost nonexistent till recent years. The main concern of the Tunisian public still remains microbial pollution and algal toxicity. Recently, concerns regarding the presence and potential effects of EDC are growing. Research and development activities have focused on EDCs in rivers, lakes (Louiz et al. 2008; Mnif et al. 2012), and lagoons (Lahbib et al. 2010; Abidli et al. 2012). However, to the best of our knowledge, there are only two studies concerning the gulf of Gabes area (Ketata et al. 2007; Kessabi et al. 2013) which is considered as one of the most productive ecosystem in Tunisia in which the bivalve *Ruditapes decussatus* represent an important economic resource.

So, as part of series of studies aimed at characterizing endocrine disruptors contamination in the Gulf of Gabes area, this study report the occurrence of compounds mimicking endogenous estrogens and androgens in Sfax sewage treatment plant and assess in vivo the biological effects of sewage effluent discharged into the Sfax coastal area (South of Tunisia) on the mollusc bivalve *Ruditapes decussatus*.

## Materials and methods

### Clam collection and exposure experiment

Clams (*R. decussatus*) (35–40 mm) were collected in March 2009 from a relatively uncontaminated site "El Hicha" (located 95 km in the south of Sfax, Tunisia). They were brought to the laboratory and allowed to acclimate to the experimental conditions 2 days before exposing them to seawater containing 10 % of treated effluent of the Sfax sewage treatment plant. This concentration was chosen on the basis of our previous data on effluent toxicity in *R. decussatus*. Exposure to effluent was performed during 30 days at 18 °C under a natural day/night cycle (12/12 h) in six distinct 20-L water tanks, aerated with a maximum of 55 individuals per tank. Clams were fed once a day with pelleted commercial food. During exposure, water was renewed every 48 h. Negative control consisted in maintaining animals in seawater without effluent under conditions strictly identical to exposure experiment. Forty-five animals were collected from each treatment group at exposure days 0, 10, 20, and 30 to assess and evaluate the effects of treated effluent on the health and integrity of clams.

### Effluent collection and analysis

The urban wastewater used in this study was sampled from the municipal wastewater treatment plant in Sfax, an industrial town 270 km south of Tunis. Effluent samples were collected in glass containers previously cleaned, rinsed with tap water, and later soaked in 10 % HCl for 24 h and finally rinsed with deionized water prior to usage. The samples were transported to the laboratory, fractioned in subsamples, and stored at −20 °C.

To determine the estrogenic and androgenic activities of the effluent, water was extracted immediately after sampling. The sample was filtered and passed through an Oasis solid-phase

extraction (SPE) cartridge (Waters Corporation Milford, MA, USA) which had been preconditioned in sequence with 5 mL of methanol and 5 mL of water containing 0.1 % (*v*/*v*) acetic acid. The sorbent was rinsed with 5 mL water and eluted with 5 mL of methanol. This extract was evaporated and redissolved in 1 mL of methanol before use in the yeast estrogen screen/yeast androgen screen test assay.

To measure the concentrations of trace metals (Cd, Cu, Zn, Ni, Cr, Mn, and Pb) in municipal sewage effluent, a sample was placed in glass tube previously washed with hydrochloric acid (10 %) and then mineralized using nitric acid. The digested sample was filtered and analyzed using an inductively coupled plasma optical emission spectrometer (ICP-AOS Thermo-Scientific iCAP 6300 DUO).

## Biological indices

**Condition index (CI)** The CI was determined as an indicator of clam physiological status. CI was calculated individually on 15 clams at each sampling time according to Lobel et al. (1991). Clams were weighted (total and soft weight), and the CI was calculated as

$$\mathrm{CI} = (\text{fresh weight of soft tissues}/\text{total weight}) \times 100.$$

**Gonado-somatic index (GSI)** GSI provides a general quantitative estimation of the reproductive status and constitutes an interesting approach in the study of reproduction from ecophysiological point of view. It was designed to evidence the proportionality between growth of germinal and somatic compartments. GSI was calculated individually on 15 clams according to Grant and Tyler (1983) as

$$\mathrm{GSI} = (\text{gonad wet mass}/\text{body mass without shell}) \times 100.$$

## Biochemical and histological analyses

### *Gill extraction*

After exposure, six gills from the clams were homogenized in 3 mL of methanol. The extract was then centrifuged at 15, 000*g*. The extraction was repeated one more time. The supernatants were combined, evaporated and then redissolved in 1 mL methanol. A fraction of every extract was analyzed by the yeast estrogen/androgen receptor transcription assay (YES/YAS assay).

### *Recombinant yeast screens (YES-YAS assay)*

The estrogenic and androgenic activities of effluent and clam extracts were determined using the YES-YAS bioassay (Routledge and Sumpter 1996). The human estrogen and androgen receptors are stably integrated in the yeast (*Saccharomyces cerevisiae*) chromosome and can activate a reporter gene system upon binding of estrogenic or androgenic compounds. Samples extracts and blanks were serially diluted in ethanol, and 20 μL was transferred to 96-well flat-bottomed multiwell plates. The ethanol was evaporated at room temperature followed by the addition of yeast and assay medium containing the chromogenic substrate to the wells. The plates were finally incubated for 3–4 days. The absorbance at 540 nm of each sample was determined after subtraction of absorbance at 620 nm to correct for differences in yeast growth. The estrogenic and androgenic activities of each sample were determined using respectively estradiol ($E_2$) and dihydrotestosterone (DHT) as a standard to each plate. The estrogenicity and androgenicity of effluent sample was expressed in terms of ng $E_2$ equivalents/L (ng $E_2$eq/L) and ng DHT equivalents/L (ng DHTeq/L) of extract, while androgenic and estrogenic activities of clam extracts were expressed in ng DHTeq/g ww and ng E2 eq/g ww, respectively.

### *Reversed phase HPLC fractionation*

In order to identify bioavailable contaminants with estrogenic and androgenic activities, samples from either clams or effluent were fractionated by high-performance liquid chromatography (HPLC). HPLC method was established and validated to screen for 11 known endocrine active substances (17α-ethynylestradiol, 5-α-androstanol, estriol, estrone, pregnenolone, testosterone, tamoxifene, bisphenol A, irgasan, 17β-estradiol, 4-nonylphenol). Extracts of gill tissues with high estrogenic and androgenic activities were combined. An aliquot of each sample (effluent and clam tissue) was injected onto a C18 column (XDB C18, 2.1×150, 1.8 micron), and separation was achieved by increasing the ratio of mobile phase solvents ultrapure water and methanol.

### *MDA analysis*

Malondialdehyde (MDA) determination was carried out in the digestive gland of 15 clams using the colorimetric method (Sunderman et al. 1985) which is based on the reaction of thiobarbituric acid with MDA. Malondialdehyde levels were estimated at 532 nm using 1,1,3,3-tetraethoxypropane as a standard. The concentration of lipid peroxidation in the digestive gland is expressed as micromoles of MDA per gram of fresh tissues.

### *AChE analysis*

For acetylcholinesterase (AChE) measurements, 15 gills were homogenized individually in phosphate buffer (0.1 M, pH 7.4) at a ratio of 3 mL of buffer for 1 g of tissues. The homogenate obtained was then centrifuged at 9000*g* for 20 min at 4 °C. An

aliquot of the supernatant was used for measuring AChE according to the method of Ellman et al. (1961), modified for microplate reading by Bocquené and Galgani (1998). The extracts were incubated in the presence of acetylthiocholine iodide as substrate (0.075 M) and 5.5-dithiobis-2-dinitrobenzoic acid (DTNB) (0.01 M). The reaction was carried out at 25 °C, and the absorption was measured by a spectrophotometer at 412 nm. The enzymatic reaction rate was quantified against a blank without substrate for each activity measurement. A second blank was performed without sample to subtract the spontaneous hydrolysis of the substrate. AChE activity is expressed as nanomoles of the product developed per minute and per milligram of proteins. The quantity of protein present in the homogenate was determined according to the method of Bradford (1976) at 595 nm, using bovine serum albumin (BSA) as a reference standard.

