

Estrogenic Endocrine Disruptors and their Possible Deleterious Effects on Marine Organisms: Use of a Novel Monitoring Bioassay

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Abstract: Estrogens are both natural and synthetic substances that mimic the effect of the female estrogenic hormone in the body and impart estrogenic activity. Human wastes, birth control pills and chemicals like detergents are a major source of estrogens in the environment. In waste water treatment plants, a major part of these estrogens are not removed after treatment and are released in the marine environment. They are suspected to interfere with the exposed aquatic species' endocrine systems. 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. A reverse phase HPLC method was used to identify the nature of estrogenic components. We focused on two marine bivalves *Ruditapes decussatus* and *Cerastoderma glaucum* recognised bioindicator organisms useful in biomonitoring. Our work is based on *in situ* and *in vivo* studies. Different compartments were used: the effluents of a wastewater treatment plant, the sea water, the sediment and the clam *Ruditapes decussatus*. Some observed histological effects showing hermaphroditic cases and parasites in the cockle *Cerastoderma glaucum* are also discussed in this paper.

Keywords: Endocrine disruption, xenoestrogens, YES, Hermaphroditism, *Ruditapes decussates*, *Cerastoderma glaucum*.

1. INTRODUCTION

Aquatic environments receive significant levels of 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 [1]. These chemicals are defined as Endocrine Disrupting Compounds (EDC), and a list of more than 500 known or suspected EDC has been established by the European Community [2], such as numerous pesticides, industrial chemicals, and commercial products that have been released into the environment. And of particular importance are those that mimic estrogens and androgens (and their antagonists), because of their central role in reproductive function.

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

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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, adenophysis 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 [3]. 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 led to sexual development and function alteration of alligators from Apopka Lake in Florida [4]. 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 [5]. Wiig *et al.* [6] 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 reversing fish exposed to estrogenic effluents have been reported by [7,8]. The earliest description of endocrine disruption is the imposex phenomenon in gastropods [9] that is induced by xenoestrogens [10]. Traduced by the presence of a penis in females, this masculinisation is due to an anti-fooling biocide, the TBT [11].

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) [12] 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.* [13]). 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 [14] extended the approach to testicular cancer, hypospadias and cryptorchidia. They introduced the notion of Testicular Digenesis Syndrome (TDS) [15] 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.

Within the invertebrates, molluscs are a particular focus of research, reflecting their abundance in nature, commercial and ecological importance and their use as bioindicators in water pollution monitoring [16, 17]. It was reported that investigations in mollusc species represented 55% of the studies performed on EDC effects with invertebrates in 2003 while they represented only 3.6% in 1998 [18]. An important effort has been made to better understand the endocrinology of this phylum although our knowledge is still fragmentary. However this is the necessary step in order to evidence any endocrine disrupting effect. In accordance with this fact, this paper attempts to briefly and critically summarise the present knowledge on the reproductive endocrinology of molluscs (for a more extensive review on the endocrine system see [19, 20]) and then to examine major reported endocrine disrupting effects and their associated mechanisms.

In the present study, we choose to work on the clam *Ruditapes decussatus*. This species is a filter-feeding

bivalve, widely distributed in the Gulf of Gabès (Tunisia). It is a natural fishery resource in this area, representing 94% of the overall exploitation of shell fish in Tunisia [21]. Moreover, it constitutes an important economic endpoint since it is mainly exposed to Europe [22]. From a biomonitoring point of view, *Ruditapes decussatus* satisfies many criteria (easy collection, sedentary habit and suitable size) required for bioindicator of pollution and is actually used in the national monitoring programs [23-25]. The second chosen bivalve is the cockle *Cerastoderma glaucum*, an important sedentary filter feeding marine bivalve, living in the superficial sediment and considered as a good bio indicator reflecting the pollution state of the Tunisian coasts.

Recent studies about endocrine disrupting effects on reproduction have dealt with some bivalves such as *Mya arenaria* [26-28], *Elliptio complanata* [29] and *Patinopecten yessoensis* [30]. Nevertheless, nothing is known about the endocrine disruption in the clam *Ruditapes decussatus*, except some *in vivo* experiments using the tributyltin [31, 32]. Therefore, the aim of the present study was to investigate *in situ* the effect of endocrine disruption on the reproductive capacities of the clam *Ruditapes decussatus* and the cockle *Cerastoderma glaucum* from the southern coast of Sfax (Tunisia).

