Universidade de Aveiro Departamento de Biologia
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Níveis e Efeitos Biológicos de Disruptores Endócrinos na Costa Portuguesa

Levels and Biological Effects of Selected EDC's in the Portuguese Coast

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Carlos Miguel Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor Shinsuke Tanabe, Professor Catedrático da Universidade de Ehime.

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palavras-chave

Disruptores endócrinos; Compostos organoestânicos; Tributilestanho; *Nassarius reticulatus*; *Mytilus galloprovincialis*; *imposex*; Costa Portuguesa

resumo

Os compostos orgânicos de estanho (OTs), de entre os quais se destaca o tributilestanho (TBT), encontram-se amplamente dispersos no meio aquático devido à sua intensa utilização como agente biocida em tintas antivegetativas. Estudos anteriores sobre a poluição por organoestanhos em Portugal demonstraram que estes compostos se encontram presentes não só na linha de costa mas também em zonas da plataforma, sendo as zonas portuárias (onde se incluem portos comerciais, portos de pesca, marinas e estaleiros navais) os principais focos de poluição. A presente tese tem como objectivo investigar o estado actual da poluição por organoestanhos na costa Portuguesa confirmando se os padrões espaciais acima descritos se mantêm, por meio da quantificação de diversos OTs, nomeadamente, butilestanhos, fenilestanhos e octilestanhos. Assim, os níveis destes compostos foram avaliados em populações de *Mytilus galloprovincialis* e *Nassarius reticulatus* ao longo da costa continental Portuguesa, com particular incidência na Ria de Aveiro onde se quantificaram os níveis de OTs em mexilhões, gastrópodes e sedimentos, recolhidos numa malha de amostragem mais densa.

A distribuição espacial dos organoestanhos foi determinada utilizando o bivalve *M. galloprovincialis* como espécie bioindicadora. Os níveis totais de estanho (Sn_T) foram quantificados nos tecidos do mexilhão e relacionados com os níveis totais de OTs nos mesmos tecidos, incluindo monobutilestanho (MBT), dibutilestanho (DBT), tributilestanho (TBT), difenilestanho (DPhT), trifenilestanho (TPhT), monoctilestanho (MOcT) e dioctilestanho (DOct). A contribuição dos OTs para os valores de estanho total (SnT) foi superior nas estações de amostragem localizadas no interior de portos onde atingiram proporções próximas dos 50%. De entre estes, os butilestanhos (BuTs=MBT+DBT+TBT) contribuíram em média com 98.6% para o valor total de OTs, tendo sido detectados em todas as amostras analisadas. Os valores mais elevados foram registados no interior ou na proximidade de portos, corroborando a ideia anterior de que constituem importantes focos de poluição. A variação das concentrações de TBT no mexilhão situou-se entre os 0,9 e 720 ng $Sn.g^{-1}$ de peso seco (ps). Estes valores são, em 69% das estações amostradas, superiores ao valor do proposto pela OSPAR (4,9 ng TBT-$Sn.g^{-1}$ ps) para tecidos de mexilhão o que sugere a forte probabilidade de ocorrência de efeitos adversos sobre os ecossistemas.

Os níveis de OTs foram também quantificados em tecidos de *N. reticulatus* recolhidos ao longo da costa em 2008.

resumo (cont.)

Os butilestanhos representaram a maioria dos compostos organoestânicos quantificados e os níveis mais elevados foram novamente detectados no interior ou nas imediações de portos. Os valores de TBT nos tecidos deste gastrópode variaram entre 3,5 e 380 ng $Sn.g^{-1}$ ps, representando uma percentagem média de 50,4% do total de butilestanhos. Simultaneamente, os níveis de *imposex* foram também avaliados e relacionados com os valores deste composto nos tecidos. As distribuições espaciais de *imposex* e de TBT seguiram a mesma tendência, sendo que em todos os locais amostrados foram encontradas fêmeas afectadas. Os valores de VDSI (índice da sequência do vaso deferente) variaram entre 0,2 e 4,4. Em 91% dos locais os valores de VDSI foram superiores a 0,3 (definido pela OSPAR como o valor de VDSI em *N. reticulatus* acima do qual o objectivo de qualidade ecológica não é atingido), confirmando a suspeição da existência de efeitos adversos nos ecossistemas.

Em todos os compartimentos analisados na Ria de Aveiro, os butilestanhos foram os principais contribuintes para estanho orgânico total. A utilização do *imposex* em *N. reticulatus* como biomarcador da poluição por TBT permitiu determinar um gradiente decrescente desde o interior da Ria (onde se situa a zona portuária) até zonas costeiras adjacentes. O mesmo gradiente foi observado relativamente às concentrações de TBT em tecidos de mexilhão. Para os sedimentos, as concentrações de TBT são bastante variáveis com valores entre 2,7 e 1780 ng $Sn.g^{-1}$ ps encontrando-se significativamente correlacionadas com o conteúdo em matéria orgânica da amostra. Em todas as amostras analisadas os níveis de TBT são elevados e superiores ao valor inferior (provisório) de EAC (critério de avaliação ambiental) proposto pela OSPAR (0,004 ng $TBT\text{-}Sn.g^{-1}$ ps).

A análise da evolução temporal da poluição por TBT ao longo da costa foi concretizada por meio da comparação entre níveis de organoestanhos em *N. reticulatus* em amostras de 2008 e 2003 e também através da comparação dos níveis de *imposex* registados em campanhas realizadas naqueles dois anos. Os resultados obtidos indicam a ocorrência de reduções significativas nas concentrações de TBT, DBT e MBT, assim como uma diminuição significativa nos valores de VDSI entre 2003 e 2008. Os resultados obtidos sugerem que a redução verificada se deve à implementação do Regulamento 782/2003 da Comunidade Europeia, que tem por objectivo a erradicação das descargas e emissões de TBT para o ambiente a partir dos sistemas antivegetativos.

resumo (cont.)

A diminuição da poluição por TBT ao longo dos últimos anos foi acompanhada por um aumento no número de fêmeas com um vaso deferente, mas sem pénis (*imposex* do tipo b): 3,5% em 2000, 11% em 2003 e 24% em 2008. Um aumento no número de locais onde se registou o fenómeno também é evidente: dois em 2000, sete em 2003 e treze em 2008. A proporção de fêmeas b no estádio 1 de VDS apresentou igual tendência com aumento de 38% em 2000 para 65% em 2008. O aumento no número de fêmeas com esta via parece estar associado à diminuição da poluição por TBT. Face à esperada diminuição da presença do composto no meio ambiente, devido à sua proibição, o aumento de fêmeas com *imposex* do tipo b é previsível.

A ocorrência de compostos xenoestrogénicos no ambiente aquático foi também estudada e os níveis de estrona (E1), 17α-e 17β-estradiol (E2), 17α-etinilestradiol (EE2), bisfenol-A (BPA) e nonilfenol (NP) foram quantificados em efluentes de estações de tratamento de águas residuais (ETARs) localizadas na região de Aveiro, bem como no efluente final descarregado no Oceano Atlântico, através de um emissário submarino (S. Jacinto), sendo amostras recolhidas na entrada da Ria e ao largo usadas como referência. Os níveis de hormonas esteróides e compostos fenólicos registados nos locais de referência são baixos. De entre as hormonas esteróides os níveis mais elevados foram registados para a estrona, com valores máximos de 85.3 $ng.L^{-1}$. Os níveis mais elevados de compostos fenólicos foram detectados em efluentes industriais (máximos de NP e BPA de 2410 $ng.L^{-1}$ e 897 $ng.L^{-1}$, respectivamente). Os resultados obtidos sugerem que os níveis de compostos xenoestrogénicos em locais de referência são baixos e não parecerem acarretar risco ecológico, no entanto o mesmo não será verdadeiro para as imediações do emissário de S. Jacinto que liberta efluentes com concentrações muito elevadas de E1, NP e BPA.

Foram realizadas experiências laboratoriais de forma a elucidar o papel do receptor retinóico X (RXR) no mecanismo de indução de *imposex* (presentemente o mecanismo que demonstra maior promessa na explicação do desencadear deste fenómeno). Fêmeas de *Nucella lapillus* e *N. reticulatus* foram injectadas com TBT em etanol ou com ácido 9-*cis*- retinóico em FBS (soro fetal bovino) tendo-se procedido à sua observação nos 30 dias subsequentes. Tanto o TBT como o 9CRA induziram o desenvolvimento de *imposex* em *N. lapillus* e *N. reticulatus*. Aumentos significativos nos valores de VDSI e FPL entre o controlo de etanol e o tratamento de TBT e o controlo de FBS e o tratamento de 9CRA foram registados. Os resultados obtidos fornecem novas provas do envolvimento da via de sinalização associada ao RXR no desenvolvimento de *imposex* em ambas as espécies.

keywords

Endocrine Disruptors; Organotins; Tributyltin; *Nassarius reticulatus*; *Mytilus galloprovincialis*; Imposex; Portuguese coast

abstract

Organotin compounds (OTs), particularly tributyltin (TBT), are ubiquitous contaminants in the aquatic ecosystem mainly due to its intensive use as biocides in antifouling (AF) paints formulations. Previous reports regarding TBT pollution in the Portuguese coast indicate its widespread presence both in inshore and offshore areas with harbors considered as hotspots of pollution. The present thesis aims to investigate the current status of organotin pollution in the Portuguese coast and seeks to confirm if the above spatial patterns are still maintained. Moreover, it intends to assess the spatial patterns of a variety of OTs that are predicted to occur in the environment, namely butyl-, phenyl- and octyltins, so that the relative importance of TBT may be rigorously determined regarding the overall OTs pollution scenario. Hence, the environmental levels of organotin compounds were assessed in mussels (*Mytillus galloprovincialis*) and gastropods (*Nassarius reticulatus*) populations along the mainland Portuguese coast, with particular focus in Ria de Aveiro (NW coast) where quantifications of OTs in mussels, gastropod's tissues and sediments were performed in a tighter sampling grid.

The spatial distribution of OTs was determined using *M. galloprovincialis* as bioindicator species. Levels of total tin (Sn_T) in soft tissues were quantified and related to the levels of total organic tin compounds including monobutyltin (MBT), dibutyltin (DBT), TBT, diphenyltin (DPhT), triphenyltin (TPhT), monoctyltin (MOcT) and dioctyltin (DOcT). Butyltins (TBT+DBT+MBT) were detected in all analyzed samples accounting, in average, for 98.6% of total OTs, and presented highest values in the vicinity of harbors, corroborating the previous idea that they represent the hotspots of OTs pollution in the Portuguese coast. The contribution of OTs to Sn_T was higher in sites located inside harbors, reaching values up to 55% of the Sn_T. TBT concentrations in mussel's tissues varied between 0.9 and 720 ng $Sn.g^{-1}$ dw. Those values, in 69% of the sampling sites, were higher than the lower-Environmental Assessment Criteria (EAC: 4.9 ng TBT-$Sn.g^{-1}$) proposed by the OSPAR Commission which indicates that, at those sites, adverse effects are possible to occur in most sensitive species.

Organotins were also quantified in *Nassarius reticulatus* soft tissues collected along the Portuguese coast in 2008. Butyltins accounted for the majority of the OTs quantified and the highest levels were, once again, detected inside or in the vicinity of harbors. TBT levels varied between 3.5 and 380 ng $Sn.g^{-1}$ dw, representing an average proportion of 50.4% of total butyltins (∑BuTs= MBT+DBT+TBT).

abstract (cont.)

Imposex was also used as a biomarker of TBT pollution and exhibited similar spatial gradients. Imposex affected females were present at all sampling sites and highly significant correlations were established between imposex indices and TBT concentrations in the whelk's soft tissues. The *vas deferens* sequence index (VDSI) varied between 0.2 and 4.4 across sampling sites. In 91% of the surveyed locations, VDSI levels were higher than 0.3 – the Ecological Quality Objective (EcoQO) set by OSPAR for imposex in *N. reticulatus* – indicating that, at those sites, there is risk of adverse effects, such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem.

Regarding Ria de Aveiro, butyltins were the major contributors to total organic tin in all the analyzed compartments. A decreasing gradient in *N. reticulatus* imposex and TBT levels from the vicinity of harbors (inside Ria de Aveiro) towards the offshore sea was observed. The same spatial gradient was observed for TBT levels in mussels. TBT concentrations detected in sediments were highly variable (from 2.7 to 1780 ng TBT-Sn.g^{-1} dw) and were significantly correlated with the sample's organic matter (OM) content. In all samples analyzed, TBT levels were much higher than the EAC proposed for sediments (provisional lower-EAC: 0.004 ng TBT-Sn.g^{-1} dw) indicating that TBT pollution stills a matter of great concern in this area as well.

The analysis of TBT pollution temporal evolution along the coast was performed trough the comparison of OTs levels in *N. reticulatus* samples collected in 2008 and in preserved samples from 2003 and also trough the comparison of imposex levels obtained in 2008 survey with the ones previously reported for 2003. The obtained results disclose significant reductions in TBT, DBT and MBT between the 5 common sites where OTs were quantified. Significant declines in VDSI levels were also observed at the majority (87%) of the sampling sites. These results clearly demonstrate that a reduction in TBT pollution occurred between 2003 and 2008, suggesting the efficacy of the EC Regulation 782/2003 on reducing this type of pollution.

The decline in TBT pollution over the last years was followed by an increase of the number of females presenting the b-type VDS=1 stages, i.e., initial stages of imposex with only *vas deferens* growth but without the typical development of a penis: from 3.5% in 2000, to 11% in 2003 and to 24% in 2008. An increase in the number of sites where this phenomenon was recorded was also evident: two sites in 2000, seven sites in 2003 and thirteen sites in 2008. The proportion of females with the b-type VDS stage 1 *versus* a-type VDS stage 1 also rose from 2000 (38%) to 2008 (65%). The emergence of this phenomenon seems to be associated with TBT pollution decline and a shift toward the b-type females is expected in the future due to the TBT global ban.

abstract (cont.)

The occurrence of xenoestrogens in the aquatic environment was also studied and the levels of estrone (E1), 17α- and 17ß-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA) and nonylphenol (NP) were quantified in effluents from WWTPs located in Ria de Aveiro (NW Portugal), as well as in the final effluent discharged into the Atlantic Ocean through the S. Jacinto submarine outfall. Reference sites, located at the estuarine system entrance and at the sea side, were also included. Levels of steroids and phenolic compounds were low in reference samples. Of the steroid hormones, E1 presented the highest levels (maximum value: 85.3 $ng.L^{-1}$), whereas the synthetic hormone EE2 was always below the detection limit. The highest levels of phenolic compounds were detected in industrial effluents (maximum NP and BPA values of 2410 $ng.L^{-1}$ and 897 $ng.L^{-1}$, respectively). The obtained results disclose levels of xenoestrogens at reference sites lower than the ones reported to pose risk for wildlife, however the concentrations of E1, NP and BPA in the final effluent released by S. Jacinto submarine outfall are very high and deserve further attention.

The mechanisms underlying imposex development were studied in more detail by performing a series of laboratory experiments aiming to understand the role of the retinoid X receptor (RXR) in the process, presently considered the most plausible hypothesis to cause the development of this phenomenon. *Nucella lapillus* and *N. reticulatus* females were injected with ethanol containing tributyltin (TBT) or with fetal bovine serum (FBS) containing 9-*cis*-retinoic acid (9CRA: the natural ligand for humans RXRs) and maintained in a flow-through system with artificial seawater for 30 days. Both TBT and 9CRA caused imposex development in *N. lapillus* and *N. reticulatus* as significant increases in VDSI and female penis length (FPL) were observed between the ethanol control and the TBT treatment and between the FBS control and the 9CRA treatment. These results provide further evidence of the involvement of RXR signalling pathway on imposex development in both species.

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Acronym list

9CRA: 9-*cis*-retinoic acid

∑BuTs: total butyltins

∑OT: total organic tin

%I: Percentage of imposex affected females

%STE: Percentage of Sterile Females

ACCI: Assessment Class Criterion for Imposex

AF: Antifouling

AFS Convention: International Convention on the Control of Harmful Antifouling Systems on Ships

AOS: Average Oviduct Stage

BCF: Bioconcentration Factor

BD: Benzophenone

BDI: Butyltin Degradation Index

BPA: Bisphenol-A

BuTs: Butyltins

CEMP: Coordinated Environmental Monitoring Program

CRM: Certified Reference Material

CSTEE: Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of the European Commission

DBT: Dibutyltin

DDD: Dichlorodiphenyldichloroethane

DDE: Dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

DHT: Dihydrotestosterone

DHTEQ: Dihydrotestosterone Equivalency

DOcT: Dioctyltin

DPhT: Diphenyltin

dw: dry weight

EAC: Environmental Assessment Criteria

EC: European Commission

EC_{50}: Median Effective Concentration

EcoQo: Ecological Quality Objective

EDCs: Endocrine Disrupting Chemicals

EEQ: Estradiol Equivalents

EEQ_{calc}: Calculated Estradiol Equivalents

EI-SIM: Electron Impact and Selected Ion Monitoring Mode

ERM: European Reference Materials

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

FPL: Female Penis Length

FSH: Female Shell Height

GC-MSD: Gas Chromatograph- Mass Spectrometer Detector

GTS: Gross Tonnage Stood

HBr: Hydrobromic acid

HRT: Hydraulic Retention Times

HNO_3: Nitric Acid

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

IMO: International Maritime Organization

KOH: Potassium Hydroxide

LC-MS/MS: Liquid Chromatography Mass Spectrometry/Mass Spectrometry

MBT: Monobutyltin

MES: Mestranol

$MgCl_2$: Magnesium Chloride

MOcT: Monoctyltin

MPL: Mean Male Penis Lenght

MPhT: Monophenyltin

MTBE: Methyl Tert-Butyl Ether

NaBr: Sodium Bromide

NIES: National Institute for Environmental Studies (Japan)

NPEO: APEO (Alkylphenol Ethoxylate) having a linear 1-nonyl chain

NPE_2C: 4-Nonylphenoxyethoxy acetic acid

OcTs: Octyltins

OM: Organic Matter

OSPAR CONVENTION: Convention for the Protection of the Marine Environment of the North-East Atlantic

OT: Organotin

PCB: Polychlorinated biphenyl

PVC: Polyvinylchloride

PhTs: Phenyltins

PTFE: Polytetrafluoroethylene

QA/QC: Quality Assurance/ Quality Control

RPLI: Relative Penis Length Index

Sn: Tin

Sn_T: Total Tin

SPE: Solid Phase Extraction

St: Station

St Dev: Standard deviation

T: testosterone

TBT: Tributyltin

TBTCl: Tributyltin Chloride

TDI: Tolerable Daily Intake

TOcT: Trioctyltin

TPhT: Triphenyltin

TPhTCl: Triphenyltin Chloride

US-EPA: United States Environmental Protection Agency

VDSI: *Vas Deferens* Sequence Index

WHO: World Health Organization

ww: wet weight

WWTPs: Waste Water Treatment Plants

YES assay: Yeast Estrogen Screen

YAS assay: Yeast Androgen Screen

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

"The only thing that can be said with certainty about the future of this field of ecotoxicology is that predicting it is foolish; the chances of being right are very slim. Instead, it seems to me likely that unexpected discoveries will probably have more influence on the field of endocrine disruption than the outcomes of all the planned experiments."

Sumpter (2003)

1.1. ENDOCRINE DISRUPTION - AN OVERVIEW

The first public concerns that man-made chemicals were affecting wildlife where raised by Rachel Carson in her book Silent Spring, a milestone work in the environmental sciences field (Carson, 1962). Carson's work disclosed the link between the exposure to DDE[1] (DDT[2] major metabolite) and egg shell thinning in birds. Since then many works reporting adverse effects of chemicals in humans and wildlife were published and in the early 90's Theo Colborn and others gathered the available information and raised one of the most important hypotheses in the environmental sciences - the "*Endocrine Disruption Hypothesis"* (Colborn et al., 1993; Colborn et al., 1996). As they refer in the book *Our Stolen Future* (1996), "for all [hormone-dependent] systems, normal development depends on getting the right hormone messages in the right amount to the right place at the right time. As this elaborate chemical ballet rushes forward at a dizzying pace, everything hinges on timing and proper cues. If something disrupts the cues during a critical period of development, it can have serious lifelong consequences for the offspring." They suggested that some man-made chemicals could be the responsible factors; designated as Endocrine Disrupting Chemicals (EDCs), they became major players in the concerns of the scientific community. Four key observations in wildlife confirmed this hypothesis (Colborn et al., 1996; EC, 2007):

[1] DDE: Dichlorodiphenyldichloroethylene

[2] DDT: Dichlorodiphenyltrichloroethane

(i) Egg-thinning effect: this phenomenon was responsible for a poor reproductive success in some bird species that were exposed to pesticides, particularly DDT. Disturbed nesting behavior and beak and skeletal abnormalities have also been noted in other species exposed to high levels of environmental chemicals (Carson, 1962; Ratcliff, 1967, 1970).

(ii) Imposex in marine gastropods: in the early seventies Blaber (1970) noticed the occurrence of a penis in *Nucella lapillus* females - a neogastropod abundant in the European Coast, at about the same time on the other side of the Atlantic, Smith (1971) also noticed the same phenomena in *Nassarius obsoletus* females. This strange occurrence was coined as imposex and defined as "*superimposition of male characters (penis and vas deferens) into functionally normal prosobranch females"* (Smith, 1971). A decade later, it was linked to tributyltin (TBT) exposure, a biocide largely used in antifouling paints (Smith, 1981a, b).

(iii) Feminized fish in rivers receiving effluents from waste water treatment plants (WWTPs): altered gonadal development, occurrence of ovotestis, induction of vitellogenesis, reproductive abnormalities and reduced reproductive success have been reported in male fish exposed to treated sewage effluents around Europe (e.g. Purdom et al., 1994; Sumpter & Jobling, 1995; Harries et al., 1997; Jobling et al., 1998; Routledge et al., 1998). Alkylphenols and natural or synthetic estrogens were suspected to be causative factors for these feminizing effects (Sumpter, 2005).

(iv) Abnormalities of the reproductive system in alligators: in the early 90's Guillette and co-workers (1994) noticed that alligators from Lake Apopka (Florida, USA) presented a variety of sexual organ anomalies, including reduced penis size in males, that lead to a tremendous population decline. Several hypotheses were raised to explain the phenomena but finally the team concluded that it was a consequence of a chemical spill that occurred in that lake. Alligators were feeding on fish contaminated with dicofol and high levels of this compound and its metabolites were detected in their eggs, serum and body tissues (Guillette et al., 1994).

(v) Marine mammals: The best evidence comes from the field studies on Baltic grey and ringed seals and from the semi-field studies on Wadden Sea harbour seals, where both reproduction and immune functions had been impaired by PCBs[3] present in the food chain. Effects upon reproduction (e.g. reduced fertility, uterine occlusions resulting from abortions, uterine smooth muscle tumors) resulted in population declines, whereas suppression of immune function has likely contributed to the mass mortalities due to morbillivirus infections (CSTEE[4], 1999).

Such cases provide a clear and unequivocal link between chemical exposure and the occurrence of deleterious effects in wildlife. In recent years, the field of endocrine disruption has deserved huge attention and therefore many other examples of endocrine disruption in wildlife are available (deFur et al., 1999; WHO, 2002; Hotckiss et al., 2008). Considering that more than 100 000 known man-made chemicals are used in everyday life (Thain et al., 2008) and that they end up in the environment, it is expected that, in the future, much more chemicals will be identified as endocrine disruptors. The European Commission has defined a provisional list of more than 500 priority substances (EC[5], 2007) and preliminary investigations have begun (for a list of EC projects related to this topic see: ec.europa.eu/research/ endocrine/index_en.html).

It is clear that endocrine disruption in wildlife is a widespread phenomenon and as Guillette (2006) refers "the questions being addressed today are not whether endocrine disruption occurs because of contaminant exposure but rather at what concentrations does it occur? Or, what is the mechanism of action driving the response? Or, is the response observed adverse at the population level?"

In the present thesis we will try to provide some answers to those questions, using mainly organotin compounds (and tributyltin in particular) as a model.