*Heavy metal content in clams*

Fifteen soft tissues at each sampling time were subjected to digestion before metal analyses. Measurement of metal concentrations in soft tissues was carried out as previously described (Geffard et al. 2010) by flameless (Cd, Cu, Ni, and Zn) atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace (SpectrAAZeeman220).

*Energy reserves*

**Total proteins** To determine acetylcholiesterase activity and Vtg-like proteins, total proteins were determined respectively in gills and gonad of each clam previously homogenized in phosphate buffer (0.1 M, pH 7.4), according to the protein-Coomassie blue dye binding principle (Bradford 1976) using bovine serum albumin (Sigma) for calibration.

**Glycogen** Glycogen was extracted from the gonad tissue and then hydrolyzed to glucose using the colorimetric method developed by Sasaki et al. (1972). The extraction of glycogen was preceded by total protein elimination step. Briefly, seven gonads from each sex were homogenized in a 2 mL of trichloroacetic acid (20 %) and then centrifuged (8000*g*, 5 min) at room temperature. The resulting supernatants were separated from the pellet (containing proteins), added to 4 mL of absolute ethanol to precipitate glycogen, and subsequently incubated for 5 min at 80 °C. The resulting pellets (containing glycogen) were dissolved in 2.5 mL of water, then incubated with 0.5 mL 30 N HCl for 60 min at 100 °C. The released glucose was quantified using the colorimetric method developed by Sasaki et al. (1972). This assay is based on the binding of the orthotoluidine reagent to glucose in a medium of acetic acid under boiling water. Glycogen concentrations were expressed in milligrams per gram of wet weight.

**Lipids** Total lipids were determined by a sulfo-phospho-vanillin reaction, based on the reaction of lipids with vanillin in a medium of sulfuric acid and phosphoric acid to form a chromogen (Frings et al. 1972). Briefly, seven gonads from each sex were homogenized in phosphate buffer 0.2 M, pH 7.0 at 4 °C. Forty microliters of homogenate was mixed with 200 μL of sulfuric acid (96 %), placed in boiling water for 10 min and transferred on ice for 5 min. Phospho–vanilline reagent (vanilline 6 g/L; phosphoric acid (85 %); $H_2O$) (35:60:5) (10 mL) was added, and tubes were incubated at 37 °C for 15 min. Tubes were kept on ice for 5 min, and absorbance was read at 540 nm within 30 min. Olive oil standards ranging between 0 and 10 mg/mL were used for standard curves, and lipid concentrations of samples were calculated from standard curves and expressed as mg/g wet mass tissue.

*Vtg-like proteins determination*

Vitellin (Vtg)-like proteins were determined in clam gonads extracts using the organic alkali labile phosphate (ALP) procedure (Gagné et al. 2003). Briefly, ten gonads from each sex were homogenized individually in 10 mM Hepes-NaOH buffer, pH 7.5, containing 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was carefully removed and adjusted to 35 % acetone and centrifuged at 10,000*g* for 5 min. The resulting pellet was dissolved in 200 μL 1 M NaOH at 70 °C for 30 min in a water-shaking bath to increase solubility. Levels of inorganic free phosphates were determined using the phosphomolybdenum method developed by Stanton (1968). A subsample of 75 μL was mixed with 125 μL trichloroacetic acid, 630 μL ultrapure water, 170 μL molybdenum reagent (0.02 M ammonium molybdatetetrahydrated and 5.25 M $H_2SO_4$ solution), and 50 μL Fiske-Subbarow reducer (Sigma, St. Louis, MO). After incubating for 10 min, the absorbance was measured at 660 nm. Total proteins in the supernatant were also determined as described by Bradford (1976). For inorganic phosphate standard curve, a series of $KH_2PO_4$ concentrations was used. ALP levels in gonads were expressed as μg phosphates/mg total proteins.

*Histological analyses*

Twenty gonads at each sampling time were fixed in aqueous Bouin's solution for 48 h.

After dehydration through increasing alcohol concentrations (from 70 to 100 %), tissues were embedded in paraffin. Thin sections (4 μm) were cut and stained with hematoxylin and eosin before observation with a light microscope (Nikon Eclipse 80i) coupled to an image acquisition (Nikon DXM1200-C) in order to determine sex and reproductive stage of animals. Five reproductive stages were described

taking into consideration the aspect of germinal cells as well as the evolution of extra-gonadic muscular fibers in the bivalve *R. decussatus* (Table 1) (Smaoui-Dammak et al. 2007).

### Statistical analyses

Data are reported as mean±standard deviation (SD), and statistical analysis was performed using SPSS software. Significant differences between groups were determined by an analysis of variance (one-way ANOVA) with the Tukey test for the post hoc muticomparison test. Significance was established at $p<0.05$. Pearson's correlation coefficients between MDA and metal levels were also measured.

## Results

### Treated wastewater quality

The treated wastewater sample was analyzed for heavy metals and endocrine active substances (Table 2). Results indicate that effluent quality was compliant with the Tunisian standards for the emission into the maritime public domain in terms of Cd, Cr, Cu, Ni, Pb, and Zn levels. Indeed, concentrations of selected metals were far below the permissible limits (NT106.02). Indeed, the effluents discharged from the Sfax sewage treatment plant to the gulf of Gabes did not pose any immediate risk to marine organisms at least for the analyzed metals. Furthermore, the screening of endocrine-disrupting chemicals in wastewater showed the presence of compounds acting as androgens and estrogens. In Tunisia, the current effluent standards for Urban Wastewater Treatment Plants still include the conventional parameters of BOD, COD, pH, suspended solids, nitrogen, phosphorus, total number of *E. coli*, metals, etc. No limits for dangerous compounds such as substances with hormone-disrupting capability exist.

**Table 1** Reproductive stages of clam *R. decussatus* according to Smaoui-Dammak et al.

| Description | Gonadic development |
|---|---|
| Sexual rest | Males: Gonads are empty. Abundant muscular fibers. |
| Start of gametogenesis | Females: Immature oogonies well defined are attached to the tubules walls. Muscular fibers are abundant. |
| | Males: Presence of numerous proliferating spermatogonia. Abundance of muscular tissue. |
| Development | Females: Gonadic tubules contain oogonies at different stages of maturity. Muscular tissue is less abundant. |
| | Males: Germinal cells from spermatogonia to spermatide were observed. |
| Maturity | Males: Densely packed bands of spermatozoa are observed. Muscular fibers absent. |
| Spawning | Males: gonadic tubules are more or less empty. Muscular fibers reappear among the gonadic space. |

From: Smaoui-Dammak et al. 2007)

**Table 2** Comparison of the average effluent quality to Tunisian standards NT106.02

| | Treated wastewater | Tunisian standards (NT106.02) |
|---|---|---|
| Cd (μg/L) | 1.9 | 5 |
| Cr (μg/L) | 12.6 | 500 |
| Cu (μg/L) | 201.4 | 1500 |
| Ni (μg/L) | 10 | 2000 |
| Pb (μg/L) | 124.2 | 500 |
| Zn (μg/L) | 265.1 | 10000 |
| Estrogenic-like substances (ng $E_2$eq/L) | 25.57±0.75 | – |
| Androgenic like substances (ng DHTeq/L) | 64.16±8.24 | – |

### Identification of endocrine-disrupting chemicals in treated effluent and *R. decussatus* tissues

In order to identify hormone-active substances, samples testing positive in the yeast assays were analyzed by LC-MS. Out of the 11 substances screened, only the nonylphenol was detected which was consistently determined as an estrogenic compound in the effluent sample and also in the clam gills.

The androgenic activity has been also measured in effluent and clam tissues; there are many unknown compounds that could contribute to the androgenic response in these compartments. However, the target compounds determined from chemical analysis could not identify these compounds' activities. Since the LC-MS analysis was selective in screening, there are potentially a multitude of other EDCs present within the effluent mixture which can individually contribute to the estrogenicity and androgenicity of tested samples.