2. MATERIALS AND METHODS

2.1. Sample Location and Collection

Sampling stations was located in the gulf of Gabès area. We choose sites where animals were available and also receiving the waste water treatment plant (WWTP) 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 \times 3 ml methanol washes which were then combined and evaporated under vacuum and redissolved in 1ml in methanol for testing for total estrogenicity on the YES assay.

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 \times 5ml of CCl₄. MeOH (2:1) extractions. Extracts were reduced under vacuum, combined and re-dissolved in 1 ml methanol before total estrogenicity was determined using the YES assay. Sediments showing estrogenic signals were fractionated by HPLC. Their estrogenic profiles and the estrogenic compounds identity were determined by GC-MS.

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 17 β -estradiol, oestrone, ethinyl estradiol as well as xenoestrogens such as alkylphenolics and bisphenol A [33]. 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 of 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 chromatogenic 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 × 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.

2.5. Histological Study of *Cerastoderma glaucum* and *Ruditapes decussatus*

The gonads of 15 individuals per sample were freshly dissected and immediately fixed in aqueous Bouin for 48 h. The tissues were subsequently dehydrated in a series of increasing concentrations of ethyl alcohol solutions, then clarified in toluene and embedded in paraffin wax (melting point at 57°C). Serial sections were cut at 4 µm using HM 315 microtome then treated with hematoxylin-eosin staining solutions. Microscopic examination under the light microscope aimed at determining the stages of the gametogenic cycle. A gametogenic scale was drawn up after examining the histological sections.

3. RESULTS 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 waste water treatment Plant (WWTP) and sediment is 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 and 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.

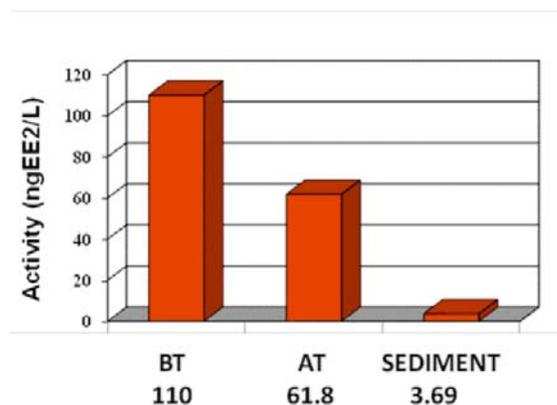


Figure 1: Estrogenic activity (ng EE2/l) in sediment and waste water.

BT: before treatment, AT: after treatment.

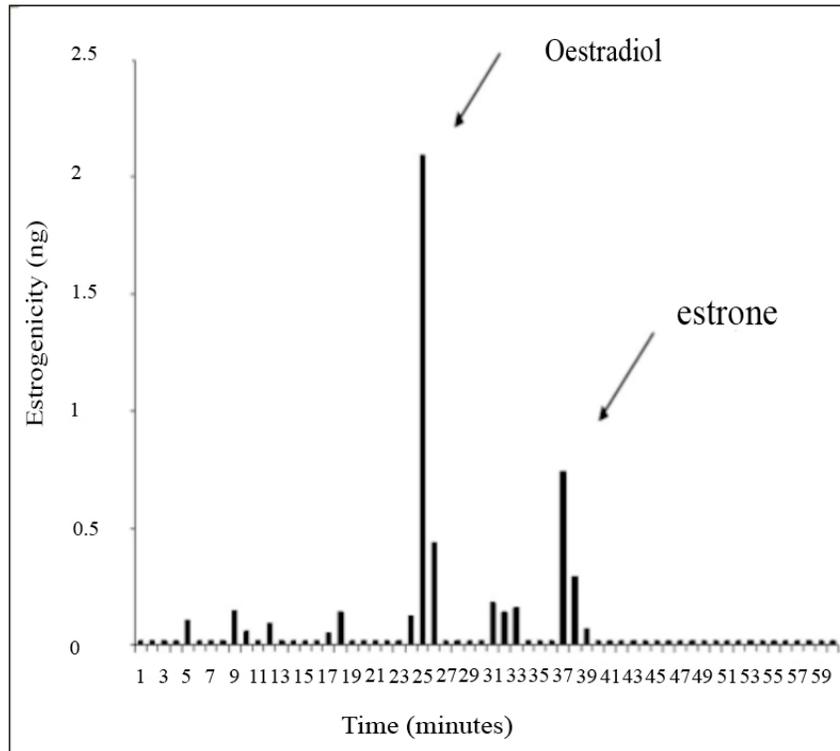


Figure 2: Estrogenic profiles of the effluent HPLC compound identification.