[3] PCBs: Polychlorinated biphenyls

[4] Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of the European Commission

[5] EC: European Commission

1.2. Endocrine Disrupting Chemicals – definition and examples

Several definitions of Endocrine Disrupting Chemicals have been proposed and here we will consider the one by the Working Group on Endocrine Disrupters under the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of the European Commission (1999):

> *"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations."*

According to the CSTEE, the alteration of endocrine function caused by an endocrine disrupter may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior. Chemicals in the environment can be endocrine disrupters that mimic, enhance (an agonist) or inhibit (an antagonist) the action of hormones. Dose, body burden, timing, frequency and duration of exposure at critical periods of life are important considerations for assessing adverse effects of an endocrine disrupter (CSTEE, 1999).

According to the European Commission (EC, 2007) the major categories of EDCs include:

(i) Natural hormones: excreted by animals and humans, they reach the aquatic environment directly or indirectly trough wastewater treatment plants (WWTPs). Estradiol for example is, among other steroid hormones, responsible for the feminization in fish downstream the release of WWTPs effluents (Jobling et al., 1998).

(ii) Natural chemicals: include toxins produced by plants and certain fungi, as for instance the phytoestrogens, such as genistein or coumestrol (EC, 2007).

(iii) Synthetic hormones: produced by man and intended to be highly hormonally active. Examples include ethinylestradiol (the ingredient of the contraceptive pill) that is usually detected in WWTP effluents and was linked to fish feminization (Jobling et al., 1998) and trenbolone, a synthetic androgen, administrated to cattle in some countries that was linked to masculinization of fish in locations receiving cattle feedlot effluent (Wilson et al., 2002).

(iv) Man-made chemicals and by-products released into the environment:

Pesticides/Insecticides/Fungicides: like DDT and metabolites (DDE and DDD); other chlorinated compounds and some organotins such as tributyltin and triphenyltin.

Industrial chemicals: such as PCBs and dioxins, brominated flame retardants (BFRs), among others.

Chemicals in some consumer and medical products: some plastic additives (Phthalates and Bisphenol-A), surfactants (alkylphenols), and personal care products in which parabens, UV-screens and synthetic musks are included.

Although the hormonal activity of synthetic chemicals referred in the previous paragraph (Section iv) is much weaker than the natural hormones, their widespread distribution renders them an important role in endocrine disruption in wildlife. Furthermore, in environmental matrices chemicals are present in complex mixtures and a “something from nothing” problem can arise. That is to say, individual chemicals are present at concentrations below their individual NOEC (No Observed Effect Concentration) level, but when put together they act synergistically rending the mixture active at endocrine level (Rajapakse et al., 2002; Silva et al., 2002). However, complex mixtures may also include chemicals with antagonistic properties that render the study of

mixtures effects even more difficult (Sumpter & Johnson, 2005). Hence, for a characterization of endocrine disruption in wildlife an integrated approach using both biomarkers as well as analytical quantification of the chemicals present in the environment is necessary.

1.3. THE CASE STUDY OF ORGANOTIN COMPOUNDS AND TRIBUTYLTIN (TBT)

"TBT is the most toxic xenobiotic ever produced and deliberately introduced into the environment" Goldberg (1986)

1.3.1. Properties and uses

Organotin compounds (OTs) are organometals characterized by a tin (Sn) atom covalently bound to one or more organic substituents (e.g. methyl, ethyl, butyl, propyl, phenyl, octyl) (Hoch, 2001). Chemically these compounds are represented by the general formula *R*Sn*X,* in which *R* is an organic alkyl or aryl group and *X* is an organic (or sometimes inorganic) ligand as, for instance, halide, oxide, hydroxide or acetate (Hoch, 2001; Sekizawa et al., 2003).

The properties of organotin compounds vary significantly, depending mainly upon the number and nature of the *R* groups, but also upon the type of ligand (*X*). The toxicity of OTs is strongly influenced by the number and nature the organic groups. In general, inorganic tin is non-toxic whereas tri-substituted compounds have maximum toxicological activity (Hoch, 2001; Sekizawa et al., 2003).

Organic forms of tin have been known since the middle of the eighteen century. Their commercial applications were widely recognized only after the 1940's when the plastic industry, especially the one linked to the production of polyvinyl chloride (PVC), started to expand. About 70% of the total annual organotin production is used as additives for thermal and light stabilization in the plastics industry as well as catalysts for polyurethane foams and silicones (Hoch, 2001).

The finding of the biocidal properties of tri-substituted OTs in the late 1950's broadened their applications and in the 1970's they became the active ingredient in

antifouling paint (AF) formulations (Sekizawa et al., 2003) which lead to its ubiquitous presence in the marine environment. However, the sources of organotin compounds are not limited to the leaching from AF paints and their use as pesticides/fungicides in agriculture is also important. Table 1.1 discloses the main applications of organotin compounds.

Table 1.1. Main commercial application of organotin compounds, adapted from Hoch (2001).

Application	Function	General Formula
PVC Stabilizers	Stabilization against decomposition by heat and light	R_2SnX_2, $RSnX_3$
Antifouling Paints	Biocide	R_3SnX
Agrochemicals	Fungicide, insecticide, miticide, antifeedant	R_3SnX
Wood preservation	Insecticide, fungicide	Bu_3SnX
Glass treatment	Precursor for SnO_2 films on glass	Me_2SnX_2
Material protection	Fungicide, algaecide, bactericide	Bu_3SnX
Impregnation of textile	Insecticide, antifeedant	Ph_3SnX
Poultry farming	Dewormer	Bu_2SnX_2

1.3.2. Adverse biological effects

"A biological effect may be defined as the response of an organism, a population, or a community to changes in its environment, man-made or natural." Thain et al. (2008).

The harmful effects of TBT were first recorded in Bay of Arcachon, an enclosed bay located in the French Atlantic coast with abundant oysters farming facilities. Oyster production was severely affected in the 1970's and early 1980's due to severe growth problems including shell calcification anomalies that were followed by a complete lack of reproduction (see review by Alzieu, 2000). Such problems were associated with the numerous marinas located on the bay that were releasing high amounts of TBT into the aquatic environment. It was found that this pollutant was responsible for the shell chambering and also for an abnormal larval growth and larval mortality (Alzieu, 2000).

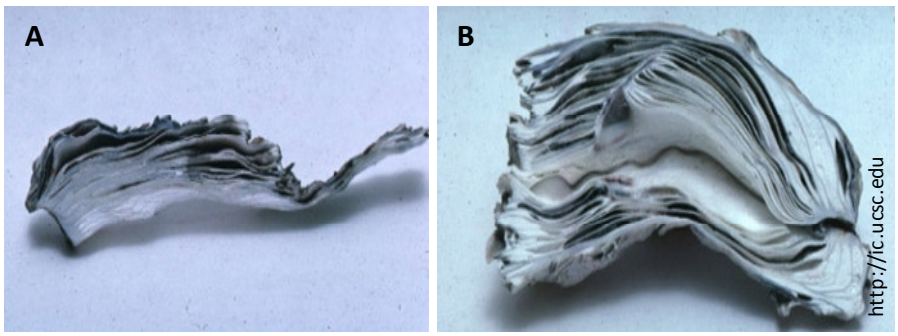

Figure 1.1. Normal oyster shell (A) and deformed one with intense chambering (B) as a result of TBT exposure.

In the beginning of the 1980's, TBT was linked to a sexual disorder recorded in some gastropods species in the United States, England and France (Blaber, 1970; Smith, 1971, 1981a, 1981b; Poli et al., 1971). Females of *Nassarius obsoletus* (in the USA) and *Nucella lapillus* (in England) were affected by imposex. In some species, the development of the *vas deferens* can block the vulva leading to female sterilization. Such was the case of *N. lapillus* females that experienced dramatic population declines that ultimately lead to its extinction at heavily TBT polluted locations (Bryan et al., 1986; Bryan et al., 1987; Gibbs et al., 1988).

Thus, imposex is regarded as an example of endocrine disruption which effects are notorious at the individual and also at the population and community levels. It has been reported to occur in more than 200 gastropod species worldwide (Shi et al., 2005) and it has been used as a fairly specific biomarker of TBT pollution. A detailed description concerning imposex expression and the possible mechanism underlying its induction/ development is provided in the next section.

Molluscs are among the most sensitive groups to TBT with the above mentioned examples as the most notorious ones; however, TBT is extremely toxic towards a wider range of organisms, from bacteria to mammals, including humans. A brief overview of some deleterious effects of TBT is presented:

Effects on bacteria: TBT is toxic to bacteria, being Gram positive usually more susceptible than Gram negative ones (WHO, 1990; Mendo et al., 2003).

Effects on phytoplankton: TBT reduce growth of marine microalgae at low concentrations, alters the photosynthetic pigment content, among many other harmfull effects that can even alter the community structure (Beaumont & Newman, 1986; Petersen & Gustavson, 1998; Sidharthan et al., 2002).

Effects on crustaceans: TBT reduces reproductive performance, neonate survival, and juvenile growth rate in crustaceans (WHO, 1990; Takeuchi et al., 2001; Aono & Takeuchi, 2008).

Effects on plants: TBT impairs the development of motile spores of some macroalgae and reduces the growth of several marine angiosperms (WHO, 1990). Recent experimental results also demonstrated that TBT bioaccumulate and induce stress in terrestrial plants used for human consumption such as onions, potatoes and lettuce (Caratozzolo et al., 2007; Lespes et al., 2009).

Effects on fish: TBT causes masculinization in fish and induces sperm abnormalities (McAllister & Kime, 2003; Shimasaki et al., 2003), it is also neurotoxic to some marine fishes (e.g. *Sebastiscus marmoratus*) through the modulation of the glutamate signalling pathway (Zhang et al., 2008; Zuo et al., 2009).

Effects on mammals: TBT reduces spermatogenesis in mice (Chen et al., 2008), causes changes in rat behavior (Ohtaki et al., 2007), suppresses the osteoclastogenesis trough the retinoic acid receptor (RAR) pathway (Yonezawa et al., 2007) and induces adipose tissue differentiation and obesity (Grün & Blumberg, 2006), among many other effects (see Antizar-Ladislao (2008) for complete review).

1.3.3. Retrospective view of regulations in Europe

France took the first restrictive initiative in 1982, motivated by the collapse of the oyster farming culture due to TBT pollution in Arcachon bay. The French government restricted the use of TBT on small boats (<25m) and four years later the United Kingdom banned the sale of TBT based antifouling paints for small boats and aquaculture structures due to the severe ecological impact of this pollutant on gastropods populations (see review by Alzieu, 1998). At that time, several reports linked TBT to the imposex phenomenon (see previous section). Such evidences drove other countries to adopt similar restrictions. In 1989, the European Union introduced the Directive 89/677/EEC banning the use of TBT and TPT on small boats (<25 m). This directive was afterwards implemented by each member state. In Portugal, for example, the Directive was transposed to the internal law in 1993. However, the first initiative was adopted one year before (1992) when TBT and TPT based AF paints were banned from the Portuguese Navy fleet.

Following the introduction of the TBT partial ban, some recovery of severely affected gastropods and oysters populations was noticed (Evans et al., 1995; Minchin et al., 1995; Evans et al., 1996; Alzieu, 1998) however this recovery was not generalized. Several reports described that imposex and TBT levels around ports were not decreasing and that those locations were hotspots of TBT pollution (Bailey & Davies, 1989; Davies & Bailey, 1991; Minchin et al., 1995; Minchin et al., 1996; Morgan et al., 1998). Further studies demonstrated that TBT pollution was not restricted to harbor areas and populations from the open ocean as well as the ones from open coastal areas were also affected (Ten Hallers-Tjabbes et al., 1994; Mensink et al., 1996; Barreiro et al., 2001; Michel et al., 2001). In Portugal, the partial ban was also not effective on reducing TBT pollution; in fact an increase on *Nucella lapillus* imposex levels was reported between 1995 and 2000 (Barroso & Moreira, 2002; Santos et al., 2002a).

Given all the scientific evidences that a partial ban was not effective on reducing TBT environmental levels and recognizing the need to protect the marine environment, the International Maritime organization (IMO) adopted, in 2001, a total ban on the use of TBT based AF paints trough the 'International Convention on the Control of Harmful

Antifouling Systems on Ships' (AFS Convention) from 2003 onwards. This convention, however, could only enter into force one year after 25 states (representing 25% of the world's merchant shipping tonnage) have ratified it. This was achieved in September 2007, when Panama became the 25th state ratifying the Convention (Gipperth, 2009).

Due to the slow ratification process and to promote the implementation of the AFS Convention in the member states, the European Union moved to the adoption of Directive 2002/62/EC and Regulation 782/2003 imposing the total interdiction of OTs AF paints application on EU ships after the 1st of July 2003 and the presence of TBT AF paints on ships' hulls from the 1st of January 2008.

The AFS convention entered into force on the 1st September 2008 and from that date onwards organotins were banned from antifouling paints formulations. A summary of the regulations concerning organotins is provided in Figure 1.2.

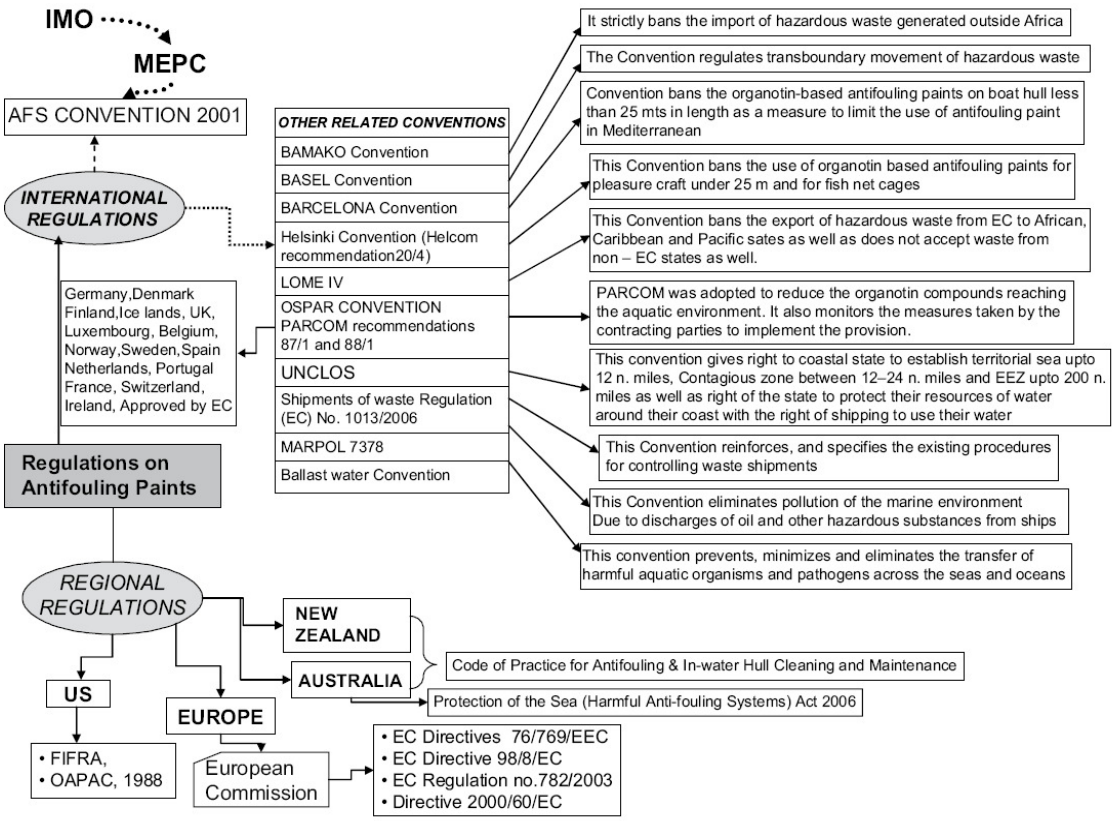

Figure 1.2 Overview of existing regulations concerning organotin compounds. Adapted from Sonak et al. (2009).

The European Commission most recent initiative regarding organotin compounds (Decision 2009/425/EC) was taken in May 2009 in order to restrict their use in other applications than antifouling paints. Dioctyltin compounds, for instance, won't be allowed after 1 January 2012, as constituent in consumer products such as textile articles, gloves, footwear, female hygiene products, childcare articles, nappies, in a concentration greater than the equivalent of 0.1% by weight of tin.

1.4. IMPOSEX AS A BIOMARKER OF TBT POLLUTION

"Imposex is the best documented example of endocrine disruption in wildlife."
Mathiessen & Gibbs (1998)

1.4.1. History and classification schemes

As referred in section 1.3.3, Blaber recorded, in 1970, the appearance of a penis in females of the gastropod *Nucella lapillus* collected in Plymouth Sound, United Kingdom (Blaber, 1970). The severity of this condition was higher inside harbors and lower further away but the causative agent was unidentified. One year later, Smith (1971) also noticed the appearance of a penis and *vas deferens* in *Nassarius obsoletus* females in the United States. He coined the phenomenon as imposex but at that time the cause was still unknown. One decade later the same author concluded that TBT was the responsible agent (Smith, 1981a, 1981b). Later, Smith's work was confirmed by Bryan and co-workers through a series of laboratory and field transplant experiments using *N. lapillus* (Bryan et al., 1987). The same team proposed the use of imposex in the dogwhelk to monitor TBT pollution (Gibbs et al., 1987). Several indices were proposed to evaluate the degree of imposex within a population: percentage of affected females (%I); relative penis size index (RPSI) defined as the ratio between female penis size (FPL) and male penis size (MPS): RPSI=(FPS*100/MPS) and the *vas deferens* sequence index (VDSI). For *N. lapillus* Gibbs and co-workers (1987) proposed a VDSI scheme with 6 stages with stage 0

corresponding to unaffected females and stage 6 corresponding to sterile ones. Table 1.2 and Figure 1.3 describe the VDSI classification scheme develop for *N. lapillus*.

Table 1.2. *Vas deferens* sequence index (VDSI) stages in *N. lapillus* according to Gibbs et al. (1987).

VDSI	Imposex development
0	Unaffected female with no apparent male characters. The genital papilla and the vulva are clearly visible with no development of the *vas deferens* tissue.
1	An infolding of the mantle cavity epithelium in the area ventral to the genital papilla marks the first development of the *vas deferens*.
2	Initiation of penis development by the formation of a ridge behind the right tentacle.
3	The ridge becomes recognizable as a small penis and development of the *vas deferens* from the base of the penis commences.
4	The two sections of *vas deferens*, originating at the genital papilla and the base of the penis, become fused into a continuous tube. The penis is larger and closer to the male penis size and shape.
5	The proliferation of vas deferens tissue (hyperplasia) about the genital papilla results in occlusion of the vulva, and the genital papilla becomes displaced, constricted or no longer visible.
6	Lumen of the capsule gland contain the material of aborted capsules (6A) that may be compressed together to form a dark brown mass (6B).

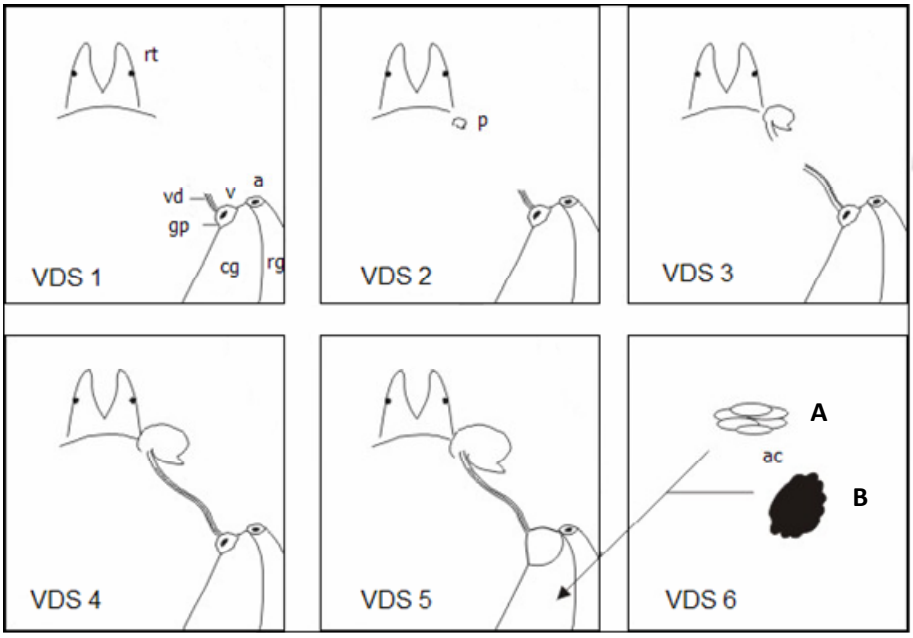

Figure 1.3. *Vas deferens* development in *N. lapillus* according to Gibbs at al. (1987). Stage 0 corresponds to unaffected females whereas stage 6 corresponds to the accumulation of aborted egg capsules in the capsule gland. A: anus; ac: aborted capsules; cg: capsule gland; gp: genital papilla; p: penis; rg: rectal gland; rt: right tentacle; v: vulva; vd: *vas deferens*.

1.4.2. TBT pollution monitoring

The OSPAR[6] Commission adopted the use of imposex in *N. lapillus* to monitor TBT pollution levels (OSPAR, 2003). Due to its absence in some coastal areas, the Commission also recommends the use of others species for monitoring the effects of TBT, namely: *Nassarius reticulatus, Buccinum undatum, Neptunea antiqua* and *Littorina littorea* (OSPAR, 2003). In Table 1.3, the criteria for *Nucella* are presented alongside with the equivalent VDSI/Intersex Index (ISI) values for those species. The relationships between species were estimated from correlations obtained from comparison of effects in sympatric populations in the field. The criteria enable consideration of the likely effects on *N. lapillus* based on effects in other species and allow the adoption of a consistent approach over the whole OSPAR region (OSPAR, 2004).

Table 1.3. Biological effect assessment criteria for TBT prepared and agreed during the technical TBT workshop, 6-7 November 2003, The Hague (OSPAR, 2003). Assessment criteria for imposex in *Nucella lapillus* are presented alongside equivalent VDSI/ISI values for sympatric populations of other relevant species. Adapted from OSPAR (2004).

Assessment class	***Nucella***	***Nassarius***	***Buccinum***	***Neptunea***	***Littorina***
	VDSI	VDSI	PCI	VDSI	ISI
A	< 0,3	< 0,3	< 0,3	< 0,3	< 0,3
B	0,3 - <2,0			0,3 - <2,0	
C	2,0 < 4,0	0,3 < 2,0	0,3 < 2,0	2,0 - 4,0	
D	4,0 - 5,0	2,0 - 3,5	2,0 - 3,5	4,0	0,3 - < 0,5
E	>5,0	> 3,5	> 3,5		0,5 - 1,2
F	-				> 1,2

As in the present thesis *N. reticulatus* is going to be used as bioindicator, a detailed description of imposex in this nassarid is provided in the next section.

[6] OSPAR: Convention for the Protection of the Marine Environment of the North-East Atlantic.

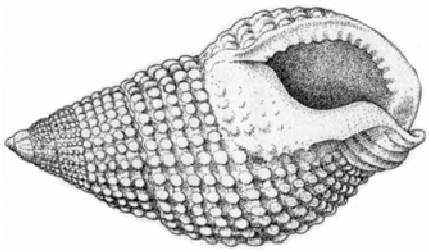

Figure 1.4. *Nassarius reticulatus* shell. Adapted from Hayward & Ryland (1995).

1.4.3. Imposex in *Nassarius reticulatus* as a biomarker of TBT pollution

The marine netted whelk *Nassarius reticulatus* (L.) is a ubiquitous nassarid gastropod in European coastal areas from the Canaries and Azores to Norway and throughout the Mediterranean and Black Seas (Fretter & Graham, 1994). It has been successfully used in several TBT biomonitoring programs along European coastlines [Table 1.4]. In Portugal, this species is commonly found at sheltered and shallow (including intertidal) sites and is particularly abundant in estuaries (Barroso et al., 2002a). It is also widespread in offshore waters and has been used to map TBT pollution in those areas (Rato et al., 2006; Rato et al., 2008).