### Effect of effluent exposure on biological indices

The clams appeared to be in good health during the 30 days of exposure. No mortality was recorded among clams either exposed or not exposed to effluent. For the GSI, no significant difference was observed between the experimental and control groups (Table 3). Nevertheless, exposure to effluent affected the CI of *R. decussatus* after 10 days of exposure. This result suggests that clams' growth may be negatively impaired after exposure to wastewater effluent.

**Table 3** Effect of treated effluent exposure on biological indices of clam *R. decussatus* (means±SD, $n$=15)

| Exposure duration (days) | | 0 | 10 | 20 | 30 |
|---|---|---|---|---|---|
| CI (%) | Control | 23±1.9 | 24.6±2 | 23.6±2.4 | 23.2±1.5 |
| | Exposed | | 18.9±1.5* | 19±2* | 19.6±1.4* |
| GSI (%) | Control | 10.6±2.7 | 9.9±1.4 | 9.1±1.6 | 9.8±1.5 |
| | Exposed | | 10.1±1.5 | 10.2±1.1 | 11±1.8 |

*Significant differences ($\alpha$=0.05)

## Accumulation and regulation of active compounds

Estrogenic and androgenic activities were determined in the gill tissues of *R. decussatus* (Figs. 1 and 2). Results indicate that estrogen content was not significantly affected during the exposure period. However, after 10 days of exposure, androgen-like substances were significantly increased in exposed clams and remained elevated reaching 20 ng DHT eq/g ww. The androgenic content in experimental groups was approximately 5 orders of magnitude higher than in the controls indicating that the animals are able to accumulate and concentrate these compounds from the water column to a large extent. This finding suggests that exposure to mixtures of natural and anthropogenic chemicals present in the effluent could be of concern for the aquatic organisms.

## Effect of effluent exposure on gametogenesis activity

Histological data of gonad development are illustrated in Fig. 3. In March, at the beginning of the study, 75 % of females were observed for being at the start of gametogenesis and 25 % of clams were already developing their gonads. After 30 days of the experiment, no significant modification of the process of gametogenesis has occurred for controls while 100 % of the exposed females were already in development stage. In the same way, all control males stayed at an early stage of gametogenesis during the 30 days of the experiment while the exposed clams reached the maturity 10 days after the beginning of the exposure and spawned at the end of the experiment. These results indicate that exposure to effluent readily affects gametogenesis activity in both sexes. This activation is more pronounced in males than in females.

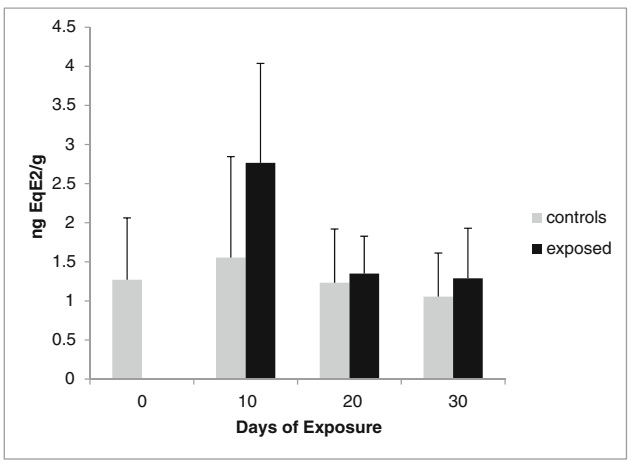


**Fig. 1** Estrogenic-like compounds levels determined in the gills of *R. decussatus* exposed to effluent for 30 days. Values are mean±SD

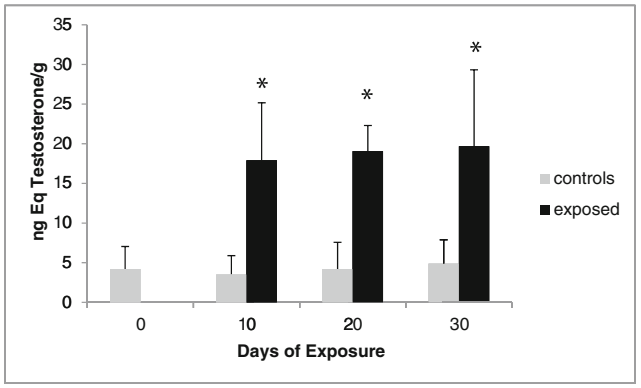


**Fig. 2** Androgenic-like compounds levels determined in the gills of *R. decussatus* exposed to effluent for 30 days. Values are mean±SD. *Significant differences ($\alpha$=0.05)

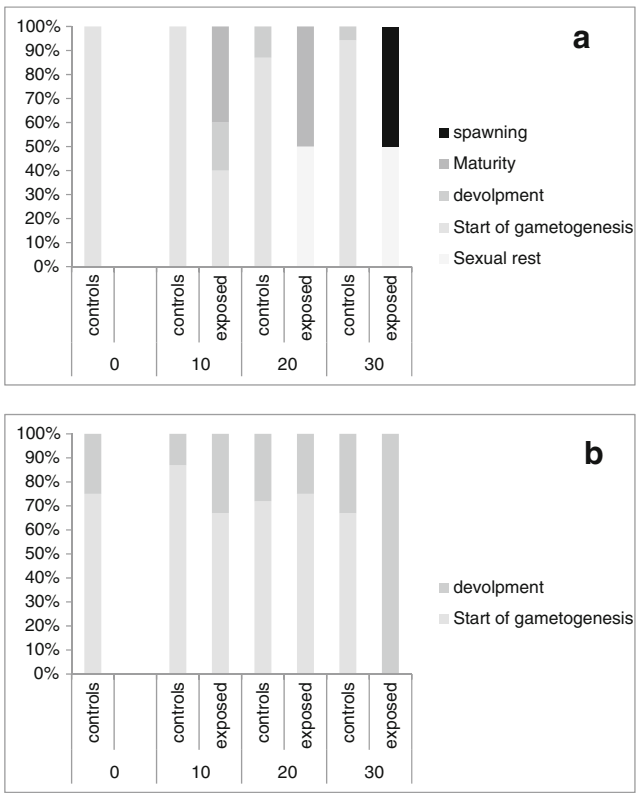


**Fig. 3** Proportion (%) of gametogenic stages of males (**a**) and females (**b**) *R. decussatus* exposed to treated sewage effluent

### Effect of effluent exposure on energy budget of *R. decussatus*

The evolution of glycogen (mg/g ww) and lipid (mg/g ww) levels in the gonad of clam *R. decussatus* are presented in Figs. 4 and 5. Significant decrease in glycogen levels was recorded in exposed males (Tukey, $P<0.05$); however, no significant variation was detected in females. It is also important to note a decrease in lipid concentrations in exposed male even if it is not statistically significant.

### Effect of effluent exposure on Vtg-like protein levels

The in vivo exposure of clams to municipal effluent yielded interesting effects. As expected, gonad Vtg-like protein levels increased significantly in exposed clams with respect to controls (Fig. 6). However, the Vtg response was more rapid in males compared to females. A significant increase was observed after 10 and 20 days of exposure respectively in exposed males and females before declining at the end of the experiment.

