Deleterious effects of estrogenic endocrine disruptors on marine organisms: Histological Observed Effects and Some Novel Useful Monitoring Bioassays

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Abstract
Aquatic environments receive significant levels of chemical contaminants generated by human activities. Among these pollutants, we noticed the xenobiotics known as reproductive toxicants and endocrine disruptors. The endocrine disruption in wildlife has been the subject of many reviews and workshops in recent years. Field observations of reproductively abnormal organisms and population declines in polluted sites stimulated major research efforts to understand links between environmental pollution and health problems. Laboratory studies evidenced that many compounds can interfere with the synthesis, secretion, transport, metabolism, mechanism of action or clearance of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes. These chemicals are thus defined as Endocrine Disrupting Compounds (EDC), and a list of more than 500 known or suspected EDC has been established by the European Community, such as numerous pesticides, industrial chemicals, and commercial products that have been released into the environment. Of particular importance are those that mimic estrogens and androgens (and their antagonists), because of their central role in reproductive function. Estrogens are substances both natural and synthetic that mimic the effect of the female estrogenic hormone in the body and impart estrogenic activity. Because of this effect, they potentially can disrupt the endocrine system in the exposed aquatic species. Human wastes are a major source of estrogens in the environment, too. These wastes are treated in wastewater treatment plants where some of the estrogens are removed, and the rest is discharged in the effluent. Other sources of estrogenic compounds include birth control pills and chemicals like detergents. It is important to be able to reduce their concentrations and it would be ideal if this can be achieved using available existing treatment processes. Both natural and synthetic estrogens released in the marine environment by the wastewater treatment plants are suspected to interfere with the exposed endocrine systems of aquatic species. In fact, they mimic the effect of the endogenous hormone and therefore, can disrupt the endocrine systems of exposed species and the reproductive systems of aquatic fauna. To understand their environmental fate, the estrogenic activity was studied by using the Yeast Estrogenic Screening (YES) bioassay. This bioassay has been validated in the detection of a wide range of estrogenic receptor agonists. The present work is based on in situ studies. Different compartments were used: the effluents of a wastewater treatment plant, the sea water, the sediment and the clam Ruditapes decussatus. A reverse phase HPLC method was used to identify the nature of estrogenic components. Some observed histological results showing hermaphroditic cases and parasites are also discussed in this paper.

Keywords: Pollution, Endocrine disruption, Xenoestrogens, Estrogens, HPLC, YES essays, Hermaphroditism, Parasites
1. Introduction

There is a growing concern about the impact of pollution on wildlife and human health. The variety of synthetic compounds released in the environment has risen during last decades. Classical toxic effects are widely studied and international regulations concerning the production and use of man-made chemicals are continuously evolving. They are currently based on the individual effects of molecules. The next great challenge is the integration of interactive effects occurring in a context of complex mixture of contaminants. Already ambitious, this objective is complicated by the existence of harmful effects caused by molecules acting by a non-classical toxicological pathway: the modulation or disruption of the endocrine system. This class of molecules is not defined by chemical properties or characteristics but by the targeted system. An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an organism or its progeny or population.

The endocrine system has many specificities and key roles in the organism, thus, its disruption has attracted more and more attention. Like the nervous system, the endocrine system is a communication system within the organism. It assumes the regulation of various functions as control of development, growth and reproduction or homeostasis. This system is composed of a variety of glands secreting chemical messengers, the hormones. They are transported by blood, free or associated with carrier proteins, towards the different targeted organs. They are then able to link receptors. Activated receptors transmit a signal that can result in gene activation leading to protein expression.

Hormones found in organisms can be classified in four main families: derived from amino-acids (as adrenalin), fatty acids (as prostaglandin), cholesterol (steroids) and protein hormones (chain of amino acids as gonadotropins). Various control and feedback control mechanisms allow the regulation of the hormonal balance in reaction to internal or external pressures. Several structured control axis are identified in vertebrates and imply hypothalamus, pituitary gland and either thyroid, adrenophysis or gonads. The hypothalamus-pituitary-gonad axis is strongly involved in reproduction events. Alteration of this main communication and regulation process can lead to dramatic consequences on individuals. Moreover, through reproduction failure or trans-generational effects, the disruption can rise to population level with long term consequences.