Table 1.4. Studies reporting the occurrence of imposex in *N. reticulatus* throughout Europe.

Country	Study area	References
England	Coastal areas in south-west England	Bryan et al., 1993
Germany	Coastal areas	Martina et al., 2007 and references therein
France	Coastal areas in Brittany	Stroben et al., 1992b, 1992a; Oehlmann et al., 1993; Huet et al., 1995; Wirzinger et al., 2007
Spain	Coastal areas North Atlantic: Galician and Basque coast	Barreiro et al., 2001; Barreiro et al., 2004; Ruiz et al., 2005; Quintela et al., 2006; Ruiz et al., 2008; Couceiro et al., 2009; Rodríguez et al., 2009
Portugal	Coastal areas along the continental Portuguese coast	Gibbs et al., 1997; Barroso et al., 2000; Barroso, 2001; Pessoa et al., 2001; Barroso et al., 2002a; Barroso et al., 2005; Sousa et al., 2005; Sousa et al., 2007; Rato et al., 2009; Sousa et al., in press
Portugal	Offshore areas	Santos et al., 2004; Rato et al., 2006; Rato et al., 2008

The use of *N. reticulatus* to monitor TBT pollution was proposed by Stroben and co-workers in the early nineteen's (Stroben et al., 1992b). The species is less sensitive than *N. lapillus* (Stroben et al., 1992a) and sterility only occurs in extreme cases. In fact, sterile *N. reticulatus* females were not detected in the first surveys and only in 1995 sterility was described for the first time (Huet et al., 1995). The following indices are used to evaluate imposex in *Nassarius reticulatus*:

(%I): Percentage of affected females, i.e., number of affected females*100/total females. This index is useful at low polluted locations; in highly polluted ones its usefulness is dubious since usually all females are affected by imposex and no distinction between locations can be performed.

FPL: Female Penis Length (mm). Important parameter in low, medium and highly polluted locations as it enables the distinction between sites with similar pollution levels, allowing for example, the distinction between heavily polluted ones.

RPLI: Relative Penis Length Index; RPLI=(FPL*100)/MPL[7]. As for FPL this parameter is very useful in the entire pollution spectrum.

AOS: Average Oviduct Stage. This index proposed by Barreiro et al. (2001) assesses the degree of oviduct masculinization. In normal females the oviduct is a straight line whereas in highly affected ones it gets convoluted resembling the male *vesicula seminalis*. Useful at moderate/ highly polluted locations since in low contaminated ones the AOS value is usually zero.

Oviduct Stage 0: females with a normal straight oviduct,

Oviduct Stage 1: females whose oviduct is slightly sinuous,

Oviduct Stage 2: females exhibiting a clearly convoluted gonadial oviduct.

VDSI: *Vas deferens* sequence index. Classifies the degree of female's masculinization and was considered as the most valid index to assess TBT pollution as, in extreme cases, it can express the reproductive capacity of the population (Stroben et al., 1992b).

[7] MPL: male penis length (mm).

Table 1.5 and Figure 1.5 provide a full description of VDS classification scheme. *N. reticulatus* exhibits two different types of imposex expression: Type a, and Type b according to the presence or absence of a penis, respectively.

Table 1.5. *Vas deferens* sequence index (VDSI) stages in *N. reticulatus* according to Stroben at al. (1992b)

VDSI Stages	Imposex development
0	Unaffected female with no apparent male characters.
1a	Tiny penis without a penis duct behind the right ocular tentacle.
1b	No penis, but a short, distal *vas deferens* tract behind the right ocular tentacle.
2	Penis with a closing or closed penis duct behind the right ocular tentacle.
3a	Penis with penis duct continuing in an incomplete distal tract of the *vas deferens* that is growing out successively towards the vaginal opening.
3b	Penis lacking; *vas deferens* running continuously from the right ocular tentacle over the botton of the mantle cavity up to the vulva (vaginal opening).
4	Penis with a penis duct and a continuous *vas deferens* from the penis up to the vulva
4+	The *vas deferens* passes the vaginal opening and runs into the ventral channel of the capsule gland.

Stage 4+ was considered as the end of imposex development in the nassarid (Stroben et al., 1992b), however some authors proposed the computation of stage 4+ to 4.5 (Barreiro et al., 2001) or to 5 (Barroso et al., 2002a) in order to better discriminate between highly polluted locations. In the present thesis Stage 4+ will be considered as Stage 5 as proposed by Barroso et al. (2002a).

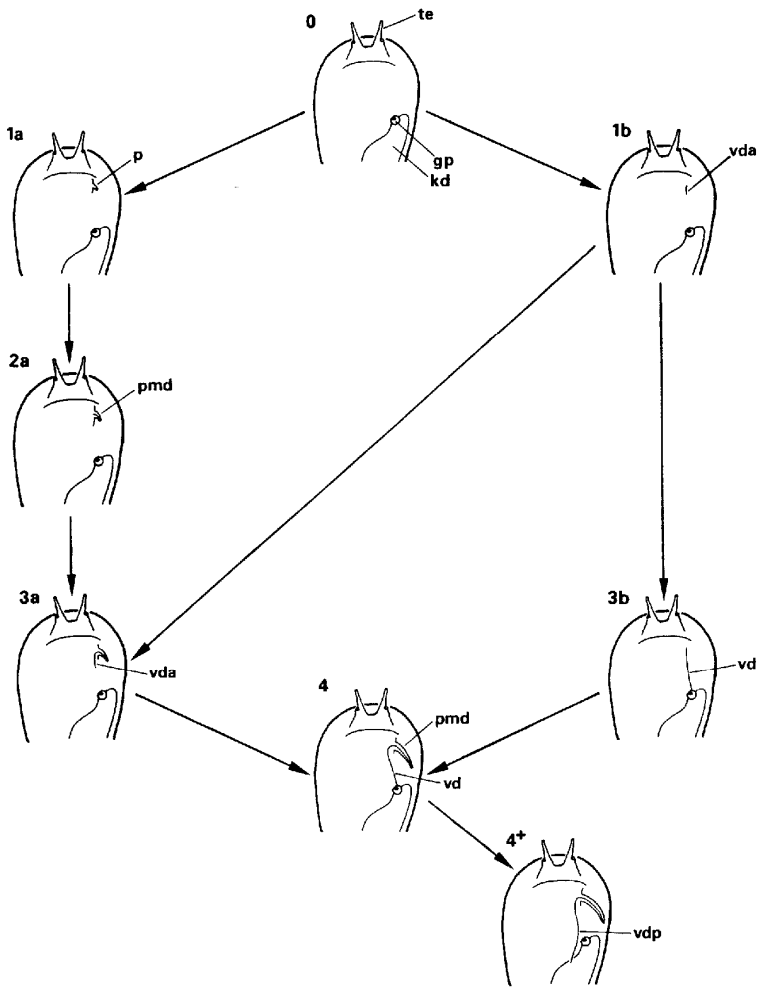

Figure 1.5. Imposex classification scheme for *N. reticulatus* according to Stroben at al. (1992b). gp: genital papilla; kd: capsule gland; p: penis without duct; pmd: penis with duct; te: tentacle; vd: *vas deferens*; vda: *vas deferens* section; vdp: *vas deferens* passes vaginal opening to run into capsule gland. Adapted from OSPAR (2008).

1.4.4. Mechanisms underlying imposex induction

Several hypotheses have been postulated in order to explain the imposex induction mechanism and three possible pathways have been identified: the neuroendocrine, the steroid and the retinoic. An overview of each pathway is given bellow.

Steroid pathway

Several studies suggested that TBT is responsible for an imbalance in testosterone/ estradiol ratio and that this disruption in hormone homeostasis could lead to imposex development. According to Castro et al. (2007) two different pathways for such disruption can be recognized: (i) interference with steroid biosynthesis, or (ii) interference with steroid excretion.

(i) Steroid biosynthesis: Spooner et al. (1991) suggested that TBT inhibits the cytochrome P450-dependent aromatase (CYP19), responsible for the aromatization of androgens to estrogens, with the consequent elevation of testosterone levels. Later, Bettin and co-workers (1996) confirmed a positive correlation between imposex stages and testosterone (T) levels in wild-caught *N. lapillus* and *N. reticulatus*. For both species, a dose- and time-dependent increase of T titers under TBT exposure in the laboratory was reported. The authors showed that the administration of an aromatase inhibitor was also able to induce imposex.

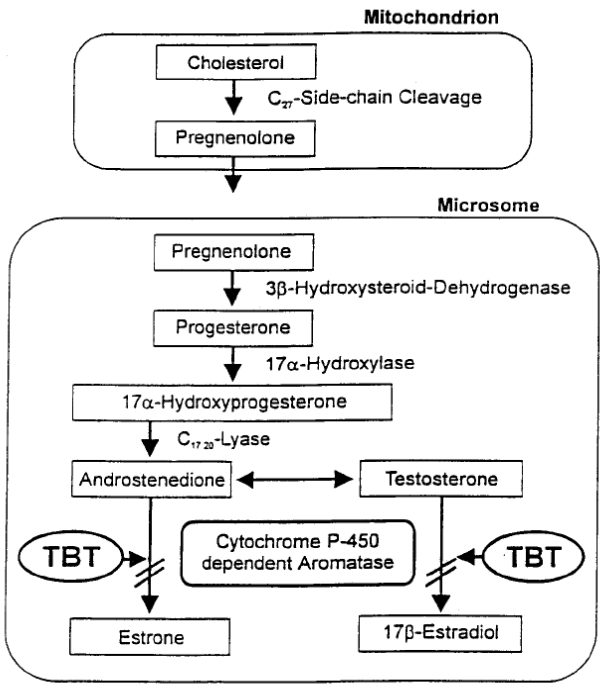

Figure 1.6. Scheme of biosynthesis of steroid hormones with possible target of TBT. Adapted from Bettin et al. (1996).

This theory has been supported by several studies that reported an elevation of testosterone and/or testosterone/estradiol ratio in snails exposed to TBT in the field and in the laboratory (Spooner et al., 1991; Bettin et al., 1996; Barroso et al., 2002b; Santos et al., 2002b; Barroso et al., 2005).

Despite such evidences some authors consider that this theory cannot explain the imposex induction as the results obtained with aromatase inhibitors are different from the ones obtained with TBT (see Horiguchi, 2006, 2009 for further details). In *N. lapillus* for instance, a selective aromatase inhibitor induces imposex to a significant lower extent than TBT, suggesting that aromatase inhibition may not be the primary mechanism involved in the imposex development (Santos et al., 2005).

(ii) Inhibition of testosterone excretion: Ronis & Mason (1996) demonstrated that TBT decreased the amount of testosterone sulphur-conjugates in *Littorina littorea* leading to an increase in levels of free testosterone in the tissues. However, this experiment was conducted with extremely high concentrations of TBT, which are already acutely toxic; furthermore the duration of the experiments was short (42 h) and the authors didn't performed analysis on endogenous testosterone levels. Some further support to this theory was provided by Gooding et al. (2001; 2003). Those authors demonstrated that TBT decreases the esterification of testosterone with fatty acids in *Ilyanassa obsoleta* leading to an increase in free testosterone which could then induce imposex. Further experimental support to this theory is provided by Santos and co-workers (2005) and Janer et al. (2006; 2007) for *N. lapillus* and *Marisa cornuarietis*, respectively.

Neuroendocrine pathway

The neuroendocrine pathway was initially proposed by Féral & Le Gall (1983). The team conducted a series of *in vitro* experiments and suggested that TBT inhibits the release of a neuroendocrine factor (Penis Regression Factor- PRF) from the pleural ganglia

which is responsible for the suppression of penis formation in females leading to imposex development. However those authors did not find any effects of TBT on the formation of Penis Morphogenic Factor (PMF), a molecule expressed in all prosobranch snails irrespective of their sex (adapted from Oehlmann et al., 2007). In 2000, Oberdörster & McClellan-Green (2000) demonstrated that the neuropeptide APGW amide induces imposex in the gastropod *Ilyanassa obsoleta* and suggested that it could the PMF. Further studies were published that support this theory (Oberdörster & McClellan-Green, 2002; Oberdörster et al., 2005) and a model for imposex induction based both on changes in peptide hormones and steroid hormones was proposed [Figure 1.7].

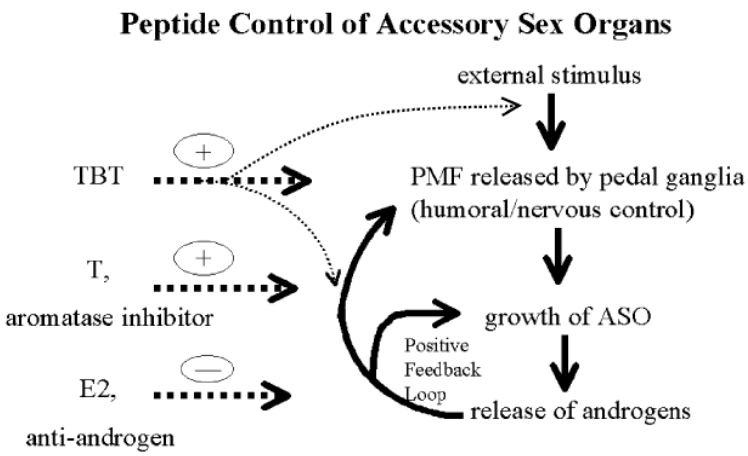

Figure 1.7. Mechanism of imposex induction by TBT and steroids. TBT acts in the nervous system, while steroids act on the positive feedback loop which maintains the Accessory Sex Organs (ASO). + indicates imposex induction while - indicates imposex inhibition. Adapted from Oberdörster & McClellan-Green (2002).

Oberdörster and colleagues (2002) suggested that: “the neurohormone, PMF, induces initial Accessory Sex Organ (ASO) growth. The ASO then releases androgens, possibly testosterone, to maintain the ASO and spermatogenesis through a positive feedback loop. TBT is a neurotoxin that can possibly cause the abnormal release of PMF, leading to ASO growth. Exogenously administered steroid hormones and their agonists could either induce or inhibit imposex by acting as components in the positive feedback loop.”

The neuropeptide hypothesis was also evaluated in other species; however APGW amide failed to promote imposex in *Bolinus brandaris* (Santos et al., 2006) and in *N. lapillus* (Castro et al., 2007).

Retinoic pathway: the involvement of Retinoic X Receptor (RXR) on imposex induction was first reported by Nishikawa et al. (2004). Those authors injected the proposed natural ligand of RXR (9-cis-Retinoic Acid: 9CRA) into *Thais clavigera* females and reported a significant induction of imposex 30 days after 9CRA injection (1 $\mu g.g^{-1}$ ww). The imposex response in 9CRA treatment was similar to the one obtained in TBT treatment. Those results were further confirmed for the same species through a series of laboratory experiments (Horiguchi et al., 2007a) and also through the assessment of RXR gene expression and protein content in wild animals (Horiguchi et al., 2007a). The involvement of the RXR on imposex induction has also been confirmed in *N. lapillus* (Castro et al., 2007).

According to the RXR theory, imposex development begins with the activation of a signaling cascade which is dependent of the RXR activation/inhibition. Horiguchi et al. (2007b) suggested that for the differentiation and/or growth of the penis and *vas deferens*, in *Thais clavigera*, an interaction between organotins and RXR in the limited tissue area behind the right tentacle (i.e., the penis-forming area) of the female gastropod is primarily important [Figure 1.8]. Such theory is supported by further studies revealing the occurrence of the RXR in that area on several gastropod species including *N. lapillus* and *N. reticulatus* (Horiguchi et al., 2008). The RXR receptor is also involved in the normal process of accessory sex organ development in gastropods (Castro et al., 2007; Sternberg et al., 2008).

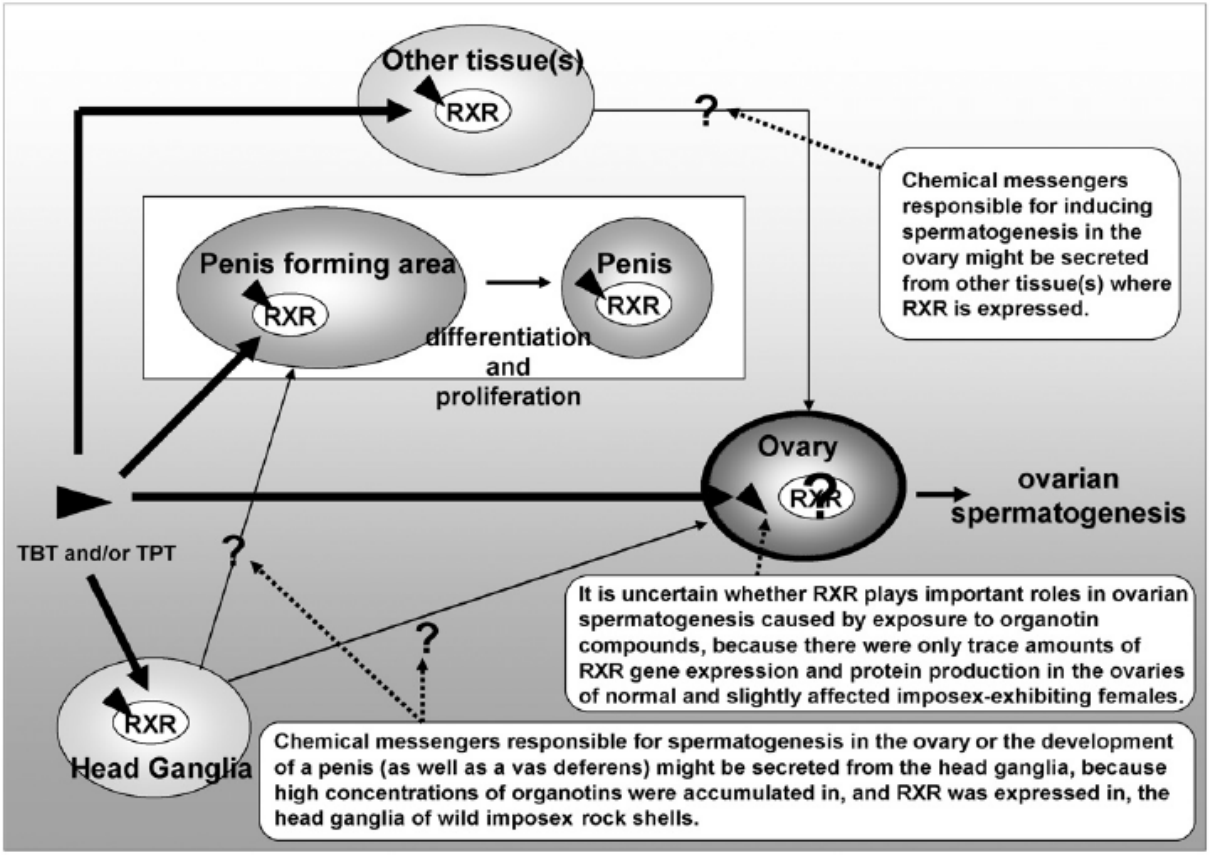

Figure 1.8. Speculative imposex induction mechanism mediated by RXR as proposed by Horiguchi et al. (2007a).

As previously mentioned, several theories attempt to explain imposex induction, however the exact mechanism is still unclear and further studies are necessary. One of the most interesting research lines is to understand the interplay between all the possible pathways in order to fully understand what triggers the imposex development. Further discussion on this topic is provided in Chapter 8 and 9.

1.5. AIMS AND RATIONALE OF THE PRESENT THESIS

The main objective of present thesis is to characterize the organotin pollution in the Portuguese coastal environment, describing both spatial and temporal trends as well as its associated biological effects. Environmental levels of organotin compounds (OTs) were assessed in mussels and gastropods along 950 Km of the Portuguese coast (from Viana do

Castelo to Faro) and also in a more restricted area - Ria de Aveiro (NW Portugal) - with the quantification of OTs in different environmental matrices: sediments, mussels and gastropods. The levels of imposex in *Nassarius reticulatus* were also assessed as it may be considered a specific biomarker for monitoring the spatial and temporal evolution of TBT pollution.

The spatial distribution of organotin pollution was studied along the coast using *Mytilus galloprovincialis* as a bioindicator species. Mussels were selected based on their unique characteristics than renders them one of the most used bioindicators of aquatic pollution throughout the world. Levels of OTs (including butyltins, phenyltins and octyltins) as well as total tin were determined in the mussel's soft tissues and tributyltin (TBT) seawater concentrations were estimated using previously reported bioconcentration factors.

Organotin levels were also quantified in the gastropod *N. reticulatus* collected along the coast and its relation with imposex levels was analyzed. The temporal evolution of TBT pollution was studied trough the comparison of the obtained results with the ones previously described for the study area, which enabled the evaluation of the effectiveness of the EC Regulation 782/2003 to reduce this pollution. Changes in imposex expression in the nassarid between 2000 and 2008 were also studied and the possible reasons for the swift towards b-type expression were analyzed.

A more detailed characterization of organotin pollution was performed in Ria de Aveiro, an estuarine system located in the NW Portuguese coast. Levels of organotins were assessed in biota (mussels and gastropods) and in sediments. Imposex in *N. reticulatus* was also used as biomarker of TBT pollution. Considering that some authors have proposed that imposex in gastropods could be affected by the presence of some xenoestrogenic compounds in the aquatic environment, we attempted to test this hypothesis. Firstly, the occurrence of such compounds in Ria de Aveiro was assessed trough chemical and biological analysis of effluents samples from waste water treatments plants discharging in the seacoast of Aveiro. After identifying the major estrogenic chemicals occurring in the aquatic environment and their levels, a series of laboratory

experiments were performed by exposing *N. reticulatus* females to a mixture of TBT and xenoestrogens (estrone, nonylphenol and bisphenol-A) in concentrations similar to those observed in the natural environment. However, the results of such experiments were highly variable and not conclusive and therefore they are not presented in this thesis. Despite the fact that no results confirming the influence of estrogens on imposex development could be obtained, we tried to study better this phenomenon by evaluating the possible biochemical mechanisms underlying the imposex development in *N. reticulatus*. Hence, a series of experiments were performed testing a new hypothesis raised in the literature regarding the involvement of retinoid X receptor (RXR) on imposex development.

1.6. ORGANIZATION OF THE THESIS

The present thesis is organized in nine chapters. The first two chapters provide an introduction to the pollution by endocrine disruptors, giving special relevance to the state of the art regarding tributyltin pollution in Portugal. Chapters 3 to 8 describe the results obtained during this study and are structured as scientific papers. Chapter 9 provides a general discussion of the results obtained. Each chapter can be briefly summarized as follows:

- **Chapter 1:** provides a brief theoretical introduction regarding the occurrence and biological effects of endocrine disrupting chemicals (EDCs) in the environment, with special relevance to tributyltin;

- **Chapter 2:** reviews the available literature concerning the status and impacts of organotin pollution in the Portuguese coast;

- **Chapter 3:** describes the concentrations of organotin compounds (OTs) in the whole tissues of mussels (*Mytilus galloprovincialis*) along the Portuguese coast and evaluates the contribution of OTs pollution to the total tin body burdens in this species;

- **Chapter 4:** describes the concentrations of organotins in different compartments (sediments, mussels and gastropods) in Ria de Aveiro and the levels of imposex in the

gastropod *N. reticulatus*. A preliminary evaluation of the effectiveness of the TBT European ban is also presented;

- Chapter 5: presents the spatial trends of imposex levels and organotin concentrations in *N. reticulatus* along the Portuguese coast in 2008 and compares this situation with 2003 in order to assess the effectiveness of the European ban to reduce organotin pollution;

- Chapter 6: describes the spatial and temporal evolution of *N. reticulatus* females exhibiting a-type *versus* b-type imposex development along the Portuguese coast between 2000 and 2008 and the possible reasons for the shift towards b-type expression are discussed.