### Metals in *R. decussatus* exposed to wastewater effluents

Metal concentrations in the whole tissues of clams exposed to wastewater effluents are represented in Table 4. Following

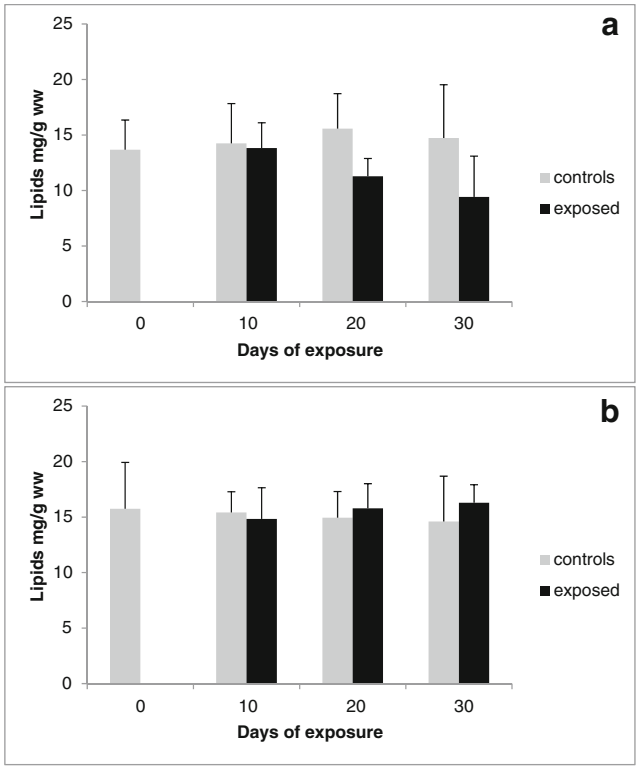


**Fig. 5** Effect of effluent exposure on lipid levels of clam *R. decussatus* (means±SD) expressed as mg/g ww (**a** males; **b** females). *Significant differences ($\alpha=0.05$)

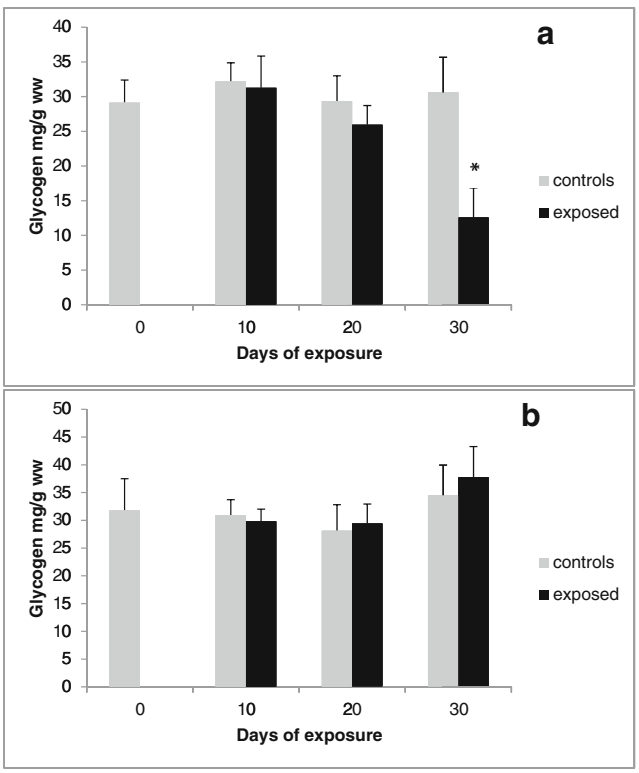


**Fig. 4** Effect of effluent exposure on glycogen levels of clam *R. decussatus* (means±SD) expressed as mg/g ww (**a** males; **b** females). *Significant differences ($\alpha=0.05$)

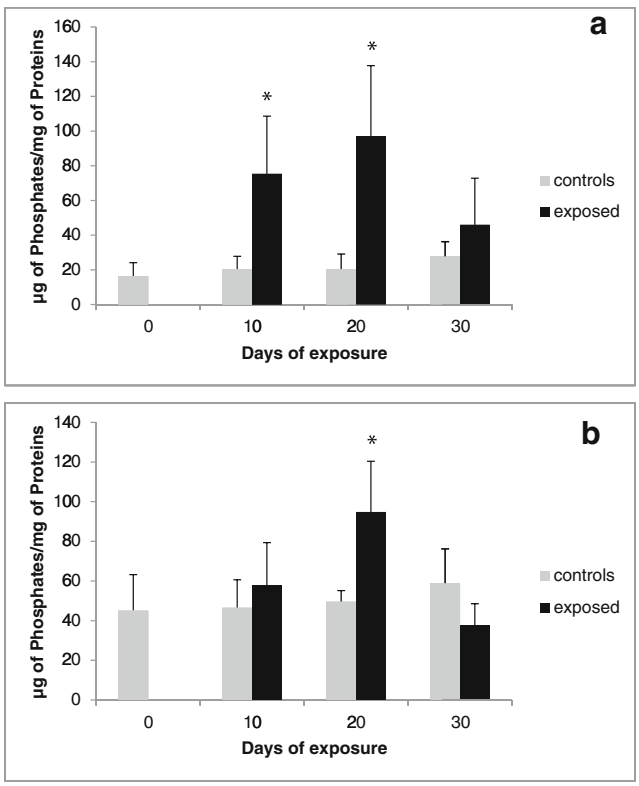


**Fig. 6** Effect of effluent exposure on vitellogenin-like protein levels in clam *R. decussatus* (means±SD) expressed as µg ALP/mg proteins (**a** males; **b** females). *Significant differences ($\alpha=0.05$)

**Table 4** Effect of effluent exposure on metal concentrations in whole tissues of *R. decussatus* (means±SD) expressed as μg/g DW

| Exposure duration (days) | | 0 | 10 | 20 | 30 |
|---|---|---|---|---|---|
| Cd | Control | 1.8±0.7 | 1.6±0.6 | 1.8±0.5 | 2±0.5 |
| | Exposed | | 2±0.6 | 2.8±0.5 | 2.6±0.5 |
| Ni | Control | 5.4±0.8 | 5±1 | 4.7±0.9 | 5±1.1 |
| | Exposed | | 12.3±6.4* | 11.6±6* | 12.8±4.3* |
| Zn | Control | 61.1±19.2 | 66.6±20.1 | 62±15 | 58.6±18.2 |
| | Exposed | | 99.7±31.3 | 108.4±20.7* | 130.5±27* |
| Pb | Control | 0.06±0.03 | 0.07±0.04 | 0.06±0.04 | 0.07±0.04 |
| | Exposed | | 0.16±0.09 | 0.21±0.1* | 0.28±0.1* |

*Cd* cadmium, *Ni* nickel, *Zn* zinc, *Pb* lead

*Significant differences (α=0.05)

exposure, the tissue cadmium concentration was increased and displayed no significant variation in time. On the contrary, exposure to effluent resulted in a significant increase of Ni, Zn, and Pb concentrations. After 30 days, metal levels in exposed clams were two times higher for Ni and Zn and four times higher for Pb compared to controls.

### Effect of effluent exposure on MDA levels and AChE activity

To assess toxic impact of treated effluent, the MDA content was determined in digestive gland of *R. decussatus* (Table 5). The MDA levels were significantly higher in exposed clams compared to controls and increased progressively in exposed throughout the experiment. At the end of the exposure, MDA concentrations in treated clams were approximately five times higher compared to the control group.

Results show also that acetylcholinesterase activity was not significantly affected during the exposure period (Table 5) whereas we notice a tendency to a decrease in the enzymatic activity compared to the control group.

Correlation between malondialdehyde levels and metal content are listed in Table 6. A statistically significant and positive relationship was revealed ($p<0.01$).

## Discussion

Sewage treatment plants are a major source of EDCs in aquatic environments (Metcalfe et al. 2001; Gauthier-Clerc et al. 2002). Indeed, municipal effluents contain a variety of natural and synthetic pollutants including heavy metals, polycyclic aromatic hydrocarbons, pesticides, health-related medicines, and industrial by-products. By disrupting the endocrine system, these substances can ultimately alter various processes such as the production, use and storage of energy and, more broadly, regulation of metabolism and development. Exposure to these substances via sewage effluent may also have other toxic effects, including reproductive damage, and may interfere with fertility or disrupt the development of the aquatic organisms (Blaise et al. 2003; Chesman and Langston 2006; Gagné et al. 2011). As municipal effluents are a potential health and environmental hazard, sensitive analyses for the detection of chemical-induced reprotoxicity and other biological impacts originating from treated effluents are required. Moreover, the biological significance of the interaction of chemicals in the effluent can only be detected using biomarkers. In this context and in order to report the ecological risk of municipal effluents discharges into the gulf of Gabes which is considered as one of the most productive area in Tunisia, specimens of clam *R. decussatus* were collected from a relatively uncontaminated site and were in vivo exposed to treated effluent from Sfax municipal plant for 30 days. This study was designed to better characterize the response of clam *R decussatus* to municipal effluent and to investigate whether the interaction of chemicals are able to disrupt the endocrine system of clams.