Endocrine disruption by xenobiotics has firstly been described in wildlife. At highly polluted sites, correlations between effects on reproduction and development and exposure to endocrine disrupting chemicals have been described for varied species such as birds, reptiles, mammals, fish as well as invertebrates (Vos et al. 2000). In birds, eggshell thinning, causing reproductive failure and so decline of populations of predator birds, has been linked to exposure to DDT. Use of pesticides (DDT) during the 80s probably leaded to sexual development and function alteration of alligators from Apopka Lake in Florida (Guillette et al. 1994). PCBs and their metabolites have been suspected to cause a disease syndrome (manifested by a serious reduction of the population) in Baltic grey and ringed seals (Hutchinson et al. 1994). Wiig et al. (1998) described a masculinisation of Polar bear associated with the presence of PCBs. Numerous studies pinpointed different endpoints of disruption (precocious maturity, intersexuality, vitellogenine induction) in marine and freshwater fish. Thus, changes in the sex of revering fish exposed to estrogenic effluents have been reported by (Jobling et al. 1998; Minier et al. 2000). The earliest description of endocrine disruption is the imposex phenomenon in gastropods (Smith et al. 1971) that is induced by xenoestrogens (Tillmann et al. 2001). Traduced by the presence of a penis in females, this masculinisation is due to an
These examples, among the most famous, demonstrate the reality and seriousness of endocrine disruption in wildlife. But one can wonder about another member of the animal kingdom: man, as it is at the top of the food chain. Our Stolen Future, probably initiated the current extend of the "endocrine disruption" field of research. Despite the dark future predicted by this book, no direct link between environmental exposition and reproduction issue due to endocrine disruption have been established yet. The only cases of clear endocrine disruption in human concern reproduction troubles and cancers in sons and daughters from women treated with diethylstilbestrol (DES) (Gill et al. 1979) and from parents professionally exposed to pesticides. Numerous studies focuses on human sperm quality or quantity, some of them concluding to drastic diminution of spermatozoids number (−40% according to Carlsenn et al. 1992). But contradictory results moderate this observation. The difficulty to conclude is mainly due to variations such as samples origin, measurement method and seasonal variations. (Sharpe and Skakkebaek, 1993) extended the approach to testicular cancer, hypospadia and cryptorchidia. They introduced the notion of Testicular Digensis Syndrome (TDS) (Skakkebaek et al., 2008) suggesting that these disruptions could have a common origin and that the etiological impact of environmental factors such as endocrine disruptors should be considered.

Consequently, European Union adopted in 2000 the directive 2000/60/CE, called "water framework", establishing a community water policy. This directive initiates a list of 32 priority molecules, 11 of them being considered as endocrine disruptors.

2. Materials and methods

2.1 Sample location and collection

Sampling stations was located in the Gulf of Gabes area. We choose sites where animals were available and also receiving the STW effluents.

For in vivo contamination by the effluent or by oestradiol, animals were placed for 40 days in aquariums filled with natural aerated sea water with photoperiod 12:12.

Animals were exposed for 40 days to effluent or 17-β-estradiol which was dissolved in methanol and added to aerated sea water.

Controls were kept under identical conditions in sea water.

Animals were fed only with natural phytoplankton contained in sea water which was renewed twice a week.

2.2 Sample extractions

Sea water samples were acidified by addition of 0.1% acetic acid and organic compounds extracted under vacuum by 5-cc (200-mg) polymeric solid-phase extraction (SPE) cartridges (Waters Oasis HLB Extraction Cartridge, Milford, MA, USA), which had been previously preconditioned with methanol (3 ml) and ultra-pure water (0.1% acetic acid, 3 ml). The columns were eluted using 2 x 3 ml methanol washes which were then combined and evaporated under vacuum and redissolved in 1 ml in methanol for testing for total estrogenicity on the YES assay (see below).

Samples were then fractionated on RP-HPLC to produce an estrogenic profile and GC-MS used to identify compounds in fractions showing estrogenic activity (see below).

Sediment samples were used either for the YES assay.

For the YES assay, replicate sediment samples (5g) from each site were serially extracted by ultrasonication in three methanol extractions (5 ml) followed by 2 x 5ml of CCl4. MeOH (2:1) extractions. Extracts were reduced under vacuum, combined and redissolved in 1 ml methanol before total estrogenicity was determined using the YES assay.
assay. Sediments showing estrogenic signals were fractionated by HPLC. Their estrogenic profiles and the estrogenic compounds identity were determined by GC-MS.

Extracts of clams Ruditapes decussatus tissue

Clams tissues have been homogenised in methanol. Then, a sonication is necessary in order to extract hormones.

Extracts have been concentrated by vacuum evaporation and redissolved in 1 ml methanol.

A fraction of every extract has been analysed by the “YES” test.

2.3 Assay of estrogenic activity using the recombinant yeast screen (YES assay)

The estrogenic activity of water, sediment, mussel extracts and HPLC fractions was determined using the YES bioassay. This bioassay has been validated in the detection of a wide range of estrogen receptor agonists including 17β-estradiol, oestrone, ethinyl estradiol as well as xenoestrogens such as alkylphenolics and bisphenol A (Routledge and Sumpter, 1996 and 1997). Briefly the human Estrogen Receptor (hER) gene was stably integrated into the yeast genome together with an expression plasmid containing the Estrogen-Response Element (ERE) which controls the reporter gene Lac-Z (expression encoding the enzyme β-galactosidase). The activation of the hER receptor, by binding of a ligand, causes binding to the ERE and consequently the production of β-galactosidase. The enzyme is then secreted into the cellular environment and metabolizes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), (normally yellow) into a chlorophenol (Red) that can be measured by absorbance at 540 nm.