- Chapter 7: describes the levels of key estrogenic compounds that occur in the effluents of WWTP discharged into the seacoast of Aveiro as well as the levels of these compounds in the water column at some selected reference sites. The assessment of potential estrogenicity in these waters is taken into consideration in the present thesis in order to evaluate interactions that might eventually occur between androgenic (ex.: TBT) and estrogenic EDCs upon the imposex expression of *N. reticulatus.*

- Chapter 8: presents the results of laboratory experiments aiming to investigate the role of Retinoid X Receptor (RXR) on imposex development in the main bioindicator used in the current thesis - *N. reticulatus.* The same experiments were also carried out with *Nucella lapillus* in order to compare responses between species and provide a broader understanding of the importance of RXR in imposex development among gastropods.

- Chapter 9: provides a general discussion of the results obtained in Chapters 3-8. As each of these chapters includes its own discussion material, here only a concise and global discussion of results in order to highlight synergies between the different chapters and to show the coherence of the work is presented.

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CHAPTER 2

HISTORICAL PERSPECTIVE OF ORGANOTIN POLLUTION ALONG THE PORTUGUESE COAST

CHAPTER 2. HISTORICAL PERSPECTIVE OF ORGANOTIN POLLUTION ALONG THE PORTUGUESE COAST

The sources, fate and distribution of organotins (OTs) in the coastal environment have been regularly studied in the Portuguese coast. This chapter summarizes the findings of those studies since the first reports on tributyltin (TBT) levels (in the 80's) until recent surveys (performed after the TBT ban enforcement). Special emphasis will focus upon imposex prevalence in gastropods populations.

2.1. FIRST REPORTS OF TBT POLLUTION AND ASSOCIATED BIOLOGICAL EFFECTS

In 1979 the populations of the Portuguese oyster (*Crassostrea angulata*) in the Tagus and Sado estuaries [Figure 2.1] were almost extinct; the phenomenon started in 1973, with sudden and extensive mortalities preceded by the onset of the widespread "shell-thickening" effect (see de Bettencourt et al., 1999). Several reports attempted to explain this mass mortality but it was only after a survey in 1982 that the possible cause was established when TBT was detected in Tagus estuary sediments, particularly near the dockyards (Andreae et al., 1983; Byrd & Andreae, 1986).

During the early 90's further studies on OTs levels in sediments and biota were conducted in Tagus and Sado estuaries as well as in Ria de Aveiro and Ria Formosa [Figure 2.1], all of them areas of traditional shellfish farming, that experienced a sudden production collapse (Quevauviller et al., 1989; Quevauviller & Donard, 1990; Cortez et al., 1993; Coelho et al., 2002). The results obtained clearly demonstrated that harbors (both commercial and fishing), and shipyards were responsible for the introduction of TBT into the environment (Cortez et al., 1993). Subsequently, other studies disclosed the link between shell calcification anomalies in oysters (including both *C. angulata* and *C. gigas*) and the presence of TBT in the water column (Phelps & Page, 1997; Almeida et al., 1998; Almeida et al., 1999; Barroso, 2001).

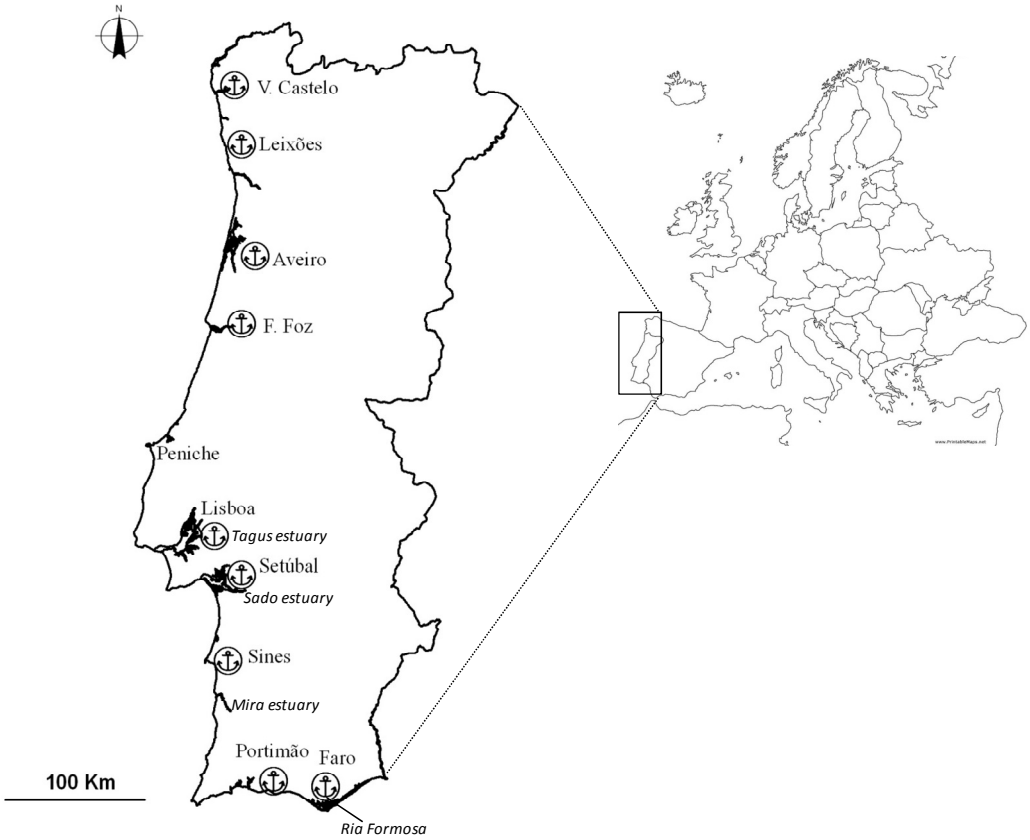

Figure 2.1. Map of the Portuguese Coast with the indication of main harbors ⚓ and some of the major estuarine systems.

2.2. IMPOSEX IN MARINE GASTROPODS

The first report of imposex in Portugal was published by Peña in 1988. The author assessed imposex levels in *Nucella lapillus* at four sites on the Northerwestern Portuguese Atlantic coast (Peña, 1988). At about the same time, Spence et al. (1990) reported the occurrence of imposex in *Thais haemastoma* populations from Azores. Later, Pessoa and co-workers published data on imposex levels and total tin concentrations in *Nassarius reticulatus* at some locations in the Sado and Mira estuaries (Pessoa & Oliveira, 1997; Pessoa et al., 2001) [Figure 2.1]. Imposex in gastropods was further investigated in the Southern coast: in 1996, Gibbs and coworkers investigated five gastropod species (*N. reticulatus*, *Hexaplex trunculus*, *Ocenebra erinacea*, *Ocinebrina aciculata* and *Columbella rustica*) at a polluted site close to Faro harbor (Ria Formosa) [Figure 2.1] and concluded

that all species exhibited imposex except for *C. rustica*, that was considered as a "zero-response" form (Gibbs et al., 1997). Langston et al. (1997) also published a report describing imposex in snails from Ria Formosa, and, one year later, Coelho (1998) published a paper about imposex in neogastropods snails from southern Portugal.

The first comprehensive study on imposex in gastropods along the coast was performed in 1995/1996 by Santos et al. (2000) that analyzed *N. lapillus* at 27 sites. The authors demonstrated that imposex in this species was widespread and that the highest levels were associated with the major commercial harbors, mainly on the northern and central coast. Later, in 1998, Barroso et al. performed an extensive survey in Ria de Aveiro, an estuarine system on the NW Portuguese coast, and adjacent coastal area (Barroso et al., 2000). Imposex levels were assessed in four gastropods species: *N. lapillus*, *N. reticulatus*, *Hydrobia ulvae* and *Littorina littorea*, and TBT concentrations were determined for water and sediment samples. All surveyed sites exhibited imposex affected females except *N. reticulatus* at some offshore locations. *N. lapillus* sterile females were found at one site. The most sensitive species was *N. lapillus* followed, in decreasing order, by *N. reticulatus*, *H. ulvae* and *L. littorea*. The same authors performed another survey between 1997-1999 assessing imposex, organotins and steroid hormone levels in *N. reticulatus* along a ship density gradient in Ria de Aveiro (Barroso et al., 2005). The authors reported that the ratio of testosterone/17β-estradiol in females tended to increase with increasing imposex and organotin contamination.

In 2000 several surveys were performed along the coast: Santos and co-workers assessed imposex levels in *N. lapillus* at the same locations studied in 1995/1996 in order to evaluate the effectiveness of the EU Regulation and concluded that TBT pollution increased over the elapsed 5 year period, indicating the inefficacy of the partial ban (Directive 89/677/EEC) (Santos et al., 2002). Barroso and co-workers sampled *N. reticulatus* at 40 sites and analyzed imposex and organotins (MBT, DBT, TBT and TPhT) in female's whole tissues (Barroso et al., 2002). Imposex was observed at 95% of the locations and sterile females were found at 8 locations with frequencies between 5-50% of the total females per site. TBT and TPhT levels varied between <20 to 1368 ng $Sn.g^{-1}$ dw and from <10 to 256 ng $Sn.g^{-1}$ dw, respectively. Highly significant correlations were

established between lnTBT and the imposex indices *vas deferens* sequence index (VDSI), average oviduct stage (AOS) and female penis length (FPL). The authors concluded that organotin pollution was, at the time, a matter of great concern, especially in estuarine systems where most harbors are located. Barroso and Moreira (2002) also evaluated the efficacy of the EEC Directive 89/677 using imposex in *N. lapillus* as a biomarker. Values obtained in the 2000 survey were compared with the ones reported in the literature and with some of the author's unpublished data from 1997. The results also demonstrated a global increase of TBT pollution, revealing the inefficacy of the legislation introduced in 1993. The authors suggested that the absence or scarcity of *N. lapillus* specimens inside harbours was probably a consequence of local extinction due to female sterilization by TBT.

Imposex and organotin levels in *N. lapillus* and *N. reticulatus* were re-assessed in 2003 by Galante-Oliveira et al. (2006) and Sousa et al. (2005), respectively. Levels of imposex in *N. lapillus* varied between 0.20-4.04, 0.0-42.2% and 16.7-100.0% for VDSI, RPSI and %I, respectively and sterile females were found at 3 sites. Levels of TBT and DBT were measured in female's tissues and varied between 23-138 ng $Sn.g^{-1}$ dw and <10-62 ng $Sn.g^{-1}$ dw, respectively (Galante-Oliveira et al., 2006). Once more, the highest imposex and TBT levels were detected at sites located in the proximity of harbors. The *N. reticulatus* survey (Sousa et al., 2005) also disclosed the same spatial pollution trend [Figure 2.2] and revealed the presence of sterile females inside Viana do Castelo and Aveiro harbors. Imposex levels varied between 0.0-5.0, 0.0-90.0%, 0.0-100.0% and 0.0-1.3, for VDSI, RPLI, %I and AOS, respectively. Butyltins (BuTs) and phenyltins (PhTs) were quantified for 10 selected locations; BuTs were detected in all analyzed samples whereas PhTs were detected at 60% of the samples. Among BuTs, TBT was dominant (47.4%) followed by DBT (27.6%) and MBT (25.0%), indicating the occurrence of recent TBT inputs.

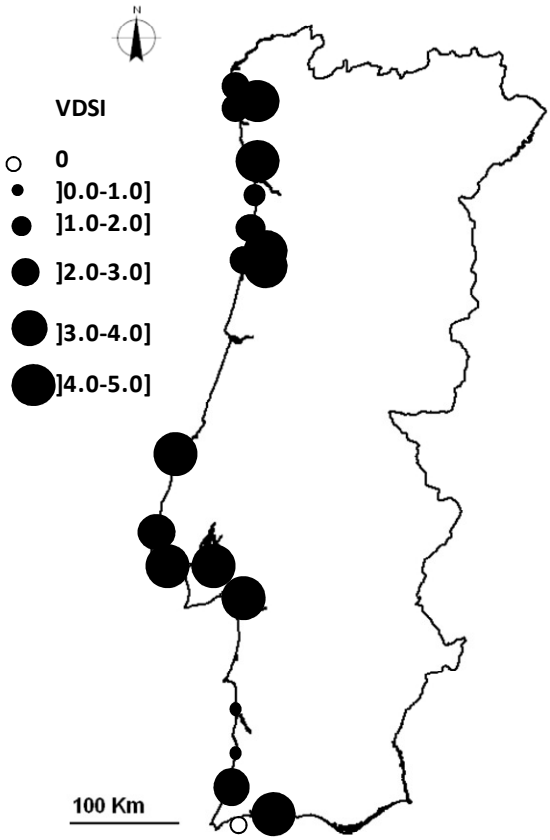

Figure 2.2. *N. reticulatus vas deferens* sequence index (VDSI) levels along the Portuguese coast in 2003. Adapted from Sousa et al. (2005).

Furthermore, the authors compared the obtained results with data from 2000 (Barroso et al., 2002) and checked whether the imposex evolution between 2000 and 2003 followed the same variation in TBT levels in females for a given site. They concluded that imposex generally accompanied the evolution of TBT concentration in the tissues and when they compared all sites the results demonstrated that imposex and TBT varied locally but did not reveal any clear global trend along the coast.

After 2003 several surveys were published for selected locations, like Ria Formosa and Ria de Aveiro. *Hexaplex trunculus* imposex was investigated in Ria Formosa by Vasconcelos et al. (2006a). Those authors examined 100 animals on a monthly basis, between March 2003 and April 2004, in order to assess imposex in this commercially

important species. The same group also developed indices for nonsacrificial sexing of imposex affected *H. trunculus* (Vasconcelos et al., 2006b). From 2005 onwards several studies were performed (Sousa et al., 2007; Rato et al., 2009; Galante-Oliveira et al., 2009; Sousa et al., in press; Sousa et al., submitted) but as some of them are parts of the present thesis they will be discussed later (Chapter 9).

2.3. Occurrence of imposex in open sea

Imposex and organotin contamination in offshore Portuguese waters was first reported by Santos et al. (2004) that studied the effect of the discharge of dredged material in the coast off Oporto (NW Portugal) during 1998. Only in 12 of the 18 sampled sites could live specimens be found. The degree of imposex was inversely related to the distance of the discharge site and varied between 0-75% and 0.0-1.5 for %I and VDSI, respectively. TBT levels in females tissues were always below the detection limit of the analytical procedure (<40 ng $Sn.g^{-1}$ dw), DBT and MBT levels varied between 50-159 ng $Sn.g^{-1}$ dw and 34-183 ng $Sn.g^{-1}$ dw, respectively. The same team performed another survey off the Iberian Peninsula between 1999-2000 (Goméz-Ariza et al., 2006). The authors analyzed imposex in different gastropods species in four sites off the southern Portuguese coast with different shipping densities (two off Lisbon, one off Sines and one off Sagres). However, only a reduced number of gastropods could be sampled at most sites and of the 8 gastropods species collected most specimens showed only early stages of imposex (Goméz-Ariza et al., 2006). Those reports didn't provide a clear picture of the offshore contamination and only in 2006 its extension was assessed by Rato et al. (2006). These authors studied imposex and organotin levels in *N. reticulatus* over a large area (735 km^2) on the coastal shelf off Aveiro (NW Portugal) and also inside the estuarine system. They analyzed over 13 000 specimens between 2002 and 2005 [Figure 2.3]. Imposex (%I and VDSI) and female´s TBT tissues concentrations decreased exponentially with distance from the mouth of the estuary in a geographic pattern reflecting both coastal currents and the jet discharge of the estuarine ebb plume, leading to the conclusion that vessels anchoring in the estuary were TBT sources to the system as well

as to the coastal shelf and consequently acted as inductors of offshore imposex (Rato et al., 2006).

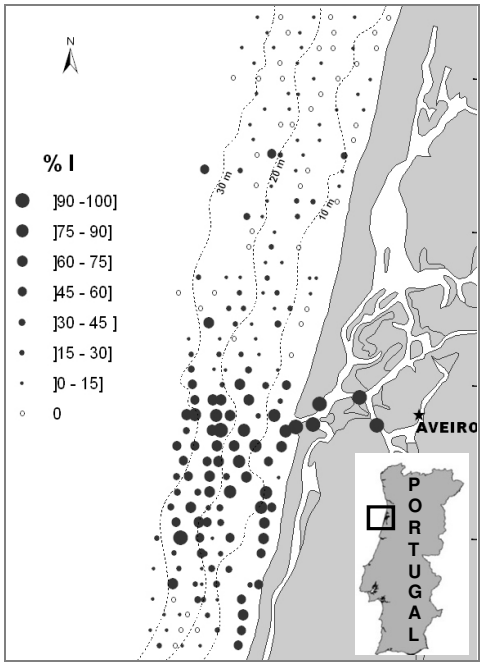

Figure 2.3. Spatial distribution of imposex (%I) in the continental shelf off Aveiro. Adapted from Rato et al. (2006).

The obtained results clearly demonstrated that offshore locations were extensively affected by imposex and that further studies were required. Subsequently, the authors extended the survey to the central and southern coast of Portugal using the same indicator species (Rato et al., 2008). Imposex and organotins were assessed at the continental shelves around the main estuaries of the central Portuguese coast -Tagus and Sado estuaries - and the main coastal lagoon in the south coast of Portugal - Ria Formosa. Imposex showed a clear gradient, decreasing from inside estuaries to the open coast, in relation to a similar gradient of TBT tissue contamination. The authors suggested that TBT is a matter of concern not only in the studied estuaries but also for the adjacent coastal shelves, regardless of the contaminants massive dilution occurring in those areas.

2.4. ORGANOTIN ENVIRONMENTAL LEVELS

As already mentioned a considerable amount of work concerning organotin in gastropods species and their correlation with imposex levels has been performed. Besides those surveys, organotins (particularly TBT) have also been quantified in mussels, water and sediments at specific locations like Tagus (Nogueira et al., 2003) and Sado estuaries (Carvalho et al., 2007), Ria de Aveiro and Ria Formosa (see section 2.1 for additional references) and also along the Portuguese coast. An overview of those surveys is presented.

The first report of butyl and phenyltin compounds in mussel samples collected along the Portuguese coastline was published in 2004 (Barroso et al., 2004). The authors analyzed those compounds in *Mytilus galloprovincialis* collected in 2000 at 17 sites positioned along the open coast in order to provide information about the background levels of pollution excluding hotspots. TBT was detected in all the analyzed samples and was predominant over its metabolites (58% on average), indicating recent contamination. TBT, DBT and MBT values varied between 11-789 ng $Sn.g^{-1}$ dw, <10-345 ng $Sn.g^{-1}$ dw and <10-605 ng $Sn.g^{-1}$ dw, respectively and TPhT was only detected at one location near Lisbon harbor. The highest butyltins concentrations were detected in the vicinity of the major Portuguese harbor (Lisbon) and at sites close to the mouth of estuaries or bays. In 2005, Díez and co-workers published a survey of organotin compounds in rivers and coastal environments in Portugal between 1999 and 2000 (Díez et al., 2005). Butyl and phenyltins levels were assessed in river water, river and marine sediments and mussels. TBT concentrations in river water ranged between 3 and 30 ng $Sn.L^{-1}$ and were associated with areas of industrial (textile, leather and paper), agriculture and urban activities. In *M. galloprovincialis* samples, TBT levels varied from 2.5 up to 490 ng $Sn.g^{-1}$ dw, and the highest concentrations were detected inside Viana do Castelo harbor [Figure 2.1]. Other highly polluted sites were located in and around dockyards and harbors. Butyltins were detected in all marine sediments whereas river sediments were not polluted at any of the analyzed locations. TBT levels in marine sediment samples varied between 4 and 12 ng $Sn.g^{-1}$ dw and were primarily related to heavy boat traffic and shipyard activities, and to a

lesser extent to urban activities. The reported concentrations were much lower than the ones previously described (Quevauviller & Donard, 1990; Cortez et al., 1993; de Bettencourt et al., 1999) for Portuguese coastal environments and the authors suggested that butyltins contamination in sediments had decreased. The same team also analyzed estuarine sediments from 11 locations along the Portuguese coast between 1999 and 2000 and they systematically detected butyltins with total concentrations ranging between 15 and 151 ng $Sn.g^{-1}$ dw (Díez & Bayona, 2009).

The occurrence of TBT and DBT in sediments was further investigated by Almeida et al. (2007). This team analyzed 104 sediment samples collected between 2001 and 2003 at eight estuaries. The target compounds were detected in the majority of the samples and the highest TBT level was reported for the Sado estuary (12.3 ng $Sn.g^{-1}$ dw). In Aveiro estuary no TBT or DBT were detected. Organotin contamination in Aveiro region has been regularly studied over the last decade and TBT levels in sediments are high (mean value of 31.5 ng $Sn.g^{-1}$ dw in 1998 survey). Such differences are probably due to different site selection as later reports (Chapter 5) also disclose high TBT levels.

Overall, those studies demonstrate that organotin compounds are ubiquitous in the Portuguese marine environment. Since OTs can accumulate through the food chain the occurrence of such compounds in seafood products was recently evaluated. Santos and co-workers (2009) quantified the levels of OTs in most relevant seafood products between 2002 and 2003 demonstrating that butyltins were present in all analyzed groups (fish, crustaceans, bivalves, cephalopods). Generally, the levels detected in the edible parts of fish, crustaceans and cephalopods were bellow 30 ng $TBT^{+}.g^{-1}$ ww (2.5 ng $Sn.g^{-1}$ dw assuming a moisture content of 80%) and therefore were much lower than the tolerable average residue levels (TARL). However, in bivalves higher concentrations were detected (22.5 ng $Sn.g^{-1}$ dw) and the authors suggested that higher bivalve consumer groups might be at risk.

Further studies on OTs environmental levels were performed but as previously referred; they will be discussed later in the present thesis (Chapter 9).

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CHAPTER 3

DISTRIBUTION OF SYNTHETIC ORGANOTINS AND TOTAL TIN LEVELS IN *MYTILUS GALLOPROVINCIALIS* ALONG THE PORTUGUESE COAST

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CHAPTER 3. DISTRIBUTION OF SYNTHETIC ORGANOTINS AND TOTAL TIN LEVELS IN *MYTILUS GALLOPROVINCIALIS* ALONG THE PORTUGUESE COAST

Abstract

Despite the huge amount of literature available on butyltins (BuTs), few studies addressed the environmental levels of phenyltins (PhTs), octyltins (OcTs) and total tin (Sn_T) in environmental samples. In 2006 a mussel watch survey was developed for the Portuguese coast (total of 29 sampling sites) in order to describe the concentrations of BuTs , PhTs, OcTs and Sn_T in the whole tissues of *Mytilus galloprovincialis*. BuTs were detected in all analyzed samples accounting, in average, for 98.6% of total organotins (∑OTs = BuTs+PhTs+OcTs), and presented highest values in the vicinity of harbors. Tributyltin (TBT) was the dominant butyltin, representing, in average, 62% of ∑BuTs (∑BuTs= TBT+DBT+MBT) suggesting that fresh inputs of TBT are still occurring in the Portuguese coast, particularly near harbors. The contribution of organotin compounds derived from antifouling paints to the total tin levels in *M. galloprovincialis* is discussed.

Keywords: Tributyltin; Total tin; *Mytilus galloprovincialis*; Portuguese coast

3.1. INTRODUCTION

Organotin compounds (OTs) are characterized by a tin (Sn) atom covalently bound to one or more organic substituent's (e.g. methyl, butyl, phenyl, octyl). The number and nature of these organic groups are the main responsible for the toxicological properties of the molecule. In general, inorganic tin is non-toxic whereas tri-substituted compounds - have maximum toxicological activity (Hoch, 2001).