To this end, androgenic and estrogenic activities were measured in the gills to assess potential accumulation and regulation of active compounds. Following exposure, the level of

**Table 5** Effect of effluent exposure on malondialdehyde levels and acetylcholinesterase activities (means±SD)

| Exposure duration (days) | | 0 | 10 | 20 | 30 |
|---|---|---|---|---|---|
| MDA (μmol/g ww) | Control | 27.7±9.7 | 36.4±12.3 | 31.6±9.9 | 39.7±11.4 |
| | Exposed | | 69.3±14.8* | 83.7±25.3* | 146.4±24.8*[c] |
| AChE activity (nmol/min/mg P) | Control | 36.9±9.2 | 43.9±19.8 | 40.2±25 | 40.1±23.1 |
| | Exposed | | 41.6±13.4 | 37.1±20 | 31±10.6 |

*Significant differences (α=0.05); **significant differences (α=0.01)

**Table 6** Pearson's correlation coefficient between metal concentrations and MDA levels in the clam *R. decussatus*

| | Cd | Zn | Ni | Pb |
|---|---|---|---|---|
| MDA | 0.261** | 0.707** | 0.507** | 0.637** |

**Significant differences ($\alpha$=0.01)

free androgenic-like substances was increased up to fivefold compared to controls and remained elevated, indicating that the animals are able to accumulate these chemicals from the effluent to a large extent. Nonetheless, exposed clams are able to maintain gill concentrations of estrogen-like substances unaltered. It is well-known that bivalves are able to rapidly metabolize steroids and thus maintain their free forms to low concentrations possibly via homeostatic mechanisms such as the conjugation with fatty acid or the formation of sulfate conjugates (Lavado et al. 2006; Labadie et al. 2007). Sulfatation of steroids may inhibit their biological activity and might regulate levels of active steroids within target tissues (Janer et al. 2005) by decreasing their affinity for steroid receptors and increasing their rate of elimination (Strott 1996). Fatty acid esterification was also recognized as a major biotransformation pathway for sex steroids in mollusks. Indeed, esterification increases significantly the lipophilicity of steroids, so they can be stored in the lipoidal matrices of the organism and their bioactivity and bioavailability will be reduced (Borg et al. 1995). Biotransformation of testosterone and estradiol to fatty acid conjugates has been reported for several species of bivalves such as *C. virginica* and *M galloprovincialis* (Janer et al. 2005). Therefore, an alteration of these activities might have consequence in terms of endogenous levels of free steroids. Our results indicate that exposure to municipal effluent has a significant effect on androgen-like substances levels. Alternative mechanisms such as the inhibition of conjugation and excretion of testosterone and or the inhibition of aromatase activity could explain the buildup of androgenic activity detected in exposed clams. Some endocrine disruptor compounds such as organotin could act by competitive inhibition of aromatase activity in mollusks (Spooner et al. 1991; Bettin et al. 1996). Moreover, the presence of nonylphenol in the effluent could have a significant effect on testosterone level in exposed clams. Indeed an increase in testosterone production following exposure to alkylphenols has been described in vertebrate (Murono et al. 2001) and in invertebrate such as Zebra mussels (*Dreissena polymorpha*) (Quinn et al. 2004).

Exposed clams to treated sewage effluent revealed significant induction of Vtg in both sexes in comparison to controls suggesting that endocrine disruption had occurred. The first question that comes to mind: how Vtg has been synthesized despite the fact that estrogenic activity was not significantly affected during the exposure period? Three hypotheses may be advanced: firstly, that the induction has been promoted before transformation of estrogen to its inactive form. Secondly, the Vtg synthesis may be related to the stress effect caused by exposure to the effluent or it is a simple reaction to the increase in gametogenesis activity. The Vtg gene in male, normally silent, can be induced by estrogen exposure (Flouriot et al. 1995). Thus, Vtg production in male *R. decussatus* could be proposed as a useful indicator of exposure to exogenous estrogens or estrogen mimics in the aquatic environment. These finding provide a strong indication that municipal effluents contain bioavailable xenoestrogens at levels sufficient to induce Vtg in clam *R. decussatus*. Nonylphenol present in effluent and detected in clam tissues is able to mimic the action of endogenous estrogens by binding to estrogen receptors (Madigou et al. 2001). However, the Vtg response was more rapid in males compared to females. A significant increase was observed after 10 and 20 days of exposure respectively in exposed males and females. Similar results have been reported in other bivalves such as manila clam (*Tapes philippinarum*) exposed to nonylphenol (Matozzo and Marin 2005), and male Zebra mussels *Dreissena polymorpha* exposed to sewage treatment work effluents (Quinn et al. 2004). In the clam *T. philippinarum* exposed to nonylphenol, Vtg-like proteins increased significantly in both hemolymph and digestive gland of male whereas no change were observed in those of female (Matozzo and Marin 2005). These results were explained by the estrogenic effect of nonylphenol (NP) which could have a particular affinity to male. Likewise, mussels exposed to PAHs significantly increased Vn-like proteins in males but not in females (Ortiz-Zarragoitia and Cajaraville 2005). In another study, males of *Mytilus galloprovincialis* collected from Venice lagoon were more susceptible to xenoestrogens than females, showing significantly higher Vtg-like protein levels in hemolymph (Pampanin et al. 2005). However, Vtg induction in male should be carefully analyzed. Indeed, Shi and collaborators (2005) revealed that Vtg could be induced in male gastropods challenged by bacteria. Male challenged with *E. coli* produced Vtg, giving a functional role of these proteins in immune reactions (Zhang et al. 2005). Some proteins involved in internal defense reactions were shown to belong to the Vtg family, thus justifying their presence not only in females but also in male of many invertebrate species (Zhang et al. 2005; Shi et al. 2006). These findings limit the use of Vtg as a specific biomarker of exposure to estrogenic compounds only. Its role may extend beyond these functions.

It was further shown that exposure to effluent had a sex-specific effect on clam's reproduction. Indeed, gametogenesis was markedly activated in exposed males as compared to the females. Male reached the maturity 10 days after the beginning of the exposure and spawned at the end of the experiment while female gonadal development remained unaffected compared to control. These effects could be associated to the

pattern of accumulation of androgenic and estrogenic compounds by *R. decussatus*. Moreover, the accelerated ripeness observed for *R. decussatus* is in agreement with the results of previous works showing that injection of estradiol, testosterone, and progesterone stimulate spermatogenesis in the scallop *Mizuhopecten yessoensis* (Varaksina and Varaksin 1991; Varaksina et al. 1992). In fact, increased gametogenesis activity in male may disrupt spawning synchronization and population maintenance in the clam *R. decussatus*. Such asynchronism in spawning between sexes has been observed in contaminated site, and it has been argued that it could affect recruitment (Smaoui-Damak et al. 2006).