The presence of nonylphenols and near compounds would result in one or picks around 50 minutes, there's nothing 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).

In situ studies showed many cases of hermaphrodite clams (Figure 4). Both simultaneous and accidental hermaphroditisms were observed by

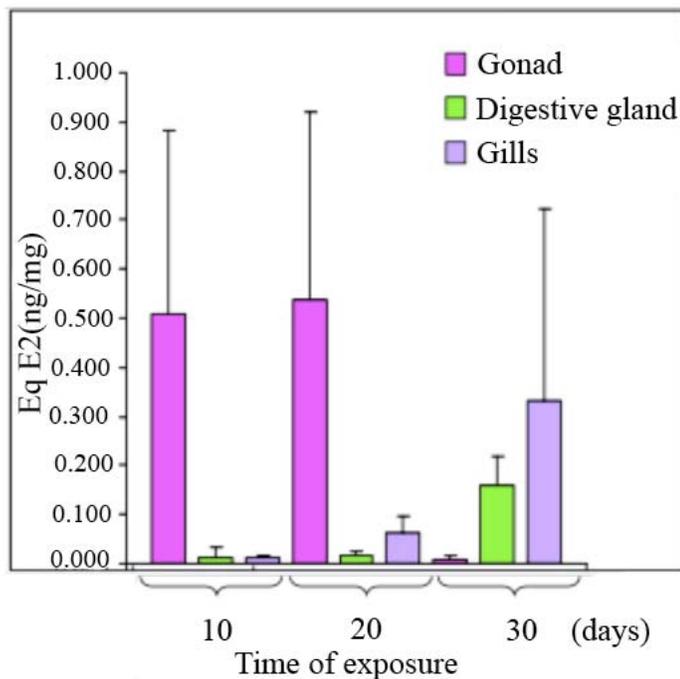


Figure 3: Estrogenic compounds in clam tissues after *in vivo* exposure to effluent.