Organometallic derivatives of tin are used in agriculture, industry and medicine, having an estimated annual production of approximately 60 000 tons (Mala, 2008). As a consequence, organotins are continuously released into the environment. Of special concern is the contamination of the marine ecosystem by tributyltin (TBT) and, to a lesser extent, triphenyltin (TPhT). Due to their effectiveness as biocides, these compounds have

been broadly used in antifouling (AF) paint formulations, leading to an ubiquitous presence in the marine environment (Fent, 2006). TBT is extremely toxic for organisms of many non-target taxonomic groups (Bryan & Gibbs, 1991), with several studies reporting the decline or extinction of bivalve and gastropod populations due to TBT pollution (Bryan & Gibbs, 1991; Alzieu, 2000a). Growing evidences about OTs high toxicity and persistence lead many countries to adopt measures limiting their usage, culminating with the adoption of the "International Convention on the Control of Harmful Antifouling Systems" (AFS convention) by the International Maritime Organization (IMO), banning the use of organotin AF paints in all ships from September 2008 onwards. However, the complete ratification of this convention was not achieved in the due date. The European Union, following the IMO recommendation to implement, as a matter of urgency, the AFS Convention, adopted the Directive 2002/62/EC and the Regulation (EC) 782/2003 prohibiting the application of OTs antifouling paints on EU ships after 1st July 2003, and forbidding the presence of TBT AF paints on ship's hulls from 1st January 2008. This study aims to create a baseline regarding organotin levels and total tin (Sn_T) concentrations in mussel's tissues along the Portuguese coast in 2006 and to discuss the contribution of synthetic organotins to total tin levels in mussels. The results obtained in the current survey are compared with the ones reported for different locations around Europe and discussed under the actual legislative framework. Possible effects in wildlife and humans are also addressed.

3.2. Materials and Methods

3.2.1. Sampling

Mussels have been used in several monitoring programs worldwide. Their sessile lifestyle, wide geographical distribution, tolerance to a considerable range of salinity, resistance to stress and high accumulation of a wide range of contaminants, together with the easy sampling make this bivalve an useful contamination bioindicator in the marine environment (Goldberg, 1975; Tanabe & Subramanian, 2006). In the present survey, the blue mussel *Mytilus galloprovincialis* (Lamarck, 1819) was collected at 29 sites

located along the Portuguese Continental coast, from Viana do Castelo to Faro, between May and August 2006 [Figure 3.1]. Sites selection aimed to recognize probable contamination gradients. Accordingly, organisms were sampled from the main harbors, their inner sections and their vicinities in the open coast. Detailed information about each sampling station is provided in Table 3.1.

Mussels were collected by hand at the midlevel of intertidal rocky shores or docksides at each station, stored in clean polyethylene bags and brought refrigerated to the laboratory where the larger specimens (30-40 animals) were selected, had their soft tissues removed, pooled and preserved at -20°C until chemical analysis.

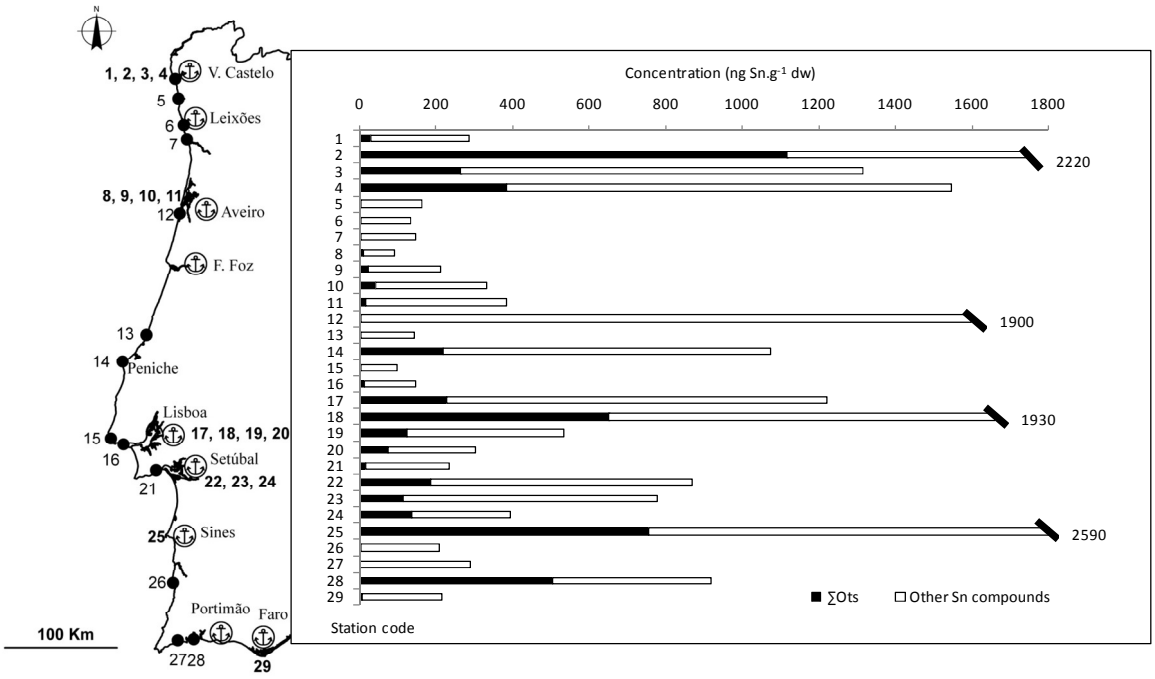

Figure 3.1. Map of the Portuguese coast depicting sampling stations and location of main harbors ⚓. The graphic presents the concentration of total tin in *M. galloprovincialis* soft tissues for each station: dark portions correspond to the sum of organotins (∑OTs= BuTs+PhTs+OcTs) whilst the white portion represents other tin compounds.

3.2.2. Chemical analysis

Organotin analyses were performed following the method described by Iwamura et al. (2000). The method was used to determine three butyltin species (BuTs): mono-(MBT), di-

(DBT) and tributyltin (TBT); two species of phenyltins (PhTs): di-(DPhT) and triphenyltin (TPhT); and two species of octyltins (OcTs): mono-(MOcT) and di-(DOcT). In brief, deuterated labeled standards (d_9-MBT, d_{18}-DBT, d_{27}-TBT, d_{10}-DPhT, d_{15}-TPhT, d_{17}-MOcT, d_{34}-DOcT) were spiked into the samples (≈2.0 g wet wt) as surrogates before the extraction. OTs in the samples were extracted by 1N HBr/methanol-ethyl acetate (1:1) and then transferred into ethylacetate/hexane (3:2) and concentrated by rotary evaporation. Afterwards, OTs were ethylated by adding 1 mL of 5% tetraethyl sodium borate. After ethylation, the extract was cleaned up by 1M KOH and SEP-PAK plus florisil cartridge (Waters Corporation, Milford, Mass, USA). OTs were then eluted by 5% diethylether/hexane. Finally, the solutions were concentrated into 1 mL and spiked with 50 ng of deuterated tetrabutyltin as a recovery standard. The final solutions were injected into a gas chromatograph-mass spectrometric detector (GC-MSD) (Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). OTs were then measured by GC-MSD in selected ion monitoring mode (EI-SIM) and quantified by isotope dilution method. OTs concentrations are reported as nanograms of Sn per gram on a dry weight (dw) basis. The procedure was validated using NIES Certified Reference Material No. 11. Results obtained from three replicate analysis of this material (1.2±0.1 $\mu g.g^{-1}$ as TBTCl and 6.3±0.1 $\mu g.g^{-1}$ as TPhTCl) are in accordance with the certified/reference values reported (1.3±0.1 $\mu g.g^{-1}$ as TBTCl and 6.3±0.1 $\mu g.g^{-1}$ as TPhTCl). Methods gave a tin detection limit (in terms of ng $Sn.g^{-1}$ dw) of 5.7 for MBT; 2.8 for DBT; 0.2 for TBT; 0.1 for DPhT; 0.1 for TPhT; 4.8 for MOcT and 3.6 for DOcT. Average recovery rates± St Dev (%) for each surrogate compound were: d_9-MBT: 60.6±11.02, d_{18}-DBT: 85.3±11.59, d_{27}-TBT: 91.0±9.84, d_{10}-DPhT: 72.6±15.76, d_{15}-TPhT: 134.8±17.14, d_{17}-MOcT: 82.3±16.02 and d_{34}-DOcT: 127.5±17.04.

The analytical procedure for total tin quantification was based on the method described by Le et al. (1999). In brief, tissue samples were dried at 80°C for 12 h and then homogenized to powder. About 0.2 g of the powdered sample was weighed into polytetrafluoroethylene (PTFE) tubes and digested with HNO_3 (ca. 69%) in a microwave oven for 30 min (MILESTONE, ETHOS D microwave laboratory system). When digestion was completed, the sample was transferred into a measuring flask and diluted with Milli-

Q water (Millipore, Bedford, MA, USA). Tin concentrations were determined using the inductively coupled plasma mass spectrometry (ICP-MS) (Hewlett-Packard, HP 4500) using yttrium as internal standard. Detection limit was 1 ng $Sn.g^{-1}$ dw. All chemicals (for ultratrace analysis) were purchased from Wako Pure Chemical Industries (Japan), except hydrobromic acid (Kanto Chemical Co., Inc., Japan) and sodium tetraethylborate (Hayashi Pure Chemical Ind., Co., Ltd., Japan). Standard solutions were purchased from Hayashi Pure Chemical Ind., Co., Ltd., Japan.

3.3. Results and Discussion

3.3.1. Organotins: levels, distribution and recent inputs

Organotin compounds were detected in all the samples analyzed. Butyltins (BuTs) were responsible for 98.6% of total organotins (∑OTs). TBT levels varied between 0.9 and 720 ng $Sn.g^{-1}$ dw representing an average proportion of 62% of total BuTs (∑BuTs= TBT+DBT+MBT); DBT levels ranged from <2.8 to 200 ng $Sn.g^{-1}$ accounting for 17% of ∑BuTs and MBT levels varied from <5.7 to 280 ng $Sn.g^{-1}$ representing 21% of ∑BuTs [Table 3.1]. Highly significant correlations were found between TBT and its metabolites (DBT and MBT) (Spearman, r=0.908, p<0.001 for MBT and r=0.959, p<0.001 for DBT) indicating that most of the DBT and MBT residues in mussels tissues are derived from TBT.

Phenyltin compounds (PhTs) represented a small fraction (0.7%) of ∑OTs and were mainly detected in the south coast, particularly near harbors. Triphenyltin was used as a pesticide in agriculture and also as a co-biocide in AF paints (Fent, 1996). The highly significant correlation (Spearman, r=0.844, p<0.001) found between TPhT and TBT [Figure 3.2] suggests that both compounds had similar sources, i.e., AF paints. The octyltins also represented a very small fraction of ∑OTs (0.7%). The major contribution was found at St. 24 where MOcT registered a value of 36 ng $Sn.g^{-1}$ dw, being negligible in the remaining locations. These compounds are mainly used as PVC stabilizers (Hoch, 2001) and the reason why MOcT registered such value at St. 24 is difficult to explain but this was the only site where construction activities were taking place during the sampling period and this might had played an important role.

Table 3.1. Description of sampling locations and concentrations of organic and total tin (ng Sn.g^{-1}dw) in *M. galloprovincialis* soft tissues. Moisture content (% of dw) of each sample is also provided. Geographical coordinates refers to WGS84 Datum. B: beach; CM: Commercial Port; E: Estuary Entrance; FB: Ferry Boat; FP: Fishing port; M: Marina; S: Shipyard; MBT: monobutyltin; DBT: dibutyltin; TBT: tributyltin; DPhT: diphenyltin; TPhT: triphenyltin; MOcT: monooctyltin; DOcT: dioctyltin; Sn_T: total tin; na: not analyzed.

Station code - name	Description	Geographical Coordinates	Moisture	MBT	DBT	TBT	DPhT	TPhT	MOcT	DOcT	Sn_T
1 - V. Castelo - Barra	E	41°40'58.79''N/8°50'18.48''W	85.4	<5.7	5.8	24	<0.1	<0.1	na	<3.6	286
2 - V. Castelo - Cais	FP	41°41'17.59''N/8°50'04.30''W	82.7	200	200	720	<0.1	3.9	na	<3.6	2220
3 - V. Castelo - Marina	M	41°41'39.08''N/8°49'15.90''W	85.8	18	26	220	<0.1	2.9	na	<3.6	1320
4 - V. Castelo - Estaleiro	S	41°41'15.73''N/8°50'20.50''W	82.5	140	61	190	<0.1	1.2	na	<3.6	1550
5 - Praia da Amorosa	B	41°38'30.55''N/8°49'26.06''W	81.8	<5.7	<2.8	3.3	<0.1	<0.1	na	<3.6	162
6 - Praia de Leça	B	41°12'08.71''N/8°42'54.60''W	81.5	<5.7	<2.8	4.3	<0.1	<0.1	na	<3.6	135
7 - Praia da Foz	B	41°09'36.32''N/8°41'11.18''W	85.1	<5.7	<2.8	2.5	<0.1	<0.1	na	<3.6	145
8 - Aveiro: Barra	E	40°30'39.26''N/8°44'55.64''W	81.3	<5.7	4.6	6.0	<0.1	<0.1	<4.8	<3.6	92.5
9 - Aveiro: M. Mira	FP	40°38'34.65''N/8°44'06.80''W	81.7	<5.7	6.3	17	<0.1	<0.1	<4.8	<3.6	211
10 - Aveiro: P. Pesca Largo	CP	40°38'08.46''N/8°41'21.28''W	79.9	10	9.0	24	<0.1	<0.1	<4.8	<3.6	331
11 - Aveiro: P. Com. Norte	CP	40°38'56.65''N/8°43'46.82''W	80.4	<5.7	3.0	13	<0.1	<0.1	<4.8	<3.6	385
12 - Praia da Vagueira	B	40°33'33.07''N/8°46'19.19''W	80.1	<5.7	<2.8	3.1	<0.1	<0.1	<4.8	<3.6	1900
13 - Nazaré	FP	39°35'00.03''N/9°04'26.02''W	81.7	<5.7	<2.8	4.8	<0.1	<0.1	<4.8	<3.6	145
14 - Peniche	FP	39°21'22.64''N/9°22'05.60''W	83.5	17	35	170	<0.1	2.1	<4.8	<3.6	1080
15 - Praia do Guincho	B	38°43'40.55''N/9°28'35.81''W	85.1	<5.7	<2.8	3.5	<0.1	<0.1	<4.8	<3.6	97
16 - Praia das Avencas	B	38°41'08.31''N/9°21'19.94''W	85.1	6.9	<2.8	4.5	<0.1	0.3	<4.8	<3.6	146
17 - Lisboa: Belém	B	38°41'32.98''N/9°12'45.26''W	79.0	49	37	140	0.26	5.1	<4.8	<3.6	1220
18 - Lisboa: Alcântara	CP/FP/M/S	38°42'07.06''N/9°09'35.29''W	81.5	110	110	430	0.15	1.7	<4.8	<3.6	1930
19 - Lisboa: Trafaria	CP	38°40'26.43''N/9°14'06.67''W	81.1	18	21	83	<0.1	1.2	<4.8	<3.6	533
20 - Lisboa: Porto Brandão	FB/ S	38°40'41.53''N/9°12'23.77''W	77.6	12	12	50	0.13	1.9	<4.8	<3.6	304
21 - Portinho Arrábida	B	38°28'33.65''N/8°59'03.51''W	84.1	<5.7	<2.8	15	<0.1	0.51	<4.8	<3.6	234
22 - Setúbal: P. Comercial	CP	38°31'06.66''N/8°52'46.15''W	83.3	26	29	130	<0.1	1.9	<4.8	<3.6	868
23 - Setúbal: Lota	FP	38°31'09.76''N/8°53'47.39''W	83.4	17	22	75	<0.1	1.8	<4.8	<3.6	779
24 - Setúbal: Tróia	FB	38°29'40.34''N/8°54'02.76''W	79.9	25	22	53	0.24	1.8	36	<3.6	395
25 - Sines	FP	37°57'12.24''N/8°52'16.54''W	82.2	77	170	500	0.26	4.8	<4.8	<3.6	2590
26 - Zambujeira do Mar	B	37°33'05.56''N/8°47'35.98''W	84.5	<5.7	<2.8	2.9	<0.1	<0.1	<4.8	<3.6	207
27 - Praia da Luz	B	37°05'08.84''N/8°43'42.83''W	83.1	<5.7	<2.8	0.9	<0.1	<0.1	<4.8	<3.6	290
28 - Lagos	FP/ S	37°06'20.08''N/8°40'26.16''W	79.0	280	39	180	<0.1	3.1	<4.8	na	918
29 - Faro	FP	37°00'44.76''N/7°56'09.64''W	84.3	<5.7	<2.8	6.8	<0.1	<0.1	<4.8	na	215

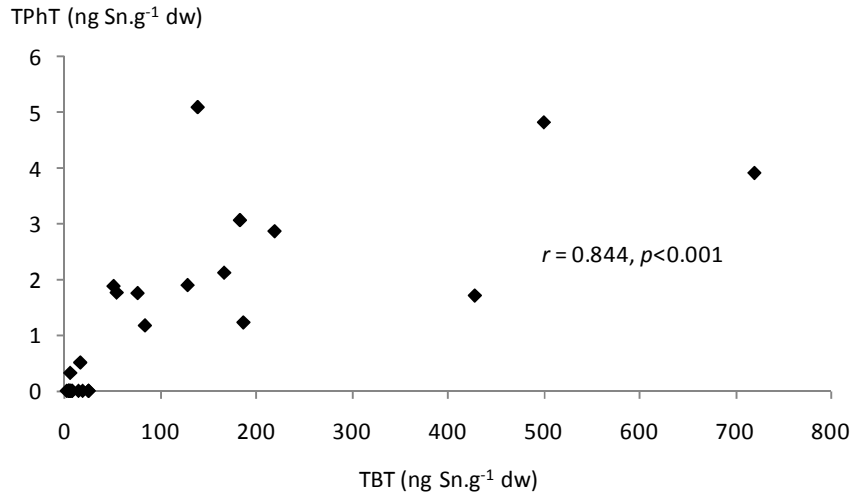

Figure 3.2. Relationship between tributyltin (TBT) and triphenyltin (TPhT) concentrations in *M. galloprovincialis* soft tissues in all the sampling stations. Null values of TPhT represent levels below the detection limit of the applied analytical procedure (<0.1 ng Sn.g^{-1} dw).

The lowest TBT levels (<5 ng Sn.g^{-1} dw) were detected in sites far from the influence of main harbors. The highest TBT levels detected in mussel samples were recorded, by decreasing order of importance, at the Viana do Castelo harbor (St. 2), Sines (St 25) and Lisboa harbor (St. 18). Samples from other locations such as Lagos fishing port (St. 28) and Peniche fishing port (St. 14) also presented high levels of TBT [Figure 3.1, Table 3.1]. Viana do Castelo, Sines and Lisboa harbors have high naval traffic that includes commercial and fishing ports, marinas, and shipyards. In 2006 ships entering both commercial and fishing ports could still have TBT-based AF paints that were applied before 2003; even inside marinas boats >25m in length could still have old TBT coatings. Furthermore, in 2006, ships with fresh TBT-based AF paints coming from countries with no regulations could still enter European Union. Finally, inside shipyards, old coatings are being removed from ship's hulls and replaced by new ones and therefore a huge amount of TBT containing wastes is expected to be produced. In fact, these facilities have been described as one of the major sources of TBT into the marine environment (Shim et al., 2002; Kotrikla, 2009). For instance, Gibbs (2009) conducted a 20 year TBT monitoring in the Fall estuary (SW England) up to 2006 and suggested that TBT pollution in this estuary in recent years

appears to be the result of shipyard discharges (mean TBT concentration of 275 ng $Sn.L^{-1}$). Since old coatings have to be sealed or removed from ships hulls from 2008 onwards, it is crucial to monitor how those activities are performed, in order to prevent the discharge of TBT into the environment. The other highly contaminated stations (St. 14 and St. 28) are not located inside commercial harbors but have similar features that might explain the high OTs levels registered: they are both placed inside important fishing ports with shipyards in the vicinity.

Hence, the main source of TBT along the Portuguese coast seems to be associated with commercial ports with high naval traffic, important fishing ports and shipyards as previously demonstrated (Santos et al., 2000; Barroso et al., 2002; Santos et al., 2002; Barroso et al., 2004; Sousa et al., 2005; Galante-Oliveira et al., 2006; Rato et al., 2008).

In order to evaluate recent TBT inputs we used the butyltin degradation index (BDI= ([MBT]+[DBT])/[TBT])) originally proposed by Díez et al. (2002) for sediments and recently used in gastropods (Ruiz et al., 2008) and mussels (Kim et al., 2008). BDI values <1 suggest recent TBT contamination, while those >1 suggest no recent inputs (Díez et al., 2002).The average BDI value in the present survey is 0.42 and in some cases values were closer to zero (BDI=0.2; e.g. St. 3). The BDI values obtained in this study confirm the fact that in 2006 fresh TBT inputs occurred on the Portuguese coast. These inputs might be due to continuous removal of TBT coatings in shipyards, as referred above, from the illegal use of TBT AF paints, or from the release of TBT from sediments that are known to act as reservoir of this compound (Sarradin et al., 1995). The remobilization from sediments can release TBT to the water column; in fact, a recent study in NW Spain reported that the bioaccumulation in two different gastropod species (*Nucella lapillus* and *Nassarius reticulatus*) implied butyltin desorption from sediments (Ruiz et al., 2008).

3.3.2. Comparison with other studies around Europe

The OTs concentration in mussel's tissues is strongly influenced by several factors such as sampling location and distance from hotspots, water temperature, salinity, oxygen

content and biological activity (Díez et al., 2005). Hence, comparisons with other studies are sometimes difficult, and should be carefully addressed. Furthermore, due to the introduction of the EU Regulation, large discrepancies in TBT levels are expected in surveys performed before and after 2003. Table 3.2 discloses the levels of butyltin compounds detected in mussel samples collected around Europe from 1999 to 2005. Generally, all studies disclose harbors as main sources of organotin contamination. Our results are similar to the ones reported after 2003 for Italy (Magi et al., 2008) and Ria de Aveiro - Portugal (Sousa et al., 2007). They are also comparable to the ones reported for Portugal in 2000 (Barroso et al., 2004; Díez et al., 2005). Nevertheless, when comparing common locations between the present survey and 2000 survey (Barroso et al., 2004), a marked decrease in TBT levels is obvious. A recent study conducted along the Adriatic Sea also reported decreasing levels of TBT in mussels collected between 2000 and 2006 (Nemanič et al., 2009). However, despite the decreasing tendency, fresh inputs were still evident (Nemanič et al., 2009). Other studies from different locations around the world also described the occurrence of TBT inputs even after the introduction of legislation banning the use of this compound (Murai et al., 2005; Inoue et al., 2006; Smith et al., 2006; Ščančar et al., 2007; Sheikh et al., 2007).

Phenytltin levels in mussels collected around Europe are typically below the detection limits of the applied analytical procedures and/or at much lower concentrations than butyltins (Nemanič et al., 2002; Bortoli et al., 2003; Ruiz et al., 2005; Ščančar et al., 2007; Magi et al., 2008; Nemanič et al., 2009; Zanon et al., 2009). In Europe, the percentage of TPhT used in AF paints formulations is relatively low when compared to TBT, and therefore lower environmental levels are observed. In the Portuguese coast, Barroso et al. (2004) found measurable levels of PhTs at only one location (P. Brandão, referred in the current study as St. 20) whereas Díez et al. (2005) did not detect such compounds in any of the 15 samples analyzed. A survey conducted in Ria de Aveiro in 2005 (Sousa et al., 2007), reported PhTs in all samples; however the detection limit of the applied analytical procedure was much lower than those in other studies. Furthermore, contribution of PhTs to total organotins was very small (1%, in average) and thus comparable to the present results. Data on octyltin levels in mollusks are scarce and the available literature

discloses concentrations below the detection limits (Mzoughi et al., 2005; Guérin et al., 2007; Sousa et al., 2007; Rato et al., 2008; Nemanič et al., 2009). Only one study reported detectable levels of OcTs in gastropods in Ria de Aveiro but their contribution to total OTs was very small (4%) (Sousa et al., 2007).

Table 3.2. Concentration range (ng Sn.g^{-1} dw) of butyltin compounds in *M. galloprovincialis* tissues collected around Europe. nd: not detected (below the detection limit of the applied analytical procedure).