What is best known is that reproduction is fully subsidized from the energy reserves. Several studies showed that exposure to chemical pollutants present in the sewage treatment effluents may impact energy balance of bivalves as a result of increased maintenance coast (De Coen and Janssen 2003; Smolders et al. 2004; Voets et al. 2006). Energy reserves are considered as biomarkers to reflect sublethal changes resulting from a stressful xenobiotic exposure (Lagadic et al. 1994). The range of the energy depletion would reflect the strength of the stress (Reddy et al. 1986; Rajalakshmi and Mohandas 1993). In the present study, the significant decrease in the glycogen levels recorded in the exposed male should be related to the increase in gametogenesis activity (maturation and gamete emission). Glycogen and lipids are important nutriment storage, usually used as energy sources during gametogenesis. Kwan et al. (2003) showed that in the zebra mussel *Dreissena polymorpha*, the level of glycogen decreased concurrently with gamete maturation and lipids decreased with gamete emission. Furthermore, the energy required to support vitellogenesis by endogenous or exogenous inputs of estrogenic contaminants could represent another major expenditure for bivalves, thus contributing to the increase in energy demands (Smolders et al. 2004).

As we said before, municipal effluents are recognized as major sources of pollution, releasing into receiving waters a complex of array of chemicals including metals. Present study shows that metals are present in municipal effluents and did not pose any immediate risk to marine organisms at least for the analyzed metals (Cd, Ni, Zn, and Pb). Following exposure, metal levels in exposed clams were two times higher for Ni and Zn and four times higher for Pb as compared to their controls. An increase in cadmium concentrations (not statistically significant) was also recorded. The metals, present in their available forms in tested effluent, were taken up and bioaccumulate by clams. Cadmium is known to have endocrine-disrupting effect in clams *R. decussatus* (Ketata et al. 2007) and in other species of bivalves (Henson and Chedrese 2004; Rodríguez et al. 2007). Potential mechanisms by which cadmium may acts as an EDC are its interference with DNA binding Zn-finger motif though the substitution of Cd for Zn or by mimicking or inhibiting the action of endogenous estrogens (Henson and Chedrese 2004). Hwang and collaborators (2010) showed that cadmium caused significant Vtg induction in copepods. Similar result was observed in female crab (*Carcinus maenas*) exposed to sediment spiked with Hg, Pb, and Zn (Martín-Díaz et al. 2008). Other studies reported the ability of certain metal ions such as Cd, Hg, Pb, and Ni to bind to estrogen receptors and to give rise to estrogen agonist responses in vitro and in vivo. Metal with androgenic activity have been also described. Ju et al. (2009) showed that exposure to zinc could skew the sex ratio towards predominance of males in *Gomphina veneriformis* (Bivalvia, Veneridae) and promote male maturity compared to female.

The biomarkers of stress such as MDA and cholinesterase activity (AChE) were also studied to assess the neurotoxic potential of treated effluent. The significant increase of malondialdehyde concentrations implies that clams have been exposed to an oxidative stress, probably due to the presence of high metal concentration in sewage effluent. Several studies have shown a lipid peroxidation increase under metal contamination in mollusks (Geret et al. 2002; Company et al. 2004; Machreki-Ajmi and Hamza-Chaffai 2008). Chang et al. (1996) showed that metals such Cd, Ni, and Pb are toxic in aquatic organisms mainly because of the oxidative potential. Nonylphenol present in treated effluent could also explain the increase of MDA levels in exposed clams. Indeed, Okai and collaborators (2004) and Gong and Han (2006) have indicated that exposure to such chemical compound is able to produce oxidative damage including enhancing reactive oxygen species (ROS). Several studies demonstrated usefulness of measurement of AChE activity in evaluating the effects of exposure to neurotoxic compounds in aquatic organisms. In the present study, no significant variation was recorded for acetylcholinesterase activity, indicating that neurotoxic chemicals in effluent are not present at sufficient level to decrease AChE activity in exposed clams. As already reported, the CI indicates the general physiological status of the animals. A decrease in CI is generally related to stress in organisms (Widdows 1985). A significant decrease in condition index of the exposed clams was observed indicating that effluent exposure was detrimental to the clams.

## Conclusion

In conclusion, the physiological status shows clear signs of disruption in clam *R. decussatus* influenced by contaminants present in the Sfax sewage treatment effluent. This study provides that environmentally relevant levels of endocrine disrupting compounds present in the treated effluent were able to alter the steroid metabolism and reproduction of clam *R. decussatus*. The biomarker data revealed a remarkable disruption in the gametogenesis activity as evidence the increase

in the gametogenesis activity and Vtg-like proteins levels in male. The increase of Vtg-like proteins in exposed males indicates that exposure to pollutant which are present in the treatment sewage effluent could change the reproductive physiology in the clam *R. decussatus*. Exposure to municipal effluent led also to increase metal concentrations in clams (Cd, Cu, Ni, and Zn) which could act as endocrine disruptors. Thus, endocrine-disrupting effects observed in clam *R. decussatus* result, in fact, from the complex interactions of different chemical pollutants present in the treatment sewage effluent. This study provides additional information regarding the toxic effect of Sfax municipal effluent in the clam *R. decussatus.* The results of this study can explain in part the weakening situation of the gulf of Gabes affected by natural and anthropogenic activities. An asynchronism in reproduction between sexes was observed in some areas and a decline of stock was also reported (Zamouri-Langar et al. 2006; Smaoui-Damak et al. 2007).

## References


Abidli S, Santos MM, Lahbib Y, Costa Castro LF, Reis-Henriques MA, Trigui El Menif N (2012) Tributyltin (TBT) effects on Hexaplex trunculus and Bolinus brandaris (Gastropoda: Muricidae): Imposex induction and Sex hormone levels insights. Ecological Indicators 13:13–21

Aerni HR, Kobler B, Rutishauser BV, Wettstein FE, Fischer R, Giger W, Hungerbuhler A, Marazuela MD, Peter A, Schonenberger R, Vogeli AC, Suter MJF, Eggen RIL (2004) Combined biological and chemical assessment of estrogenic activities in wastewater treatment plants. Anal Bioanal Chem 378:688–696

Barber LB, Lee KE, Swackhamer DL, Schoenfuss HL (2007) Reproductive responses of male fathead minnows exposed to wastewater treatment plant effluent, effluent treated with XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds. Aquat Toxicol 82:36–47

Bateman KS, Stentiford GD, Feist SW (2004) A ranking system for the evaluation of intersex condition in European Flounder (Platichthys flesus). Environ Toxicol Chem 23:2831–2836

Bettin C, Oehlmann J, Stroben E (1996) TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helogander Meeresunters 50:299–317

Blaise C, Gagné F, Salazar M, Salazar S, Trottier S, Hansen PD (2003) Experimentally-induced feminization of freshwater mussels after a long- term exposure to a municipal effluent. Fresenius Environmental Bulletin 12:865–870

Blankvoort BMG, Rodenburg RJT, Murk AJ, Koeman JH, Schilt R, Arts J (2005) Androgenic activity in surface water samples detected using the AR-LUX assay: indications for mixture effects. Environmental Toxicology and Pharmacology 19(2):263–272

Bocquené G, Galgani F (1998) Biological effect contaminants: cholinesterase inhibition by organophosphate and carbamate compounds. ICES Tech Mar Environ Sci 22:1–12

Borg W, Shackleton CH, Pahuja SL, Hochberg RB (1995) Long-lived testosterone esters in the rat. Proc Natl Acad Sci USA 92(5):1545–1549

Bradford MM (1976) A dye binding assay for protein. Anal Biochem 72: 248–254

Burkhardt-Holm P (2010) Endocrine disruptors and water quality: A state-of-the-art review. Water Resour Dev 26(3):477–493

Chang LW, Magos L, Suzyki T (1996) Toxicology of Metals. CRC Lewis, Boca Raton, 1232 pages

Chesman BS, Langston WJ (2006) Intersex in the clam Scrobicularia plana: a sign of endocrine disruption in estuaries? Biol Lett 2:420–422

Colborn T, Vom Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384

Company R, Serafim A, Bebianno MJ, Cosson R, Shillito B, Fiala-médini A (2004) Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymdiolus azoricus*. Mar Environ Res 58:377–381

De Coen WM, Janssen CR (2003) The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed Daphnia magna and corresponding population characteristics. Environ Toxicol Chem 22(7):1632–41