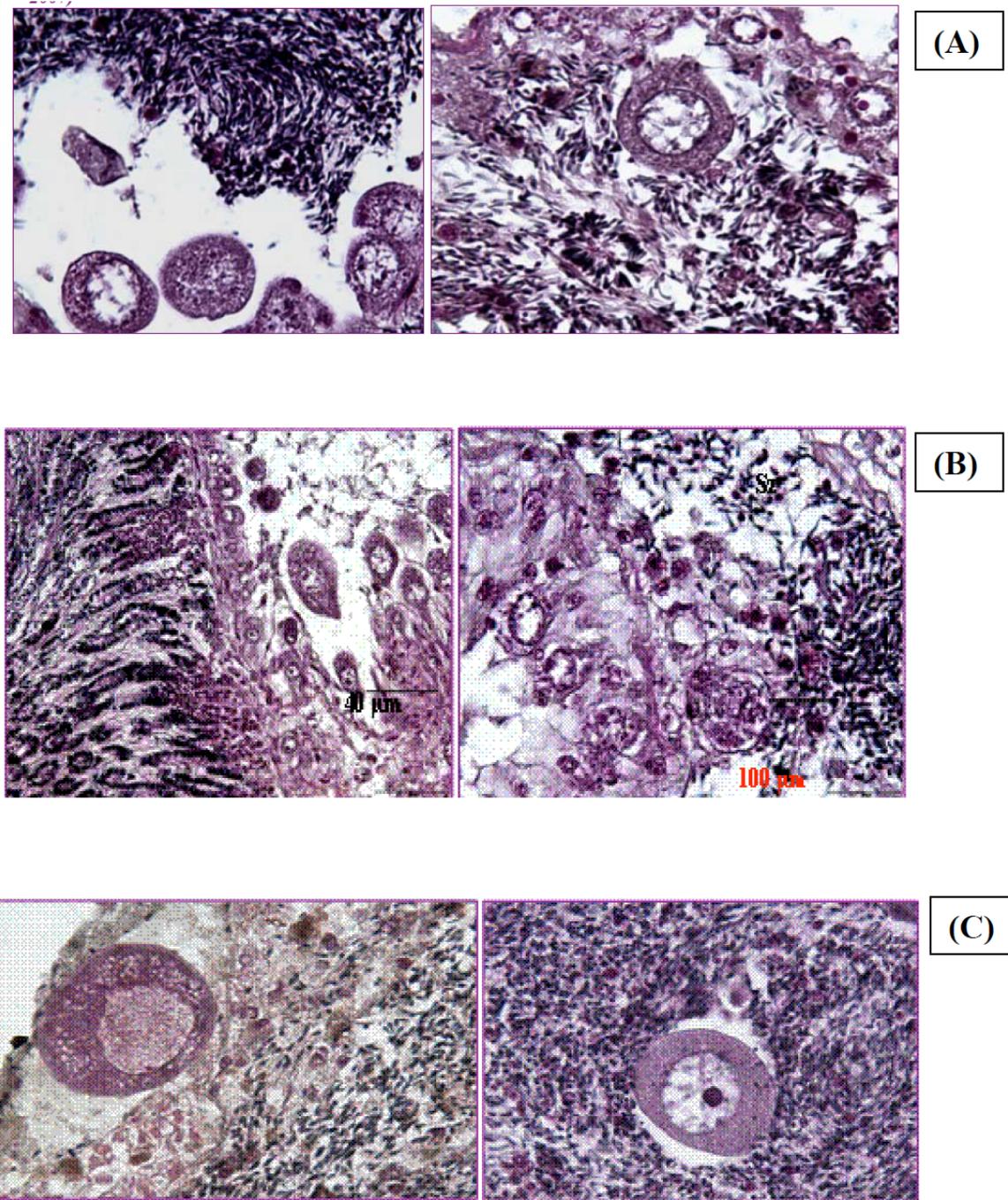


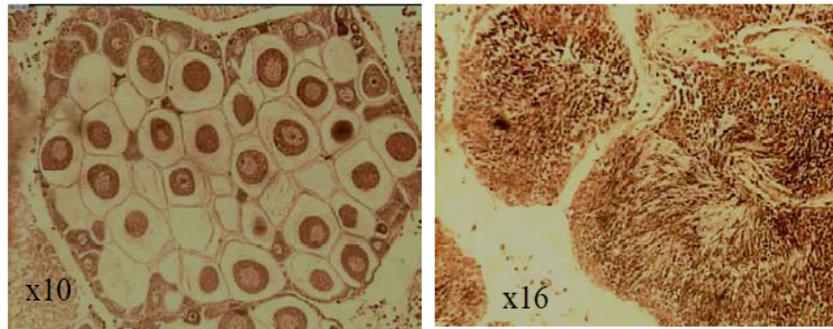
Figure 4: Histology of *Ruditapes decussatus* gonads showing hermaphrodite cases. (A) and (B) Simultaneous hermaphroditism, (C) Superposed hermaphroditism [34].

histological study of the gonads. We observed at the same time both male and female, gonadal tubules arranged in separate zones (Figure 4B). In males gonadal tubules, spermatozoa were forming concentric bands or were free in the lumen and ready to be spawned. Whereas, in females gonadal tubules oocytes appeared at different stages of vitellogenesis along the wall. However, in May and December male gonadal tubules occupied the majority of the gonadal

area with mature spermatozoa, whereas few residual ripe oocytes were identified in the lumen or along the wall (Figure 4C). Muscular tissue occupied a large space between gonadal tubules. It seems that we are face to two kinds of hermaphroditism, the first one is simultaneous (Figures 4A and B) and the second one is accidental (Figure 4C).

The Figure 5 shows the hermaphrodite cases in the cockle as well as the presence of parasites.