Location	Year	MBT	DBT	TBT	Reference
Venice lagoon, Italy	1999-2000	nd-1800	nd-1300	nd-4500	Bortoli et al., 2003
Venice lagoon, Italy	1999-2003	nd-1259	nd-2136	16-2732	Zanon et al., in press
Portugal	1999-2000	nd-41	nd-18	nd-489	Díez et al., 2005
Bay of Piran, Slovenia	2000	44-691	174-2170	510-3456	Nemanič et al., 2002
Galicia, NW Spain	2000	9-1243	121-2094	155-1856	Ruiz et al., 2005
Portugal	2000	nd-605	nd-345	11-789	Barroso et al., 2004
Slovenia	2000-2006	nd-1335	15-2660	36-6434	Nemanič et al., 2009
Slovenia	2001-2002	nd-600	nd-2200	340-7900	Ščančar et al., 2007
Italy (Mussel farm)	2005	16	107	193	Magi et al., 2008
Italy (Harbor)	2005	264	209	570	Magi et al., 2008
Ria Aveiro, Portugal	2005	nd-163	11-107	24-500	Sousa et al., 2007
Portuguese Coast	2006	nd-280	nd-200	1-720	current study

3.3.3. Tributyltin: possible impacts on wildlife and humans

Biomonitoring using mussels is a common approach to estimate environmental levels of pollutants. In 2004 the OSPAR Commission updated the Environmental Assessment Criteria (EAC) values of TBT in water, sediments and biota and proposed a lower-EAC of 4.9 ng TBT-Sn.g^{-1} and an upper-EAC of 71.7 ng TBT-Sn.g^{-1} for mussels (OSPAR, 2004). Values below the lower-EAC don't give rise to biological effects; the ones between the lower and the upper-EAC indicate that possible biological effects can occur (e.g. impaired growth and reproduction); for concentrations above the upper-EAC long-term biological effects are likely to happen (e.g. impaired growth, reproduction and survival) and acute biological effects (survival) are possible to occur (OSPAR, 2004). Our results disclose TBT concentrations between 0.9 and 720 ng Sn.g^{-1} dw. Only 31% of the sampling stations

exhibited levels below the lower-EAC and are all located away from harbors. The remaining sites exhibited levels between the lower and upper-EAC (31%) and above the upper-EAC (38%); hence TBT pollution is of great concern at those locations and further monitoring for assessing ecotoxicological impacts are necessary.

Several studies calculated TBT concentrations in seawater and in blue mussel tissues and established a bioconcentration factor (BCF) for this species. However, BCF values exhibited huge temporal and spatial variations. BCF, defined as the concentration in mussel tissues divided by the concentration in seawater, ranged from 2,000 to 90,000 in Slovenia (Nemanič et al., 2009), whereas in mussels transplanted to Arcachon harbor the values ranged between 280,000 and 1,300,000; being the highest BCFs reported so far (Devier et al., 2005). Estimation of seawater levels based on BCF should therefore be carefully addressed and can only provide indicative data. Considering, for example, the BCF of 11,000 reported by Takahashi et al. (1999b) the estimated seawater levels across our sampling stations varied from 0.014-11 ng $Sn.L^{-1}$(as the BCF described by those authors was on a wet weight basis and by considering TBT as a cation, our data was converted accordingly). In 93% of the locations, estimated seawater levels are above the lower Environmental Assessment Criteria (EAC) set for TBT (0.04 ng $Sn.L^{-1}$) by OSPAR Commission (OSPAR, 2004) and therefore biological effects are possible.

Although they should be considered only as indicative values our estimated TBT concentrations seems to be reasonable when comparing with measured ones: Galante-Oliveira el al. (in press) reported total extractable tin in subsurface water samples from Ria de Aveiro in 2006 between <0.6 and 39 ng $Sn.L^{-1}$; Díez et al. (2005) reported TBT levels in Portuguese river samples between 3 and 30 ng $Sn.L^{-1}$ in samples collected in 1999/ 2000, and Barroso et al. (2000) reported TBT levels between 9 and 42 ng $Sn.L^{-1}$ in subsurface water samples from Ria de Aveiro collected in 1998.

Mollusks populations are among the most sensitive to TBT pollution. Concentrations of 0.82 ng TBT- $Sn.L^{-1}$ induce shell calcification anomalies in the oyster *Crassostrea gigas* (Alzieu, 2000b) with severe impacts on the oyster farming. Mussels are also negatively affected by the concentration range found in the study area: a chronic toxicity value of 7 ng TBT- $Sn.L^{-1}$ was reported for larval survival (Lapota et al., 1993). Gastropods are also

very sensitive to these levels of TBT in seawater: values of 0.4 ng TBT- $Sn.L^{-1}$ induce imposex development in *N. lapillus* (Bryan & Gibbs, 1991) and sterility occurs at concentrations as low as 2 ng TBT- $Sn.L^{-1}$ (Gibbs et al., 1988). In the Portuguese coast imposex has been reported in several species of gastropods like, for instance, *N. lapillus* (Santos et al., 2000; Barroso & Moreira, 2002; Santos et al., 2002; Galante-Oliveira et al., 2006), *N. reticulatus* (Barroso et al., 2002; Sousa et al., 2005; Rato et al., 2009), *Littorina littorea* (Barroso et al., 2000), *Hexaplex trunculus* (Vasconcelos et al., 2006) and *Hydrobia ulvae* (Barroso et al., 2000).

Mussels are commonly consumed in Portugal as food and thus organotin residues are transferred to humans *via* dietary uptake. According to FAO, the daily average intake of mollusks (excluding cephalopods) in Portugal in 2005 was 7 g (on a wet weight basis) *per capita*, corresponding to 1.4 g on a dry weight basis (assuming mussel's moisture content as 80%). Considering the tolerable daily intake (TDI) of 100 ng $Sn.kg^{-1}$ body weight day^{-1} for TBT, DBT, TPhT and DOcT (6000 ng $person^{-1}day^{-1}$, considering a person with 60 kg) set by the Scientific Panel on Contaminants in the Food Chain (EFSA, 2004; Guérin et al., 2007), the estimated maximum daily intake (considering hypothetically that mussels compose 100% of the mollusks diet) varies between 0.9 and 1293 ng $Sn.person^{-1}.day^{-1}$. Those values are much lower than the TDI (6000 ng day^{-1}, considering a person with 60 Kg). However, two considerations have to be made: firstly, the TDI value was calculated based only on consumption of mollusks and the real intake is probably higher considering all the seafood items together, as Portugal is one of the world largest seafood-consuming nation with a consumption of 56.5 kg per capita per year (EC, 2006); secondly, the TDI proposed by the panel was based on chronic immunotoxicity studies but recent works point out the possible effect of low doses of TBT on the retinoic acid receptor-dependent signaling pathway (Kanayama et al., 2005; Grün & Blumberg, 2006; Yonezawa et al., 2007). Therefore, these compounds may have more effects on humans than previously reported but further research is essential to clarify the possible link between OTs and some biological disorders (Grün & Blumberg, 2006). Furthermore, humans are exposed to OTs not only through dietary fish intake but also through other food items and other consumer products (Takahashi et al., 1999a) and also indoor dust (Fromme et al., 2005).

Therefore, an integrated risk assessment of OTs is needed, taking into account human exposure from all possible sources, as suggested by the Scientific Panel on Contaminants in the Food Chain.

3.3.4. Total tin and the role of anthropogenic sources

Environmental levels of total tin depend both on natural sources and anthropogenic inputs. Natural sources include inorganic tin that occurs in the earth's crust, with an average concentration of ≈2–3 mg kg^{-1}; however, higher levels can occur in areas of high tin deposits (Howe & Watts, 2005). In Portugal massive tin deposits can be found both in the Northern and Southern parts of the country. During 1990 Portugal was the largest tin producer in Europe and, in 1997, the production was about 6511 tons (IGM, 2000). Organic tin compounds are mainly of anthropogenic origin, except methyltins that can also be produced naturally by biomethylation (Hoch, 2001).

In order to understand the contribution of synthetic OTs to the total amount of tin in mussels, values of total tin (Sn_T) concentrations obtained by ICP-MS were compared to the total organotin (∑OTs) quantified by GC-MSD. Table 3.1 and Figure 3.3 summarize Sn_T and ∑OTs concentrations in mussels along the Portuguese coast. It is clear that OTs, particularly BuTs, represents an important fraction of the Sn_T in mussels, particularly at sites near the main harbors, where OTs can reach up to 55% of the Sn_T. In addition, a highly significant correlation (r=0.77, p<0.001) was observed between ∑OTs and Sn_T across all sampling stations [Figure 3.3]. In Figure 3.3, the presence of an outlier at St. 12 (∑OTs=3.1 ng $Sn.g^{-1}$ dw and Sn_T=1900 ng $Sn.g^{-1}$ dw), is clearly noticeable. After its removal, the Spearman correlation improved from r=0.77 to 0.90 maintaining the significance level (p<0.001). The relationship between ∑OTs and Sn_T represented in Figure 3.3 shows that when ∑OTs tends to zero the Sn_T tends to 260 ng $Sn.g^{-1}$ dw and when ∑OTs tends to its maximum value (1120 ng $Sn.g^{-1}$ dw) the Sn_T tends to 2220 ng $Sn.g^{-1}$ dw); these results suggest that environmental contamination by OTs derived from AF paints may constitute an important direct/indirect source of the tin accumulated in mussels.

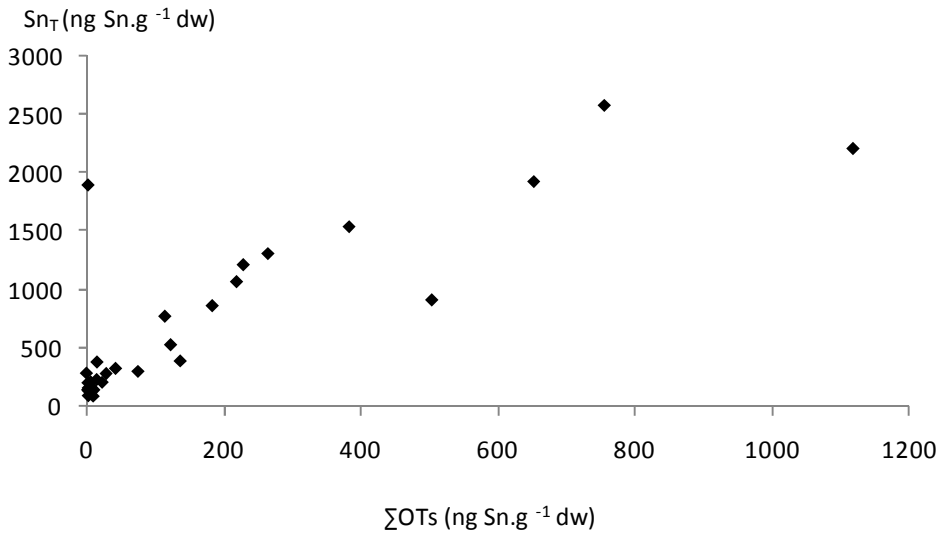

Figure 3.3. Relationship between total organic tin (∑OTs= BuTs+PhTs+OcTs) and total tin (Sn_T) in *M. galloprovincialis* soft tissues in all the sampling stations.

Several studies analyzed the contribution of OTs to total tin levels in various environmental samples. High percentages of butyltin compounds in relation to total tin levels were detected in fish, mussels and algae from the German North Sea and River Elbe (Shawky & Emons, 1998); in marine mammals from Asian countries (Takahashi et al., 2000), including cetaceans collected in Japanese coastal waters (Le et al., 1999); in mussels from Asian developing countries (Sudaryanto et al., 2002); in skipjack tuna collected in the offshore waters of Asian countries (Ueno et al., 2004); and in bivalves and fish from Malaysia (Sudaryanto et al., 2004). In mussels collected from Indonesian coastal environments (Sudaryanto et al., 2005) lower percentages of butyltins in relation to total tin levels were detected (<50%) being comparable to our results.

Besides OTs, other forms of tin contributing to Sn_T quantified in mussel tissues in the current work include: (i) inorganic tin compounds that are derived from natural sources or by the degradation of OTs into inorganic tin by microbial processes (Landmeyer et al., 2004) and then incorporated by mussels (Zuolian & Jensen, 1989); (ii) methylated compounds produced by bacteria and incorporated by mussels (Fent, 1996), or (iii) OTs metabolites produced by mussels themselves during metabolization process. It has been

accepted that mussels have limited ability to metabolize TBT due to the low mixed-function oxygenase activity (Lee, 1996). However, Suzuki et al. (1998) demonstrated that, under natural conditions, *Mytilus graynus* and *Mytilus edulis* could rapidly metabolize TBT after being accumulated. This metabolization process includes the formation of hydroxylated and oxygenated compounds. The presence of high levels of those compounds has already been reported for another species of blue mussels (Shiraishi et al., 1992). Hydroxylated and oxygenated TBT metabolites can therefore account for a considerable proportion of total tin, but further research on these metabolites is required.

It seems therefore plausible to conceive that a considerable portion of total tin levels observed in our samples might have been derived exclusively from anthropogenic inputs (either directly or indirectly through degradation products). Since one of the most important anthropogenic sources of OTs comes from the extensive use of OT-based AF paints, it is expected that levels of total tin will decrease in future after the effective ban on OTs.

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CHAPTER 4

INTEGRATIVE ASSESSMENT OF ORGANOTIN CONTAMINATION IN A SOUTHERN EUROPEAN ESTUARINE SYSTEM: TRACKING TEMPORAL TRENDS IN ORDER TO EVALUATE THE EFFECTIVENESS OF THE EU BAN

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CHAPTER 4. INTEGRATIVE ASSESSMENT OF ORGANOTIN CONTAMINATION IN A SOUTHERN EUROPEAN ESTUARINE SYSTEM: TRACKING TEMPORAL TRENDS IN ORDER TO EVALUATE THE EFFECTIVENESS OF THE EU BAN

Abstract

Organotin (OT) compounds have been used as biocide agents in antifouling paints since the mid 1960s and are now ubiquitous in the marine environment. Due to their high toxicity to non-target species they were banned from antifouling paints in the European Union in 2003 (2002/62/EC directive). The aim of the present work is to assess any obvious decline of the OT environmental levels at Ria de Aveiro (NW Portugal) after the ban. The organotins - monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), monophenyltin (MPhT), diphenyltin (DPhT), triphenyltin (TPhT), monoctyltin (MOcT), dioctyltin (DOcT) and trioctyltin (TOcT) – were quantified in the gastropod *Nassarius reticulatus*, in the mussel *Mytilus galloprovincialis* and in sediments. Imposex (imposition of male characters on females of gonochorist gastropods) in *N. reticulatus* was additionally used as a biomarker of TBT pollution. Time comparisons show a slight decrease of imposex between 2003 and 2005 probably as a consequence of the EU ban, though in some cases this trend seems to have started earlier since 2000. The fraction of TBT relatively to its metabolites has been decreasing over the last years but still remains high suggesting that there are recent inputs of this compound into the study area.

Keywords: *Nassarius reticulatus*; *Mytilus galloprovincialis*; Sediments; Organotins; Imposex; 2002/62/EC directive.

4.1. INTRODUCTION

Organotin compounds (OTs), particularly tributyltin (TBT), have been widely used since de mid 1960s as biocides in antifouling paints. Although very effective against biofouling they are extremely toxic to a wide range of non target organisms and have been considered as one of the most toxic xenobiotics ever produced and deliberately introduced into the environment (Goldberg, 1986). One of the most remarkable effect of TBT pollution is 'imposex' - the superimposition of male characters onto females of gonochorist gastropods (Smith, 1971) - being described for more than 150 gastropod

species worldwide (Horiguchi et al., 2006). The harmful effects of TBT in marine ecosystems lead governments to adopt restrictions on the use of organotin antifouling paints. Regardless the restrictions, TBT pollution was still high at many sites of coastal and deep-sea waters of countries that adopted TBT regulations (Minchin et al., 1996; Morgan et al., 1998; Michel et al., 2001; Barroso & Moreira, 2002; Santos et al., 2002; ten Hallers-Tjables et al., 2003). As a consequence, in 2001 the International Maritime Organization (IMO) formulated the International Convention on the Control of Harmful Antifouling Systems, which ratification is not completed yet and, consequently, is still not effective. However, the European Union (EU) followed the rational of this convention and introduced the Directive 2002/62/EC that bans the application of organotin antifouling paints on EU boats after 1 January 2003 and forbids its usage by any boats after 2008. The recent concern now is to check the effectiveness of the ban in terms of how fast the OT environmental levels decline in the EU after 2003. Hence, the main objective of the present study is to evaluate the short term effectiveness of the Directive 2002/62/EC at Ria de Aveiro (NW Portugal) through: (i) the assessment of the current levels of OT contamination in biota, water and sediments, (ii) the evaluation of recent inputs of OTs into this area and (iii) the comparison of the present situation with the pre-ban period.

4.2. Materials and Methods

4.2.1. Study area

Ria de Aveiro is an important estuarine system located in the NW Portugal. It is formed by a complex system of channels connected permanently to the sea trough an artificial inlet [Figure 4.1]. The potential sources of organotin contamination in Ria de Aveiro are ports, dockyards and marinas. The main ports and dockyards are located along the main navigation channel. Of major relevance is the deep sea fishing port (St. 1), the Aveiro dockyards (next to St. 1), the commercial port (St. 2), the chemical port and the coastal fishing port (see Barroso et al., 2000). Additionally there are two small marinas in the North Channel (St. 8 and St. 9) plus two in the south. Also spread along the Ria are small local docking sites for fishing and recreational boats. In the adjacent coastal area the

traffic lane for commercial ships lies parallel to the coastline, some 40 km offshore, from which an east-to-west navigational channel runs to the entrance of Ria de Aveiro.

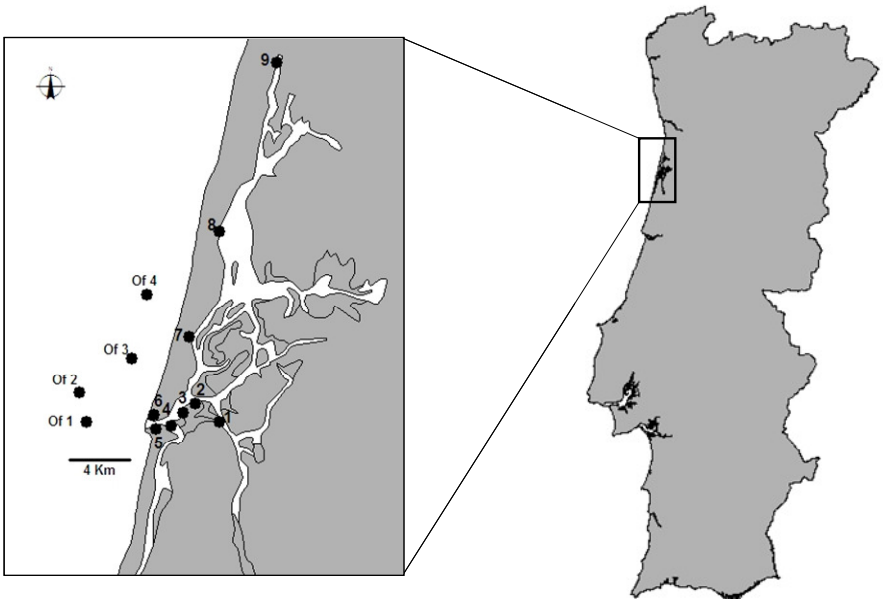

Figure 4.1. Map showing the study area and the sampling locations: Deep sea fishing port (1), North Commercial port (2), Forte Barra (3), M. Mira (4), Barra (5), S. Jacinto (6), Muranzel (7), Torreira (8), Marina Ovar (9) and Offshore locations (Of. 1 - Of. 4).

4.2.2. Sampling procedure

Sampling was performed between May and June 2005. The whelk *Nassarius reticulatus* (Linnaeus, 1758) was collected along a ship traffic gradient from the ports inside Ria de Aveiro (7 sites) towards the offshore coast (4 sites). Whenever possible the mussel *Mytilus galloprovincialis* (Lamarck, 1819) and sediments were collected inside Ria de Aveiro at the same sites as *N. reticulatus* [Figure 4.1]. Results from our previous surveys (Barroso et al., 2000; Barroso et al., 2002a; Sousa et al., 2005) on imposex and TBT levels at common locations are also included in the present study to allow temporal comparisons and further discussion.

The whelk was collected following the methodology and site selection described in our previous studies (Sousa et al., 2005; Rato et al., 2006). Mussels were collected by hand at

the mid intertidal level. In the laboratory, the bigger sized specimens were selected, measured and the whole soft tissues were removed, pooled together, transferred into clean glass bottles and preserved at -20°C until analysis. Biometric data and number of pooled specimens *per* sample are shown in Table 4.1. Sediments were sampled following the methodology and site selection described elsewhere (Barroso et al., 2000). In brief, the superficial layer of the sediment (*ca* 1 cm) was removed with a spatula, at about the mid-tide level, placed in polyethylene bags and transported to the laboratory under cold conditions. About 200 g of sediment were preserved at -20°C for chemical analysis and the remaining was reserved for granulometric composition and total organic matter analysis. The organic matter content was obtained by measuring the stable loss of weight of the dried sediment sample at 450°C.

Table 4.1. Sampling locations, number of pooled specimens and biometric data of *M. galloprovincialis*.

Station	Code	No.	Shell length (mm)
Deep Sea fishing Port	1	45	47.6±5.21
North commercial Port	2	53	41.6±7.47
Forte Barra	3	22	61.9±4.25
M. Mira	4	26	62.6±6.56
Barra	5	47	48.2±3.41
S. Jacinto	6	26	61.1±4.69
Muranzel	7	41	48.1±4.51
Torreira	8	25	62.7±6.67
Marina Ovar	9	32	60.9±7.44

4.2.3. Imposex analysis

The following imposex indices were determined for *N. reticulatus*: mean female penis length (FPL), *vas deferens* sequence index (VDSI), incidence (percentage of females affected by imposex = % I) and average oviduct stage (AOS). The penis length was measured to the nearest 0.5 mm using 1 mm graduated graph paper under a stereo microscope. The VDSI was classified according to the scoring system proposed by Stroben

et al. (1992) with minor alterations proposed by Barroso et al. (2002a); throughout the paper the VDSI values refers to the modified scale (values 4+ computed to 5) but the values of $VDSI_{(4)}$ computed after Stroben et al. (1992) are given in Table 4.2 to allow comparisons with other works. The degree of oviduct convolution (AOS) was ranked according to the 3-stage scale proposed by Barreiro et al. (2001).

4.2.4. Organotin analysis

The organotin analyses were performed following the method described by Iwamura et al. (2000) with some modifications. For biological samples, approximately 2 g of wet tissue of mussels or gastropods was spiked with 50 ng of internal standards including deuterated butyltins (d_9-MBT, d_{18}-DBT and d_{27}-TBT), phenyltins (d_5-MPhT, d_{10}-DPhT and d_{15}-TPhT), and octyltins (d_{17}-MOcT, d_{34}-DOcT and d_{51}-TOcT) and homogenized with 1N HBr/methanol-ethyl acetate (1:1) solution using a Polytron homogenizer. For sediment samples, approximately 2.5 g of freeze-dried sediment was spiked with the same internal standards and 1g of ascorbic acid with ethyl acetate was added, the sample mixture was then homogenized with 1N HBr/methanol-ethyl acetate (1:1) solution by ultrasonication.

The homogenate of biological/sediment samples was centrifuged (15 min at 3000 rpm), and the supernatant was transferred to decantation balloons with 50 mL of NaBr saturated water and 15 mL of ethylacetate/hexane (3:2). After extraction (by shaking for 10 min, twice) 100 mL of hexane was added into the extract and water phase was discarded. Then the organic layer was dehydrated with anhydrous sodium sulphate and concentrated using a rotary evaporator near to dryness. The concentrate was solved into small amount of ethanol and mixed with 5 mL of 1M acetate buffer (pH 5.0). Organotins in the extract were then ethylated by adding 1 mL of 5% tetraethyl sodium borate. After ethylation (by shaking for 15 min) 40 ml of 1M KOH was added to the mixture that was shaken one hour to decompose the fat. After saponification the ethylated organotins were re-extracted by hexane. Afterwards the extract was dehydrated by sodium sulphate, concentrated by a rotary evaporator, and cleaned up using a SEP-PAK plus florisil cartridge. The final solution was concentrated under a gentle nitrogen flux to 1 mL and

spiked with 50 ng of deuterated tetrabutyltin as a recovery standard. The final solutions were then injected into a gas chromatograph.