Ellman GL, Courtney KD, Andres VJ, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Falconer IR, Chapman HF, Moore MR, Ranmuthu G (2006) Endocrine-disrupting compounds: A review of their challenge to sustainable and safe water supply and water reuse. EnvironToxicol 21:181–191

Flouriot G, Pakdel F, DucouretB VY (1995) Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. Journal of Molecular Endocrinology 15:143–151

Frings CS, Fendly TW, Dunn RT, Queen CA (1972) Improved determination of total serum lipid by sulpho-phosphovanillin reaction. Clin Chem 18:673–674

Gagné F, Blaise C, Aoyama I, Luo R, Gagnon C, Couillard Y, Campbell P, Salazar M (2002) Biomarker study of a municipal effluent dispersion plume in two species of freshwater mussels. Environmental Toxicology 17(3):149–159

Gagné F, Blaise C, Pellerin J, Pelletier E, Douville M, Gauthier-Clerc S, Viglino L (2003) Sex alteration in soft-shell clams (Mya arenaria) in an intertidal zone of the Saint Lawrence River (Quebec, Canada). Comp Biochem Physiol Part C 134:189–198

Gagné F, Bouchard B, André C, Farcy E, Fournier M (2011) Evidence of feminization in wild Elliptio complanata mussels in the receiving waters downstream of a municipal effluent outfall. Comp Biochem Physiol C 153(1):99–106

Gauthier-Clerc S, Pellerin J, Blaise C, Gangé F (2002) Delayed gametogenesis of Mya arenaria in the Saguenay fjord (Canada): A consequence of endocrine disruptors? Comp Biochem Physiol Part C131: 457–467

Geffard A, Sartelet H, Garric J, Biagianti-Risbourg S, Delahaut L, Geffard O (2010) Subcellular compartmentalization of cadmium, nickel and lead in *Gammarus fossarum*: comparison of methods. Chemosphere 78:822–829

Geret F, Serafim A, Barreira L, Bebianno MJ (2002) Effect of cadmium on antioxident enzyme and lipid peroxidation in the gills of the clam (*Ruditapes decassatus*). Biomarkers 7:242–256

Gong Y, Han XD (2006) Nonylphenol- induced oxidative stress and cytotoxicity in testicular sertoli cells. Reprod Toxicol 22:623–630

Grant A, Tyler PA (1983) The analysis of data in studies of invertebrate reproduction. I. Introduction and statistical analysis of gonad indices and maturity indices. Int J Invert Reprod 6:259–269

Gravato C, Santos MA (2003) *Dicentrarchus labrax* biotransformation and genotoxicity responses after exposure to a secondary treated industrial/urban effluent. Ecotoxicol Environ Saf 55:300–306

Gross MY, Naycock DS, Thorndyke MC, Morritt D, Crane M (2001) Abnormalities in sexual development of the amphipod Gammarus pulex (L.) found below sewage treatment works. Environmental Toxicology and Chemistry 20:1792–1797

Guillette LJ, Craine A (2000) Environmental Endocrine Disruptors. Taylor & Francis, New York

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N (1997) Estrogenic Activity in Five United Kingdom Rivers Detected by Measurement of Vitellogenesis in Caged Male Trout. EnvironToxicol Chem 16:534–542

Hemming JM, Allen HJ, Thuesen KA, Turner PK, Waller WT, Lazorchak JM, Lattier D, Chow M, Denslow N, Venables B (2004) Temporal and Spatial Variability in the Estrogenicity of a Municipal Wastewater Effluent. Ecotoxicology and Environmental Safety 57(3):303–310

Henson MC, Chedrese PJ (2004) Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. Exp Biol Med 229:383–392

Horrigan L, Lawrence RS, Walker P (2002) How sustainable agriculture can address the environmental and human heath harms of industrial agriculture. Environ Health Perspect 110(5):445–456

Hwang DS, Lee KW, Han J, Park HG, Lee J, Lee YM, Lee JS (2010) Molecular characterization and expression of vitellogenin (Vg) genes from the cyclopoid copepod, Paracyclopina nana exposed to heavy metals. Comp Bioch Physio C 151:360–368

Janer G, Msia-Vela S, Le Blanc GA, Porte C, Kauffman FC (2005) Esterification of vertebrate-type steroids in the Eastern oyster (Crassostrea virginica). Steroids 69:129–136

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP (1998) Widespread sexual disruption in wild fish. Environ Sci Technol 32:2498–2506

Johnson AC, Aerni HR, Gerritsen A, Gibert M, Giger W, Hylland K, Jurgens M, Nakari T, Pickering A, Suter MJF, Svenson A, Wettstein FE (2005) Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Res 39:47–58

Ju SM, Park JJ, Lee JS (2009) Induction of Intersex and Masculinization of the Equilateral Venus, Gomphina veneriformis (Bivalvia: Veneridae) by Zinc. Animal Cells and Systems 13(3):339–344

Katsiadaki I, Scott AP, Mayer I (2002) The potential of the three-spined stickleback (Gasterosteus aculeatusL.) as a combined biomarker for oestrogens and androgens in European waters. Marine Environmental Research 54:725–728

Kessabi K, Annabi A, Ibn Hadj Hassine A, Bazin I, Mnif W, Said K, Messaoud I (2013) Possible chemical causes of skeletal deformities in natural populations of Aphanius fasciatus collected from the Tunisian coast. Chemosphere 90:2683–2689

Ketata I, Smaoui-Damak W, Guermazi F, Rebai T, Hamza-Chaffai A (2007) In situ endocrine disrupting effects of cadmium on the reproduction of Ruditapes decussatus. Comp Biochem Physiol 146C: 415–430

Kwan KHM, Chan HM, De Lafontaine Y (2003) Metal contamination in zebra mussels (Dreissena polymorpha) along the St. Lawrence River. Environ Monit Assess 88:193–219

Labadie P, Peck M, Minier C, Hill EM (2007) Identification of the steroid fatty acid ester conjugates formed in vivo in Mytilus edulis as a result of exposure to estrogens. Steroids 72:41–49

Lagadic L, Caquet T, Ramade F (1994) The role of biomarkers in environmental assessment (5). Invertebrate populations and communities Ecotoxicol 3:193–208

Lahbib Y, Abidli S, Chiffoleau JF, Averty B, Trigui El Menif N (2010) Imposex and butyltin concentrations in snails from the lagoon of Bizerta (Northern Tunisia). Marine Biology Research 6:600–607

Lavado R, Sugni M, Carnevali MDC, Porte C (2006) Triphenyltin alters androgen metabolism in the sea urchin Paracentrotus lividus. Aqua Tox 79:247–256

Lobel PB, Bajdik CD, Belkhode SP, Jackson SE, Longerich HP (1991) Improved protocol for collecting mussel watch specimens taking into account sex, size, condition, shell shape and chronological age. Arch Environ Contam Toxicol 21:409–414

Louiz I, Kinani S, Gouze ME, Ben-Attia M, Menif D, Bouchonnet S, Porcher JM, Ben-Hassine OK, Aït-Aïssa S (2008) Monitoring of dioxin-like, estrogenic and anti-androgenic activities in sediments of the Bizerta lagoon (Tunisia) by means of in vitro cell-based bioassays: Contribution of low concentrations of polynuclear aromatic hydrocarbons (PAHs). Sc tot env 402:318–329

Machreki-Ajmi M, Hamza-Chaffai A (2008) The effect of in situ cadmium contaminations on some biomarkers in *Cerastoderma glaucum*. Ecotxicology 17:1–11

Madigou T, Le Goff P, Salbert G, Cravedi JP, Segner H, Pakdel F, Valotaire Y (2001) Effects of nonylphenol on estrogen receptor conformation, transcriptional activity and sexual reversion in rainbow trout (Oncorhynchus mykiss). Aquatic Toxicol 53:173–186

Martín-Díaz ML, Blasco J, Sales D, DelValls TA (2008) Field validation of a battery of biomarkers to assess sediment quality in Spanish ports. Environ Pollut 151:631–640