Normal case female male(A)



Hermaphrodite case: presence of an ovocytein male

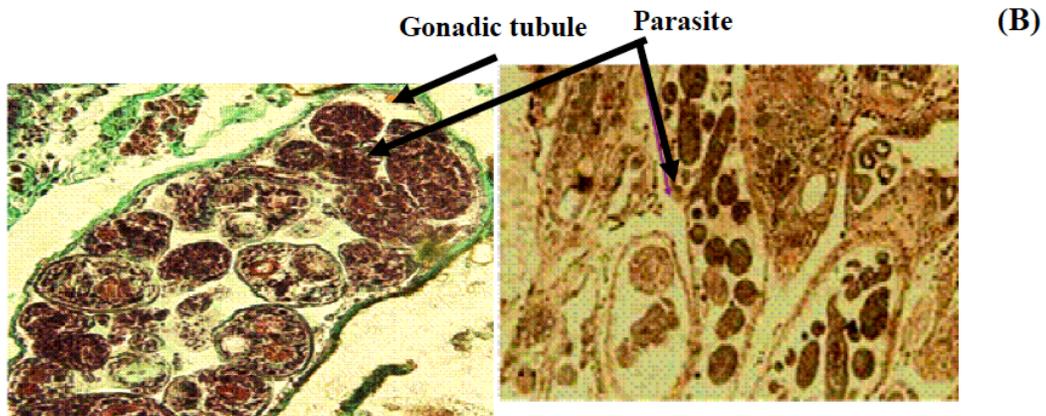
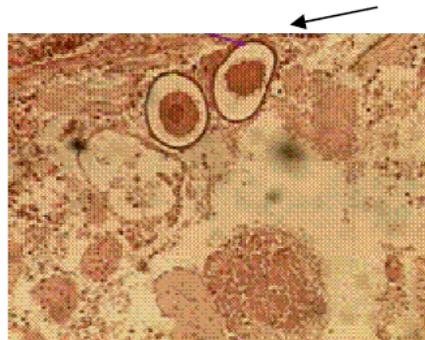


Figure 5: The in the cockle *Cerastoderma glaucum*.

(A) Hermaphrodite cases [35].

(B) Presence of parasites [36].

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 (hermaphroditism, 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.

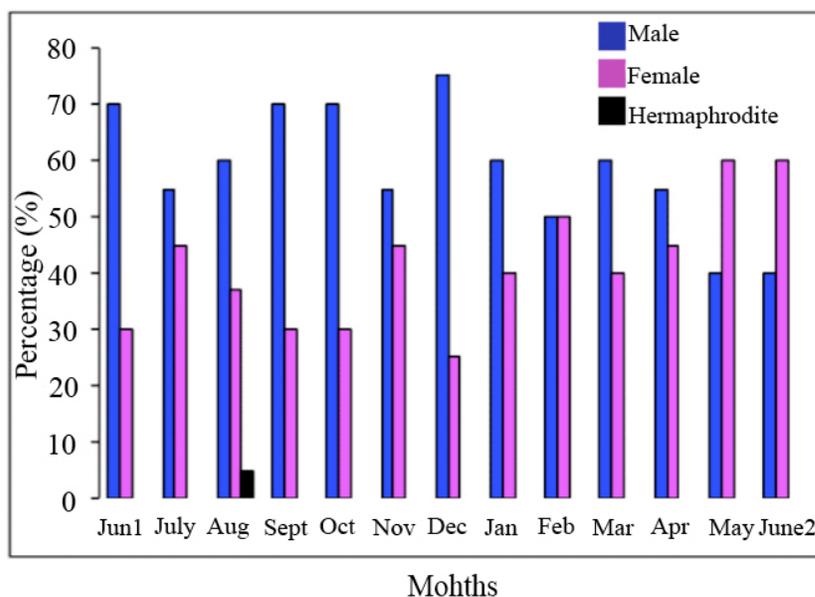


Figure 6: Sex ratio of *Cerastoderma glaucum* showing the dominance of male.

4. CONCLUSION

This paper presented some observed effects of endocrine disruptors. It also analyzed both *in situ* and *in vivo* results about the effect of endocrine disruptors from waste water treatment plant on marine bivalves. The originality of our approach was based on contamination with the effluent instead of chemicals. Our aim was to attempt an understanding of the situation in field conditions. We focused on two strategic marine bivalves validated by our previous work, as Bioindicators reflecting the pollution status of one site and used in the Tunisian monitoring program. Yeast estrogen screening test (YES) was used. It is an efficient tool for large scale screening which is widely used in environmental studies and now adapted to the analysis of various matrices. High estrogenic contamination and significant observed effects in bivalves gave us a global picture is alarming. The extrapolation of the observed effects to population level cannot be easy, but the observed disruption on fertility and reproduction success is uncertain. But this "uncertainty" certainly is worthy of attention.

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