The quantification of organotin compounds was conducted by a gas chromatograph equipped with a mass spectrometer (GC-MS) (Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). GC-MS was equipped with a fused silica capillary column (0.25 mm i.d. x 30m length consisted of DB-1: 100% dimethylpolysiloxane, 0.25 µm bounded phase) and operated in electron impact and selected ion monitoring mode (EI-SIM). The concentrations of organotin compounds were calculated based on the peak areas of target compounds and their deuterated surrogates as internal standards following an internal standard isotope dilution method. Calibration curves for MBT, DBT, TBT, MPhT, DPhT, TPhT, MOcT, DOcT, TOcT, were made from the analysis of standard solutions showing 4 levels of native compound concentrations (5, 10, 50 and 250 $ng.mL^{-1}$) with a constant concentration of internal and recovery standards (50 $ng.mL^{-1}$). Recoveries of internal standards through the whole analytical procedure were estimated based on the peak areas of internal and recovery standards. Monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), monophenyltin (MPhT), diphenyltin (DPhT), triphenyltin (TPhT), monoctyltin (MOcT), dioctyltin (DOcT) and trioctyltin (TOcT) average recovery rates ±St Dev (%) were: 43.5±5.84, 81.2±4.54, 83.7±4.27; 13.8±11.09, 59.6±12.44, 107.8±13.79; 62.3±12.41, 107.5±10.09 and 113.6±11.37, for biological samples. Concentrations of MPhT were not estimated because recoveries of the internal standard for MPhT were less than 10% in almost all the analytical batches. Recoveries of internal standards for MBT were around 30 to 50 %, meaning that the concentrations of this compound must be considered as reference values. For sediments MPhT was not analysed and average recovery rates (±St Dev) for MBT, DBT, TBT, DPhT, TPhT, MOcT, DOcT and TOcT were 38.0±7.46, 70.7±9.14, 71.7±13.26; 62.8±5.57, 122.7±28.38; 62.0±10.81, 90.8±13.20 and 83.7±30.34, respectively.

To assess the QA/QC of measurements in this study, certified reference materials of fish tissue (NIES CRM No.11) and sediment (NIES CRM No.12) were analyzed by the method described above. The CRM No.11 and No. 12 have the certified concentration values for TBT at 1.3±0.1 $\mu g.g^{-1}$ and 0.19±0.03 $\mu g.g^{-1}$, respectively (both concentrations

are dry weight basis as chloride foam), and the (non-certified) reference values for TPhT at 6.3 $\mu g.g^{-1}$ and 0.08 $\mu g.g^{-1}$, respectively. Our data obtained from the analysis of these CRMs showed a good agreement: TBT concentrations in CRM No.11 (*n*=3) and No. 12 (*n*=3) were 1.2±0.06 $\mu g.g^{-1}$ and 0.18±0.003 $\mu g.g^{-1}$, respectively, and TPT concentrations in CRM No.11 (*n*=3) and No. 12 (*n*=3) were 6.3±0.13 $\mu g.g^{-1}$ and 0.070±0.001 $\mu g.g^{-1}$, respectively. In addition, a procedural blank was included with each analytical batch to check for interfering compounds and to correct sample values, if necessary. The detection limits of each organotin compound were calculated based on deviation (3σ) of each peak area when the standard solutions containing low levels of native compounds (1 or 5 $ng.mL^{-1}$) were measured by GC-MSD. If any peak was detected in the blank sample, detection limit was determined as quantities of three times those peak areas. In this study, concentrations of organotin compounds were described in terms of ng $Sn.g^{-1}$ dry weight (dw). Our methods gave a tin detection limit (in terms of ng $Sn.g^{-1}$ dw) of: 30 for MBT, 7.1 for DBT, 0.42 for TBT, 9.7 for MPhT, 0.16 for DPhT, 0.08 for TPhT, 3.1 c, 3.6 for DOcT and 0.43 for TOcT in biological samples; and 3.2 for MBT, 0.05 for DBT, 0.05 for TBT, 0.03 for DPhT, 0.03 for TPhT, 0.6 for MOcT, 0.3 for DOcT and 0.1 for TOcT in sediment samples.

4.2.5. Statistical analysis

All statistical analysis were performed using the software Statistica 6.0. The correlation analysis refers to the non parametric Spearman rank order correlation. Comparison of VDSI, FPL and female shell length values between the different years was made through the non parametric Mann-Witney *U* Test.

4.3. Results and Discussion

4.3.1. *Nassarius reticulatus*: imposex levels and OTs tissue concentrations

Levels of imposex for *N. reticulatus* showed a clear decreasing gradient from the vicinity of ports and dockyards inside Ria de Aveiro towards the offshore sea [Figure 4.2; Table 4.2]. This spatial pattern follows a clear ship traffic gradient as already noticed in previous surveys (Barroso et al., 2000; Barroso et al., 2005; Sousa et al., 2005; Rato et al., 2006). VDS stages type b, corresponding to the absence of a penis (Stroben et al., 1992), were observed at St. 2 (3%), St. 5 (6%) and St. 7 (80%). Female's penis length (Log_{10}FPLI) across all sampling sites was highly correlated with VDSI (r=0.82, p<0.001). For stations located inside Ria de Aveiro oviduct convolution was only found in females with VDSI stages ≥4 and the Log_{10}AOS was highly significantly correlated with VDSI (r=0.91, p<0.001). No sterile females were found in the present survey. Female's *N. reticulatus* organotin concentrations are shown in Table 4.3. Butyltins (BuTs) represent by far the highest fraction of organotin compounds with about 95% in relation to total organic tin (∑OTs) quantifiable by our method. TBT represented 38% of the total BTs (∑BT), followed by MBT (37% of ∑BuTs) and DBT that (25% of ∑BuTs). The gradient of BuTs contamination from the Ria de Aveiro towards the offshore sea is not so notorious as observed for imposex, though they were significantly correlated (see below). Remarkably, females from St. Of.1 exhibited high TBT tissue concentrations that were similar to the most polluted sites inside the Ria. This may be partially explained by the contaminated water that flows in the ebb tide from the Ria into the adjacent coastal zone in a west-southwest direction and by the navigational route that runs close to this station, but other causes such as butyltin accumulation and retention in sediments should be considered.

Phenyltin compounds represented only 1% of ∑OTs. Triphenyltin was quantifiable in all stations analysed and the highest levels were found in the offshore locations though with very low values. Triphenyltin compounds are commonly used as co-biocides in antifouling paints - although at much lower concentrations than TBT - and as pesticides in agriculture (Fent, 1996). The highly significant correlation found between TPhT and TBT tissue concentration in the present survey (r=0.87, p<0.001) suggests that both compounds may

have the same source (i.e. antifouling paints). Octyltin compounds are mainly used in PVC as stabilizers against decomposition by heat and light (Hoch, 2001) and are almost negligible in the study area.

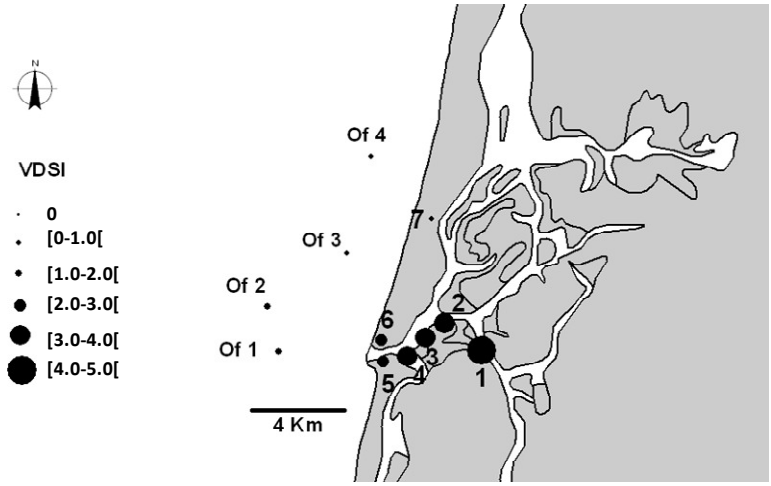

Figure 4.2. *N. reticulatus*. Spatial distribution of VDSI across the sampling stations.

TBT tissue concentration in *N. reticulatus* was highly significantly correlated to the imposex indices VDSI (r=0.867, p<0.001), FPL (r=0.869, p<0.001) for all sampling stations and to AOS index (r=0.871, p<0.001), for the sampling stations located inside Ria de Aveiro. The clear relationship between imposex and TBT found in the current study has also been observed in other field monitoring surveys (Bryan et al., 1993; Barreiro et al., 2001; Barroso et al., 2002a; Sousa et al., 2005; Rato et al., 2006) and laboratory experiments (Stroben et al., 1992; Bettin et al., 1996; Barroso et al., 2002b), which reinforces the robustness of the imposex for monitoring TBT pollution.

4.3.2. Organotin body burden in *Mytilus galloprovincialis*

Organotin concentrations in whole tissue of *M. galloprovincialis* are show in Table 4.3. Octyltin compounds were not detected in mussel samples and phenyltin compounds were not relevant since they represented only 1% of ∑OTs. Levels of BuTs in *M. galloprovincialis*

showed a marked gradient that follows the general pattern described for imposex and OTs in *N. reticulatus*. TBT was the most abundant compound (58% of ∑BuTs), followed by MBT (24% of ∑BuTs) and DBT (18% of ∑BuTs). The percentage of TBT in relation to the ∑BuTs in *M. galloprovincialis* is higher (54%) than reported for *N. reticulatus* (37%). Despite possible differences in their capacity for metabolizing TBT, the major reason to explain the different proportions of this compound could be the different uptake routes of OTs related to the habitat and food items. In fact, the gastropod may accumulate TBT preferentially via sediment and mussels from the water column (Devier et al., 2003).

4.3.3. Estimation of TBT water concentration

Measurements of TBT water concentration in Ria de Aveiro typically present large daily and weekly variability (unpublished data) due to the highly dynamic hydrology of this estuarine system related to tides and different sea and river inputs (Dias et al., 2000; Lopes et al., 2005; Vaz et al., 2005). Therefore, we estimate TBT levels in water based on TBT tissue concentrations found in *M. galloprovincialis* and the bioconcentration factor (BCF) of 11000 (Takahashi et al., 1999) for this species. Estimated seawater levels varied from 0.42 ng TBT-Sn.L^{-1} in St. 7 to a maximum value of 6.0 ng TBT-Sn.L^{-1} in St. 4 [Table 4.4]. All stations located inside ports or marinas exhibited levels above the PNEC (predicted no effect concentration) for TBT [3.0 ng TBT-Sn.L^{-1}(EPA, 2003)], indicating that the risk is high at those locations (mean risk value of 1.5). However, if we consider stations away from point sources, like St. 5-8, the risk factor drops to 0.33. When comparing the estimated levels in the present survey with the ones quantified directly from water (tide cycle average) in 1998 (Barroso et al., 2000), we found that they are one order of magnitude lower in 2005.

Table 4.2. *N. reticulatus.* Data relative to each sampling site with the indication of: numbers of males (♂ N) and females (♀N) with respective mean shell heights (mm) ± StDev; male penis length (MPL); female penis length (FPL); relative penis length index (RPLI), *vas deferens* sequence index (VDSI); female oviduct convolution (AOS); percentage of affected females (%I) and percentage of parasitized specimens (%PRZ). [(a)] For St. 7 *vas deferens* development didn't follow the usual scheme; instead the alternative b-way was observed in 80% of the affected females.

Station	Code	N♂	♂Shell height	MPL(mm)	N♀	♀Shell height	FPL(mm)	VDSI	$VDSI_{(4)}$	AOS	%I	% PRZ
Deep Sea fishing Port	1	7	24.3±4.46	15.3±3,24	12	25.0±2.23	7.8±1.21	4.8±0.39	4.0±0.00	1.3±0.75	100	0.0
North commercial Port	2	30	21.8±1.26	13.4±3.43	30	22.8±1.53	3.7±1.84	3.9±0.86	3.5±0.73	0.1±0.43	100	9.8
Forte Barra	3	30	24.2±1.53	15.5±3.03	30	24.4±3.14	2.1±1.18	3.8±0.57	3.7±0.47	0.1±0.25	100	0.0
M. Mira	4	32	22.9±3.13	10.4±3.93	32	24.6±2.53	4.5±2.32	3.6±0.70	3.5±0.51	0.1±0.35	100	1.7
Barra	5	28	25.6±2.16	13.8±2.50	35	26.6±2.38	1.1±1.84	2.2±1.33	2.2±1.33	0.0±0.00	91.4	0.0
S. Jacinto	6	23	24.3±1.68	13.2±4.43	42	25.8±1.93	0.9±1.24	2.4±1.04	2.4±1.04	0.0±0.00	90.5	3.3
Muranzel	7	26	22.0±1.19	13.0±3.01	26	22.9±1.75	0.1±0.23[(a)]	1.2±0.83[(a)]	1.2±0.83[(a)]	0.0±0.00	84.6	0.0
40°38´50 H	Of.1	14	28.1±1.53		20	30.6±1.73	0.1±0.40	1.70±1.174	1.70±1.174		85.0	9.5
40°39'50 K	Of.2	14	28.4±2.04		20	30.6±1.30	0.3±0.29	1.45±1.276	1.45±1.276		75.0	4.7
40°41'00 D	Of.3	12	26.4±1.02		23	27.8±2.10	0.0±0.07	0.13±0.344	0.13±0.344		13.0	0.0
40°43'00 D	Of.4	10	26.6±2.26		26	29.1±2.33	0.0±0.00	0.04±0.200	0.04±0.200		3.85	0.0

Table 4.3. Organotins concentrations (ng Sn.g^{-1} dw) in *N. reticulatus* and *M. galloprovincialis* across the sampling stations with indication of the moisture content for biological samples (% of dw) and organic content (%) in sediment samples.

Station	Coordinates	Code	Moisture & OM	MBT	DBT	TBT	MPhT[1]	DPhT	TPhT	MOcT	DOcT	TOcT
Nassarius reticulatus												
Deep Sea fishing Port	40º38'24N-8º43´59W	1	67.6	51	50	40	<9.7	<0.16	0.32	<3.1	<3.6	<0.43
North commercial Port	41º39'06N-8º43´76W	2	72.5	71	40	60	<9.7	<0.16	0.4	5.4	8.1	<0.43
Forte Barra	41º38'56N-8º43´59W	3	-	-	-	-	-	-	-	-	-	-
M. Mira	41º38'65N-8º44´06W	4	69.8	32	28	73	<9.7	<0.16	1.3	<3.1	7.4	<0.43
Barra	41º38'71N-8º44´82W	5	73.7	54	18	25	<9.7	<0.16	0.34	11.8	<3.6	<0.43
S. Jacinto	41º39'84N-8º43´56W	6	73.5	37	25	38	<9.7	<0.16	0.66	<3.1	<3.6	<0.43
Muranzel	40º43'13N-8º41'55W	7	67.4	<30	11	15	<9.7	<0.16	0.34	<3.1	<3.6	<0.43
Offshore 1	40º38´50N-8º47'40W	Of. 1	75.7	53	43	66	<9.7	<0.16	2.5	<3.1	<3.6	<0.43
Offshore 2	40º39'50N-8º47'96W	Of. 2	69.0	<30	19	27	<9.7	<0.16	1.5	<3.1	<3.6	<0.43
Offshore 3	40º41'00N-8º45'06W	Of. 3	71.5	32	19	28	<9.7	<0.16	0.98	<3.1	<3.6	<0.43
Offshore 4	40º43'00N-8º44'45W	Of. 4	72.4	33	18	33	<9.7	<0.16	0.88	<3.1	5.4	<0.43
Mytilus galloprovincialis												
Deep Sea fishing Port	40º38'24N-8º43´59W	1	80.7	115	94	310	51	<0.16	0.15	<3.1	<3.6	<0.43
North commercial Port	41º39'06N-8º43´76W	2	80.1	163	66	175	<9.7	<0.16	0.13	<3.1	<3.6	<0.43
Forte Barra	41º38'56N-8º43´59W	3	-	-	-	-	-	-	-	-	-	-
M. Mira	41º38'65N-8º44´06W	4	86.9	125	107	500	<9.7	<0.16	0.54	<3.1	<3.6	<0.43
Barra	41º38'71N-8º44´82W	5	80.0	<30	13	45	<9.7	<0.16	0.36	<3.1	<3.6	<0.43
S. Jacinto	41º39'84N-8º43´56W	6	78.8	72	29	31	<9.7	<0.16	0.21	<3.1	<3.6	<0.43
Muranzel	40º43'13N-8º41'55W	7	80.4	<30	11	24	23	<0.16	<0.08	<3.1	<3.6	<0.43
Torreira	40º45'37N-8º41'58W	8	74.3	32	44	95	12	<0.16	0.12	<3.1	<3.6	<0.43
Marina Ovar	40º51'35N-8º39'26W	9	75.4	<30	38	149	<9.7	<0.16	1.4	<3.1	<3.6	<0.43
Sediments												
Deep Sea fishing Port	40º38'24N-8º43´59W	1	0.83	18	21	307	na	<0.03	<0.03	<0.06	<0.3	<0.1
North commercial Port	41º39'06N-8º43´76W	2	-	-	-	-	-	-	-	-	-	-
Forte Barra	41º38'56N-8º43´59W	3	-	-	-	-	-	-	-	-	-	-
M. Mira	41º38'65N-8º44´06W	4	1.6	19	5.9	23	na	<0.03	<0.03	<0.06	<0.3	<0.1
Barra	41º38'71N-8º44´82W	5	-	-	-	-	-	-	-	-	-	-
S. Jacinto	41º39'84N-8º43´56W	6	1.8	<3.2	0.4	2.7	na	<0.03	<0.03	<0.06	<0.3	<0.1
Muranzel	40º43'13N-8º41'55W	7	2.0	18	19	184	na	<0.03	<0.03	<0.06	<0.3	<0.1
Torreira	40º45'37N-8º41'58W	8	2.4	442	658	1780	na	<0.03	<0.03	<0.06	<0.3	1.0
Marina Ovar	40º51'35N-8º39'26W	9	2.6	17	9.9	65	na	<0.03	<0.03	<0.06	<0.3	<0.1

Table 4.4. Estimated seawater concentrations across the sampling stations, considering the bioconcentration factor described for *M. galloprovincialis* in field studies of 11 000.

Station	Code	Seawater (ng TBT-Sn.L^{-1})
Deep Sea fishing Port	1	5.4
North commercial Port	2	3.2
Forte Barra	3	-
M. Mira	4	6.0
Barra	5	0.8
S. Jacinto	6	0.6
Muranzel	7	0.4
Torreira	8	2.2
Marina Ovar	9	3.3

4.3.4. Organotin levels in sediments

Granulometric analysis of sediment samples revealed that St. 1 presented muddy sands (fraction 63 µm<25%) whilst the remainder stations presented sandy muds (fraction 63 µm between 25 to 75%). The organic matter (OM) content of each station varied between 0.84 – 2.6 (% of dw).

Phenyltin compounds were not detected in sediment samples and octyltin compounds were only detected in St.8 (MOcT: 10 ng Sn.g^{-1} dw), therefore BuTs represents the major group of OTs in sediments. TBT represented 75.6% of ∑BuTs, followed by DBT (12.6% of ∑BuTs) and MBT (11.8% of ∑BuTs). ∑BuTs in sediments was highly correlated with organic matter across the sampling stations (r=0.869, p<0.001). The highest levels of BuTs in biological samples were detected in St. 1 and St. 4 whilst in sediments St. 8 is by far the most contaminated site (the OM content for this station (2.5%) is higher than the ones reported for St. 1 and St. 4: 0.8 and 1.6%, respectively).

4.3.5. Temporal variation of imposex and organotin body burden

Temporal comparisons of imposex levels for the same locations between 1998 (Barroso et al., 2000), 2000 (Barroso et al., 2002a), 2003 (Sousa et al., 2005) and the

present survey are shown in Figure 4.3. In all occasions the spatial pattern of imposex was similar, with the highest levels inside ports (St. 1, St. 2). This supports the idea that the distribution of imposex levels does not change randomly over time and space - an important prerequisite when assessing temporal and spatial patterns of pollution. Statistical comparisons of imposex indices were performed only between 2003 and 2005 [Figure 4.3] as we are particularly interested on the recent evolution of TBT pollution after the implementation of the EU Directive in 2003. We found no significant differences in the VDSI for all stations between 2003 and 2005. In what regards to FPLI, and considering that no significant differences between female's shell length was observed between both surveys (which could affect penis size), a significant decrease in the female penis length was registered in Sts. 2, 3 and 5 between 2003 and 2005. Comparisons of OTs tissue concentrations also show a slight decrease in TBT tissue concentrations from 2000 to 2005 [Figure 4.4A].

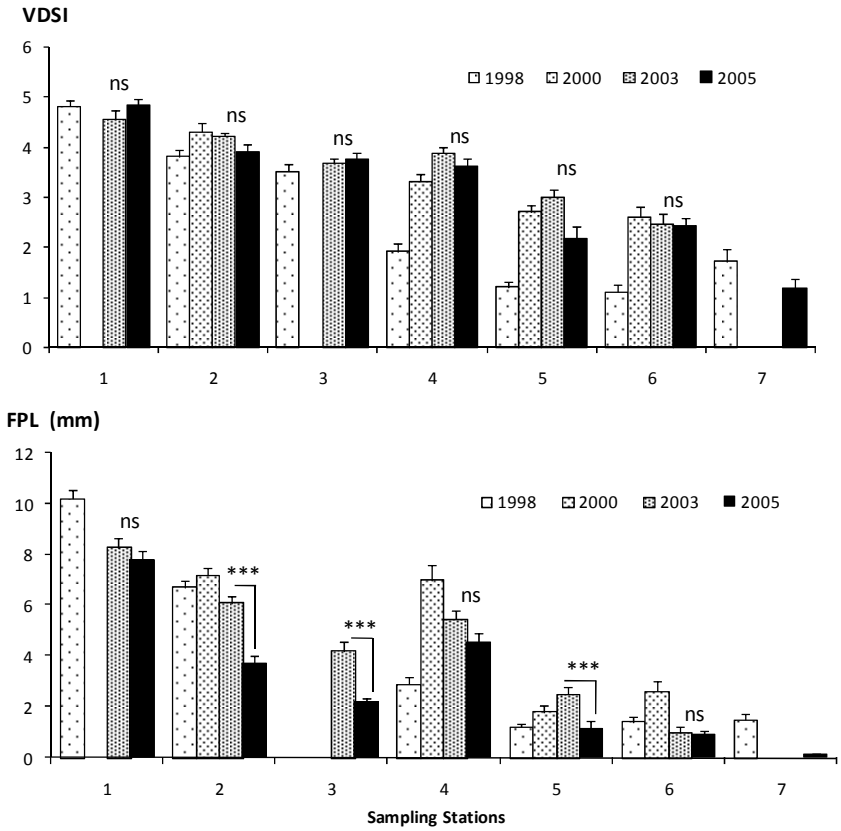

Figure 4.3. Temporal comparisons of VDSI and FPL imposex indices between 1998 and 2005. Bars represent standard error. Statistical comparisons were performed for 2003 (the year of the EU ban) and the present survey [ns: not significant; *: *p<0.05*; **: *p<0.01*; ***: *p<0.001*)]. The low value of FPL in St. 7 is due to the high proportion of VDSI type b (see text).