Matozzo V, Marin MG (2005) Can 4-nonylphenol induce vitellogenin-like proteins in the clam Tapes phillipinarum? Environ Res 97:43–49

Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T (2001) Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with japanese medaka (Oryzias latipes). Env Toxicol Chem 20:297–308

Milla S, Depiereux S, Kestemont P (2011) The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology 20:305–319

Mnif W, Zidi I, Ibn Hadj Hassine A, Gomez E, Bartegi A, RoigB BP (2012) Monitoring Endocrine Disrupter Compounds in the Tunisian Hamdoun River using *In Vitro* Bioassays. Soil and Sediment Contamination 21:815–830

Moore CG, Stevenson JM (1994) Intersexuality in benthic harpacticoid copepods in the Firth of Forth, Scotland. J Nat History 28:1213–1230

Murono EP, Derk RC, De León JH (2001) Differential effects of octylphenol, 17[beta]-estradiol, endosulfan, or bisphenol A on the steroidogenic competence of cultured adult rat Leydig cells. Reproductive Toxicology 15(5):551–560

Okai Y, Sato EF, Higashi-Okai K, Inoue M (2004) Enhancing effect of the endocrine disruptor para-nonylphenol on thegeneration of reactive oxygen species in human blood neutrophils. Environ Health Perspect 112:553–556

Ortiz-Zarragoitia M, Cajaraville MP (2005) Effects of selected xenoestrogens on liver peroxisomes, vitellogenin levels and spermatogenic cell proliferation in male zebrafish. Comp Biochem Physiol C 141:133–144

Pampanin DM, Maragon I, Volpato E, Campesan G, Nasci C (2005) Stress biomarkers and alkali-labile phosphate level in mussels (Mytilus galloprovincialis) collected in the urban area of Venice (Venice Lagoon, Italy). Environ Pollut 136:103–107

Parks LG, Lambright CS, Orlando EF, Guillette LG, Andley GT, Gray LE (2001) Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. Toxicol Sci 62(2):257–267

Parrot JL, McMaster ME, Hewitt LM (2006) A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: development and application of fish bioassays. J Toxicol Environ Health B Crit Rev 9:297–317

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP (1994) Estrogenic effects of effluents from sewage treatment works. Chem Ecol 8:275–285

Quinn B, Gagné F, Costello M, McKenzie C, Wilson J, Mothersill C (2004) The endocrine disrupting effect of municipal effluent on the zebra mussel (Dreissena polymorpha). Aquat Toxicol 66: 279–292

Rajalakshmi P, Mohandas A (1993) Effect of heavy metals on tissue glycogen levels in the freshwater mussel, lamellidens corrianus (Lea). Proc 2nd European Con Ecotoxicology. Science of the total environment 134:617–630

Reddy JK, Reddy MK, Usman MI, Lalwani ND, Rao MS (1986) Comparison of hepatic peroxisome prolifer ative effect and its implication for hepatocarcinogenicity of phthalate esters, di(2-ethylhexyl)phthalate, and di(2-ethylhexyl) adipate with a hypolipidemic drug. Environ Health Perspect 65:317–27

Rodríguez EM, Medesani DA, Fingerman M (2007) Endocrine disruption in crustaceans due to pollutants: a review. Comp Biochem Physiol Part A Mol Integr Physiol 146:661–671

Routledge EJ, Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15:241–248

Salo HM, Hébert N, Dautremepuits C, Cejka P, Cyr DG, Fournier M (2007) Effects of Montreal municipal sewage effluents on immune responses of juvenile female rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 84(4):406–414

Sasaki T, Matsy S, Sorae A (1972) Effect of acetic acid concentration on the colour reaction in the O-toluidine boric acid method for blood glucose estimation. Rinsho Kagarku 1:346–353

Schultz MM, Furlong ET, Kolpin DW, Werner SL, Schoenfuss HL, Barber Blazer VS, Norris DO, Vajda AM (2010) Antidepressant pharmaceuticals in twoU.S.effluent-impactedstreams: occurrenceand fate in water and sediment, and selective uptake in fish neural tissue. Environ Sci Technol 44:1918–1925

Shi HH, Huang CJ, Zhu SX, Yu XJ, Xie WY (2005) Generalized system of imposex and reproductive failure in female gastropods of coastal waters of mainland China. Mar Ecol Prog Ser 304:179–189

Shi XD, Zhang SC, Pang QX (2006) Vitellogenin is a novel player in defense reactions. Fish Shellfish Immunol 20:769–772

Smaoui-Damak W, Mathieu M, Rebai T, Hamza-Chaffai A (2007) Histology of the reproductive tissue of the clam Ruditapes decussates. Inverterbrate Reproduction and development 50(3):117–126

Smaoui-DamakW RT, Berthet B, Hamza-chaffai A (2006) Does cadmium pollution affect reproduction in the clam Ruditapes decussates A one-year case study. Comparative Biochemistry and Physiology 143:252–261

Smolders R, Bervoets L, De Coen W, Blust R (2004) Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ Pollut 129:99–112

Spooner N, Gibbs PE, Bryan GW, Goad LJ (1991) The effect of tributyl tin upon steroid titres in the female dogwhelk, Nucella lapillus and the development of imposex. Marine Environmental Research 32:37–50

Stanton MG (1968) Colorimetric determination of inorganic phosphate in the presence of biological material and adenosine triphosphate. Anal Biochem 22:27–34

Strott CA (1996) Steroid sulfotransferases. Endocrine Reviews 17:670–697

Sumpter JP (1998) Xenoendocrine disrupters-environmental impacts. Toxicol Lett 102–103:337–342

Sumpter JP, Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103:173–178

Sunderman FW Jr, Marzouk FW, Hopfer SM (1985) Increased lipid peroxidation in tissues of nickel chloride-treated rats. Annals of Clinical and Laboratory Science 15(3):229–236

Svenson A, Allard AS (2004) Occurrence and some properties of the androgenic activity in municipal sewage effluents. J Environ Sci Health A Tox Hazard Subst Environ Eng 39:693–701

Thornton J, McCally M, Orris P (1996) Hospital and plastics. Public Health Reports 11:298–313

Tilton F, Benson WH, Schlenk D (2002) Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. Aquatic Toxicology 61:211–224

Tyler CR, Jobling S, Sumpter JP (1998) Endocrine disruption in wildlife: A critical review of the evidence. Crit Rev Toxicol 28:319–361

Vajda AM, Lopez EM, Woodling JD, Barber LB, Norris DO (2008) Reproductive disruption of fish by an estrogenic wastewater effluent. Environ Sci Technol 42:3407–3414

Varaksina GS, Varaksin AA (1991) Effects of estradiol, progesterone, and testosterone on oogenesis of yezo scallop. Biol Morya 3:61–68

Varaksina GS, Varaksin AA, Maslennikova LA (1992) The role of gonadal steroid hormones in the spermatogenesis of the scallop Mizuhopecten yessoensis. Biol Morya Mar Biol 3:77–83

Voets J, Talloen W, De Tender T, Van Dongen S, Covaci A, Blust R, Bervoets L (2006) Microcontaminant accumulation, physiological condition and bilateral asymmetry in zebra mussels (Dreissena polymorpha) from clean and contaminated surface waters. Aquatic Toxicology 79:213–225

Widdows J (1985) Physiological measurements, and physiological procedures. In: Bayne BL (ed) The effects of stress and pollution on marine animals. Praeger Publishers, New York, pp 3–45, 161–178

Zamouri-Langar N, Chouba L, Jarboui O, Mrabet R (2006) Présence du bivalve veneridae Ruditapes philippinarum (Adams & Reeve, 1850) sur le littoral sud de la Tunisie. Bull Inst Natn Scien Tech Mer de Salammbô 33:23–27

Zhang SC, Sun YN, Pang QX, Shi XD (2005) Hemagglutinating and antibacterial activities of vitellogenin. Fish Shellfish Immunol 19:93–95