Extracts of samples and blanks were added in a series of dilutions to a test multiwell plate and the ethanol allowed evaporating at room temperature. Concentrations of E2 were analyzed in parallel as a positive control. Yeast and assay environment containing the chromogenic substrate was added to the wells and the plate incubated for 3-5 days. The absorbance of each sample was determined, corrected for untreated controls and yeast growth, and compared with that of the E2 standard. The estrogenicity measured in water, sediment, mussel samples or fish bile was expressed as E2 equivalent values (E2Eq) which were determined from the linear range of concentration-response curves (recorded between 10 pM and 1 nM for E2). In agreement with published values the median effect concentration of E2 was typically around 100 pM. The total estrogenicity of each sample was determined in terms of ng E2Eq/L or kg sample.

To unconjugate biotransformed compounds in fish bile, samples were first incubated with 25 U/mL glucuronidase, 25 U/mL sulfatase and 5 U/mL de glucosidase for 12 h at 37°C then acidified with 2 μL/mL acetic acid.

2.4 HPLC analyses

Normal phase HPLC: Mussel and fish metabolites were separated on a Waters Ltd HPLC system comprising a model 600 pump and controller, model 717 auto-sampler and a photodiode array detector (Model 996, Waters Ltd) and Flow scintillation Analyzer (Packard 500TR) in series. In this system standards of Oestradiol (E2), of Estrone (E1) and nonylphénol (NP) were used. An aliquot of each sample (100 µL) was injected onto a normal phase column (Nucleosil Silica 5µ 4.6 x 250 mm; Alltech). Mobile phase solvents were isopropanol (A) and hexane (B) in an initial ratio (A: B) of 0:100. Separation was achieved at room temperature using a flow rate of 2.0 ml/min with the following gradient programme: 0 minutes (0:100); 5 minutes (10:90); 45 minutes (30:70); 65 minutes (30:70). The HPLC eluent was split at a ratio of
10:1 where the majority of the eluent fractions were collected for analysis by GC-MS to identify metabolites. The remainder was mixed with scintillation fluid and analysed by the flow through scintillation analyser.

3. Result and discussion

To assess the extent of estrogenic disruptor chemicals in the marine environment we used an “in vitro” short term test such as the recombinant yeast reporter gene assay. Both in situ and in vivo evaluations were made. The estrogenic activity measured in the SWT and sediment are shown in figure 1. These results show that effluent contains chemicals able to mimic oestrogen hormones activity.

HPLC analysis of the effluent was used in order to determine the compounds present in the effluent. We have noticed the presence of compounds eluted in the fraction 25, 26, 37 et 38 min. corresponding to estradiol (E2) (25 min) and to estrone (E1) (37 min) (referring to retention time of known standards) (figure 2).

The small picks between 31 and 35 minutes can correspond to the ethinyl estradiol (EE2) but quantities are weak and near of the bottom noise.

The presence of nonylphenols and near compounds would result in one or picks around 50 minutes, there’s no thing in this zone for the sewage effluent.

In vivo contamination of clams R.decussatus showed that estrogenic compounds are accumulated in gonads after 20 days. After 30 days they were found in gills (figure 3) (Mezghani, 2008; unpublished)

![Compound Identification by HPLC](image)

**Fig 2- Estrogenic profiles of the effluent**

![Estrogenic activity in the SWT effluent and sediment](image)

**Fig 1- Estrogenic activity in the SWT effluent and sediment**

![Estrogenic compounds in clam tissues after in vivo exposure to effluent](image)

**Fig 3- Estrogenic compounds in clam tissues after in vivo exposure to effluent**

In situ studies showed many cases of hermaphrodite clams (figure 4). Both simultaneous and accidental hermaphroditism were observed by histological study of the gonads.

The figure 5 shows the hermaphrodite cases in the cockle as well as the presence of parasites.
Simultaneous Hermaphroditisme in *R. decussatus* (Damak-Smaoui et al, 2007)

Superposed Hermaphroditisme (Damak-Smaoui et al, 2007)

Fig 4- Histology of the gonads showing hermaphrodite cases
Fig 5- shows the hermaphrodite cases in the cockle as well as the presence of parasites.
Parasites (a) and Hermaphroditism (b) cases in Cerastoderma glaucum (Ajimi-Machrek, 2008)

Parasites in Cerastoderma glaucum (Karray 2008)

Cerastoderma glaucum (Ajimi, 2008)

Fig 6- Sex ratio of the cockle showing the dominance of males
4. Conclusion

The result of this study emphasizes the importance of monitoring aquatic environment to control the presence of estrogenic chemicals which contribute to endocrine disruption in marine organisms.

The yeast reporter gene assay is a highly reliable methodology for a first level screening to assess surface water quality as it is a sensitive tool to detect marine pollutant with estrogenic activity. This assay allows to design the pollution degree and to state a scale of evaluation and risk assessment (low, medium, high) for the aquatic ecosystem.

The observed preliminary effects (hermaphrodism, parasites, sex ratio…) need greater and deeper investigations in order to evidence the relationship Dose/effect between the ED (endocrine disruptor) and the organism.

Both in vivo and in situ studies are needed to understand the effect of ED on reproduction. The first approach could allow the control of exposure and the second will be reliable to monitor the reproductive cycle.

5. References