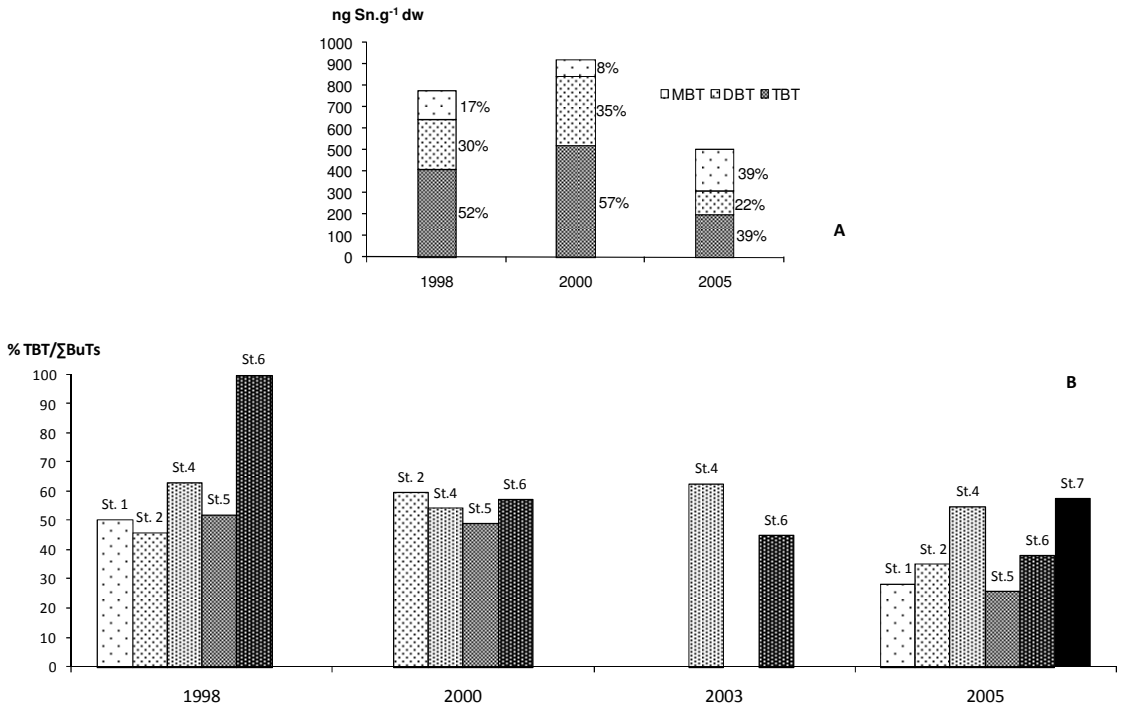

Figure 4.4. Relative proportions of mean *N. reticulatus* butyltins tissue concentrations in relation to total butyltins (∑BuTs) observed at common locations (St. 2-6) each year inside Ria de Aveiro from 1998 to 2005 (A) and relative proportion of TBT in each sampling station along the same period (B). The samples were collected between May and July for all surveys. The butyltins were quantified by GC-MSD in two different laboratories: Servicios Xerais de Apoio á Investigatión – Coruña University – in 1998 and 2000 and CMES – Ehime University – in 2005. In both laboratories the procedure was validated using certified reference material of fish tissue (NIES CRM No.11). Values bellow detection limit were considered as zero.

This evolution over such short time period may reveal a decrease in TBT pollution and therefore lower levels of imposex due to population renovation or to a limited degree of imposex reversibility, a phenomenon already reported in previous studies (Bryan et al., 1993; Sousa et al., 2005). It is not possible to know if the above decrease occurred as a direct consequence of the 2003 EU ban. As a matter of fact, in some cases a reduction in imposex is apparent from 2000 onwards which could be due to a decline on the use of TBT paints just before 2003 as some companies started to phase out the commercialization of these paints due to the upcoming ban, but other causes should be checked as well. The major conclusion that can be drawn from the current

work is that there is an apparent decreasing trend in the level of TBT pollution in the area, and the EU ban may had contributed for this tendency after 2003.

The reduction of TBT pollution in Ria de Aveiro over the last years does not mean that the emissions of TBT have stopped. Figure 4.5 shows the average proportions of butyltins in gastropods, mussels and sediments from all stations analysed in the present survey. The highest proportion of TBT in relation to total butyltins (TBT/∑BuTs) was detected in sediments followed by mussels and gastropods, respectively. This can be easily explained by the much higher TBT half life in sediments (1.85 to more than 10 years) than in molluscs (2-3 months) (Batley, 1996; OSPAR, 1998). The relative proportions of butyltins may provide an additional insight about the recent contamination history of a given location.

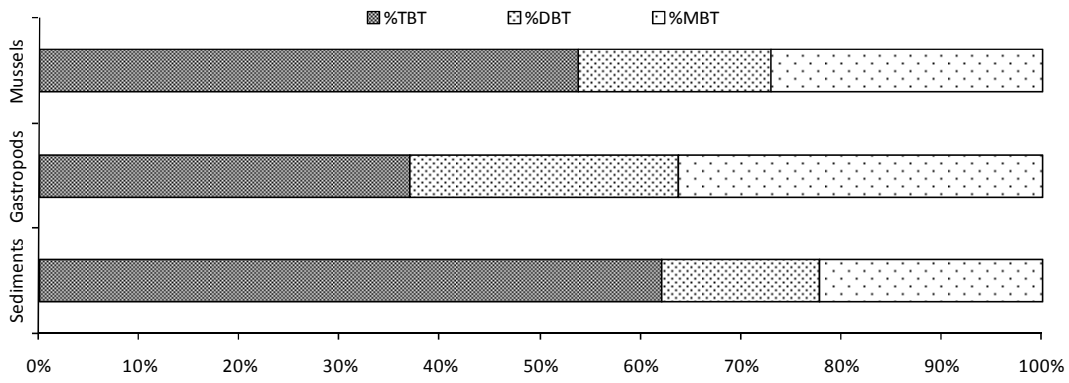

Figure 4.5. Relative composition of mean butyltins across all sampling stations in the different compartments analysed: sediments, gastropods and mussels.

The high proportion of TBT in relation to ∑BuTs observed in 2005 in all analysed compartments indicates that there are still recent inputs of this contaminant to the area and that OT pollution is still a matter of great concern. This should be expected since the use of TBT based antifouling paints is allowed in all boats untill 2008. Furthermore, boats can still be painted with TBT outside the European Union and in countries were the AFS convention has not been signed yet. Figure 4.6 shows the number of commercial ships that entered the Ria de Aveiro over the last years and

indicates their provenience since 2003, when the European ban took place. In 2003 about 50% of the ships came from countries were TBT could still be applied whereas in 2005 that percentage decreased to 23%. Although there have been recent inputs of TBT into the area, the total amount of the inputs may have decreased lately since we observed a slight decline in both OTs, TBT proportion and imposex levels in Aveiro between 2003 and 2005 [Figures 4.3-4.5]; this may be related to the fact that local dockyards cannot apply anymore OT antifouling paints since 2003 and also because an increasing number of boats that call the port of Aveiro have not been repainted with TBT.

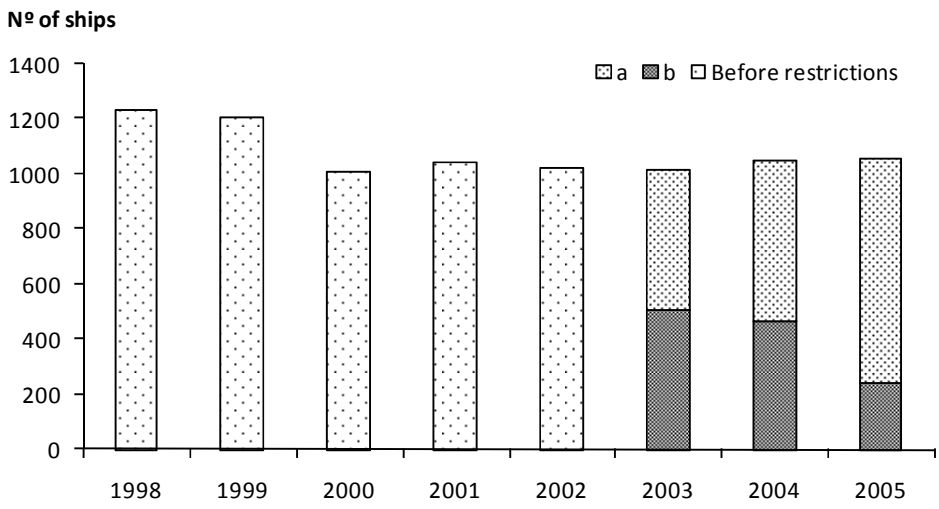

Figure 4.6. Number of ships entered in Aveiro Port each year since 1998 with reference to the number of ships entered between 2003 and 2005 from: (a) countries where the application of TBT is forbidden (European Union and other countries that have already ratified the AFS Convention), (b) countries with no restrictions on TBT use.

The present work denotes a decrease in TBT pollution in Aveiro over the last years, particularly after 2003, for which the EU ban could had played an important role. If consistent, this tendency should become more evident through the upcoming years. Hence, this survey forms an important baseline and time series on TBT pollution in the

region in order to assess the impact of a global ban on TBT antifouling, due to be in place by 2008.

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CHAPTER 5

IMPOSEX AND ORGANOTIN PREVALENCE IN A EUROPEAN POST-LEGISLATIVE SCENARIO: TEMPORAL TRENDS FROM 2003 TO 2008

Chemosphere (in press)

CHAPTER 5. IMPOSEX AND ORGANOTIN PREVALENCE IN A EUROPEAN POST-LEGISLATIVE SCENARIO: TEMPORAL TRENDS FROM 2003 TO 2008

Abstract

Imposex levels and organotin tissue concentrations were assessed in *Nassarius reticulatus* females collected between June and August 2008 at 23 sites along the Portuguese coast. Females with imposex were present at all sampling sites with highest levels inside main harbors. Imposex parameters across sampling stations varied between 6.3 and 100% for %I, 0.2-4.4 for VDSI, 0.1-7.8 mm for FPL, 0.3-88.9% for RPLI and 0.0-1.1 for AOS. TBT levels varied between 3.5 and 380 ng $Sn.g^{-1}$ dw, representing an average proportion of 50.4% of total butyltins (∑BuTs= MBT+DBT+TBT). Sterile females were detected at two locations. Highly significant correlations between imposex and TBT levels were found. The efficacy of the EU legislation banning the use of TBT-based antifouling paints since 2003 (EC Regulation 782/2003) was evaluated by comparing the levels of imposex observed in 2008 with those reported for 2003. OTs tissue concentrations were also determined in preserved samples collected in 2003. There was a decrease in imposex and TBT tissue contamination between 2003 and 2008 indicating that a decline in TBT pollution has occurred in the Portuguese coast since the implementation of the legislation. Considering that the EC Regulation 782/2003 is an anticipation of the IMO AFS Convention, a global scale decrease in TBT pollution can be expected in the near future. Despite the rapid amelioration in the Portuguese coast, TBT pollution is still a problem as the Ecological Quality Objective (EcoQO) proposed by OSPAR Commission was not achieved in 91% of the surveyed sites.

Keywords:

Nassarius reticulatus; Tributyltin; Portuguese coast; Sterility; EC Regulation 782/2003

5.1. INTRODUCTION

Tributyltin (TBT) is a biocide that enters the marine environment mainly through the leaching of antifouling (AF) paints from ship's hulls and other immersed structures. It is a well known endocrine disruptor capable of inducing sexual anomalies in marine gastropods as well as many other deleterious effects in wildlife and humans (for reviews see Bryan & Gibbs, 1991; Fent, 2006; Antizar-Ladislao, 2008). Imposex (defined as superimposition of male characters such as a penis and a *vas deferens* onto gastropods

females) is a powerful biomarker of TBT pollution and has been used worldwide to map its distribution in the marine environment. Whilst initial reports focused mainly on spatial pollution gradients, in recent years, an evaluation of temporal pollution trends and population imposex recovery are the two principal monitoring targets in order to evaluate the effectiveness of the successive legislative measures. In Europe, the ban on the use of TBT AF paints on small boats (Directive 89/677/EEC) adopted in the late 1980s and early 1990s (in Portugal this directive was introduced in 1993) revealed itself ineffective in reducing TBT pollution (e.g. Barroso & Moreira, 2002; Santos et al., 2002). With the proven inefficacy of the partial restrictions, attention is now focused on the TBT global ban adopted by the International Maritime Organization (IMO) in 2001 that entered into force in September 2008. In the European Union (EU), the global ban was enacted by EC Regulation 782/2003 that adopted a total interdiction on the application of TBT-based formulations to vessel's hulls after 1st of July 2003 and the prohibition on the presence of those paints on vessel's hulls from 1st of January 2008. As this prohibition was extended to all vessels entering EU ports, a decline in TBT pollution in the EU is expected. It is now essential to monitor these changes in order to understand the degree of imposex recovery in gastropod indicator species, with 2003 and 2008 being benchmark years for this purpose.

The present work aims to create a base-line of imposex and organotin levels in *Nassarius reticulatus* (Linnaeus, 1758) for the Portuguese coast in 2008 and compare this baseline with that reported for 2003 (Sousa et al., 2005) in order to assess the effectiveness of EC Regulation 782/2003.

5.2. Materials and Methods

5.2.1. Study area

The Portuguese coastline extends for around 950 Km and encompasses several estuarine areas where main harbors are located [Figure 5.1]. Those harbors include commercial and fishing ports, marinas and dockyards. Table 5.1 provides a summary of

the evolution of commercial ship traffic in Portuguese harbors during 2003-2008. Recreational and fishing vessels also contribute to the naval traffic over the study area (for a more detailed description see Rato et al., 2009). Parallel to the coastline a maritime traffic line is positioned 40 Km offshore. This is one of the main maritime traffic lines linking the Mediterranean, the north of Europe, Africa, and America, with an average of 100 ships crossing the line, per day (Delfaud et al., 2005). Several studies performed over the last two decades identified harbors as hotspots of TBT pollution in Portugal and also revealed that outside these areas the pollution is diffuse with lower levels along the entire coastline and in offshore waters (e.g. Santos et al., 2002; Sousa et al., 2005; Galante-Oliveira et al., 2006; Rato et al., 2006; Rato et al., 2008).

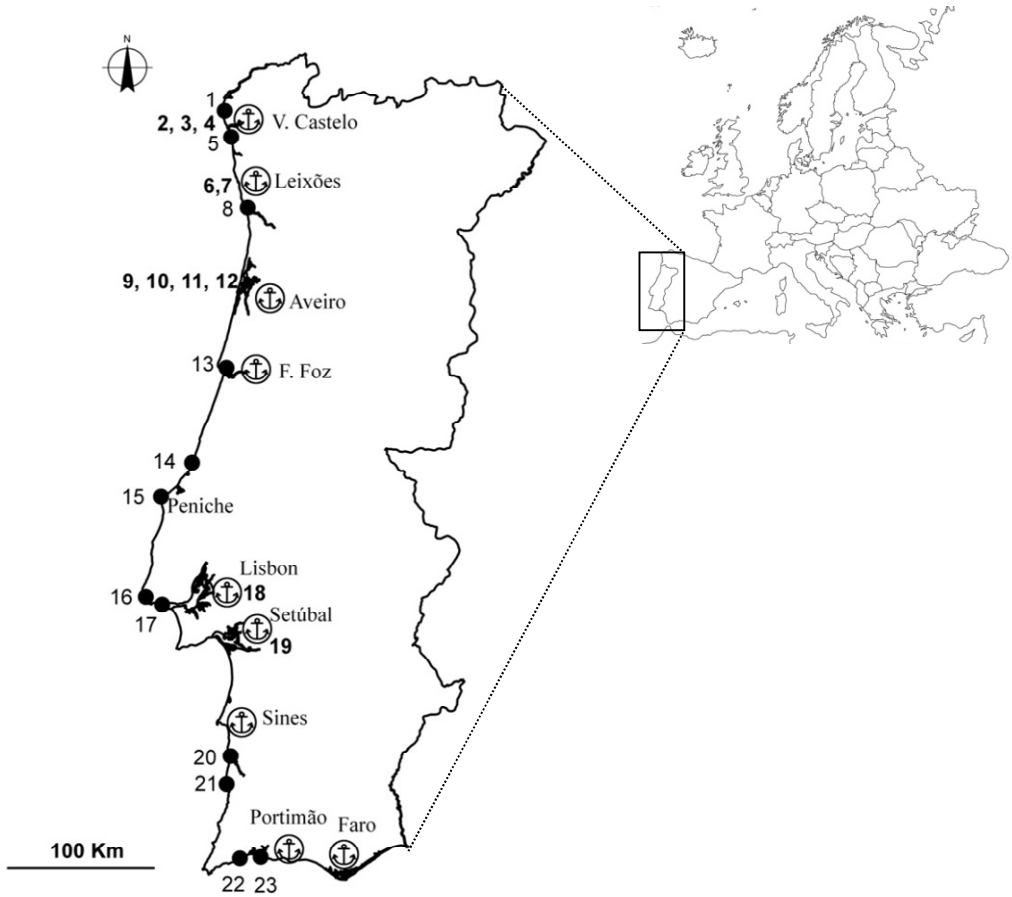

Figure 5.1. Map of the Portuguese coast indicating the sampling stations and the main harbors ⚓.

Table 5.1. Commercial ship traffic activity in Portuguese harbors between 2003 and 2008 expressed in terms of total numbers of ships entered at each port (No.) and total gross tonnage stood (GTs). Data obtained from *IPTM - Instituto Portuário e dos Transportes Marítimos* website (www.imarpor.pt), T: tons, (-) data not available.

Harbor	2003		2005		2006		2007		2008	
	No.	$GT.10^{-3}T$	No.	$GT.10^{-3}T$	No.	$GT.10^{-3}T$	No.	$GT.10^{-3}T$	No.	$GT.10^{-3}T$
V. Castelo	261	866	197	872	211	923	228	990	189	884
Leixões	2690	20519	2739	20009	2725	20415	2739	21661	2625	22860
Aveiro	1002	2732	1057	2850	1064	3141	977	3069	1010	3325
F. Foz	269	597	299	750	320	823	363	969	409	1052
Lisboa	3522	40219	3351	38569	3336	36777	3281	38271	3455	43621
Setúbal	1617	16715	1508	16923	1498	16202	1446	14324	1397	14202
Sines	751	13155	1192	22916	1351	29693	1465	31671	1489	32887
Portimão	33	401	74	1439	24	90485	14	31016	-	-
Faro	49	141	38	96	23	67	23	66	-	-

5.2.2. SAMPLING CAMPAIGN

5.2.2.1. Sampling

Thirty-one locations spread along the Portuguese coast were surveyed between June and August 2008, but *N. reticulatus* was obtained only in 23 of those locations [Figure 5.1; Table 5.2]. The sampling campaign aimed to evaluate spatial and temporal evolution of imposex and organotin tissue content in the netted whelk *N. reticulatus* in the mainland Portuguese coast between 2003 and 2008; hence the sampling sites were the same as the ones previously selected (Sousa et al., 2005), with the exception of St. 19 that was deviated 250 m in the current campaign compared to the 2003 campaign. Whelks were captured by hand at the intertidal shore whereas at sublittoral sites baited hoop nets were used. The animals were brought to the laboratory and observed within two days. During that time they were maintained in permanently aerated seawater collected from respective sampling sites.

5.2.2.2. Imposex analysis

Wherever possible, 60 adults from each site were selected. Shell heights (distance from shell apex to lip of siphonal canal) were measured with vernier calipers to the nearest 0.1 mm. After narcotization (40 min; 7% $MgCl_2$ in distilled water), shells were cracked open with a bench vice and the animals gently removed, sexed and dissected under a stereo microscope. The following imposex parameters were assessed: percentage of imposex affected females (% I), *vas deferens* sequence index (VDSI), mean female penis length (FPL), mean male penis length (MPL), relative penis length index (RPLI= (FPL/MPL)*100), average oviduct stage (AOS) and percentage of sterile females (%STE). The penis length was measured (to the nearest 0.14 mm) with a stereo microscope graduated eyepiece. The VDSI was classified according to the scoring system proposed by Stroben et al. (1992a) except that the VDSI stage 4+ was converted to stage 5 in order to better describe spatial gradients, as proposed by Barroso et al. (2002a). Throughout the paper the VDSI values refers to this modified scale, however the values of $VDSI_{(4)}$ according to Stroben et al. (1992a) are given in Table 5.2 to allow comparisons with other works. The average oviduct stage (AOS) was ranked according to the 3-stage scale proposed by Barreiro et al. (2001). Parasitized animals were discarded from analysis.

5.2.3. Laboratory experiments

In order to understand the degradation of TBT in *N. reticulatus* over short time periods, animals collected from two locations with distinct pollution levels (St. 4 and St. 10) were allowed to depurate in the laboratory in artificial seawater (Crystal Sea® Bioassay Formulation, Marine Enterprises International) (salinity 30‰) for 60 days. The experiments were conducted in an acclimatized room (18 ^{o}C ± 1 ^{o}C) under a 16 h light: 8 h dark photoperiod. The animals were maintained in 15 L aquaria with an internal power filter and permanently aerated. For each location two aquaria were used with approximately 100 animals each. Whelks were fed once a week with mussels ($[TBT]_{mussels}$<5 ng $Sn.g^{-1}$ dw) and seawater was changed every two days. Sub-samples of 24 animals were taken at the beginning of the experiment (Day 0), at Day 30 and at the end

of the experiment (Day 60). Imposex evaluation followed the same protocol described in section 6.2.2.2 and female's tissues of each treatment were pooled together and kept frozen at -20°C for later organotin (OTs) analysis.

5.2.4. Organotin analysis

Chemical analyses were performed in pooled whole soft tissues of 14-18 females. The tissues were freeze dried, ground into powder and kept at -20°C in dark conditions. Since one of the objectives of the present study is to evaluate temporal trends in TBT pollution, five samples of pooled *N. reticulatus* female's tissues collected in 2003 campaign (preserved as described above) were also analyzed to compare OTs tissue concentrations.

Organotin compounds in gastropod samples were quantified according to the method described by Sousa et al. (2007) based on the method proposed by Iwamura et al. (2000). In short, deuterated labeled standards (d_9-MBT, d_{18}-DBT, d_{27}-TBT, d_{10}-DPT, d_{15}-TPT, d_{17}-MOT, d_{34}-DOT) were spiked into the samples (0.5-1.0 g dry weight (dw)), as surrogates, before extraction. OTs in the samples were extracted by 1 N HBr/methanol-ethyl acetate (1:1) and then transferred to ethyl acetate/hexane (3:2) and concentrated by rotary evaporation. OTs in the extracts were ethylated by adding 1 mL of 5% tetraethyl sodium borate. After ethylation, the extracts were cleaned up by 1 M KOH and SEP-PAK plus florisil cartridge (Waters). OTs were eluted by 5% diethylether/hexane, then solutions were concentrated to 1 mL and spiked with 50 ng of deuterated tetrabutyltin used as a recovery standard. The final solutions were injected into a gas chromatograph-mass spectrometric detector (GC-MSD) (Hewlett-Packard 6870 GC system with 5973 mass selective detector and 7683 series auto sampler). OTs were measured by GC-MSD in selected ion monitoring mode (EI-SIM) and quantified by isotope dilution method. Average recovery rates ± St. Dev (%) for each surrogate compound in the 16 analyzed samples were: d_9-MBT: 73.2±29.5, d_{18}-DBT: 125.9±24.5, d_{27}-TBT: 95.2±11.3, d_{10}-DPT: 89.5±15.1, d_{15}-TPT: 109.7±8.7, d_{17}-MOT: 63.4±18.4 and d_{34}-DOT: 130.5±40.7. To assess the QA/QC of measurements in this study, two certified reference materials - fish tissue (NIES CRM No.11) and mussel tissue (ERM-CE 477) - were also analyzed by the method

described above. Results obtained from three replicate analysis of NIES CRM No. 11 (1.1±0.1 µg.g^{-1} as TBTCl) and ERM-CE 477 (2.0±0.1 µg.g^{-1} as TBT^{+}, 1.4±0.1 µg.g^{-1} as DBT^{2+}, 2.2±0.1 µg.g^{-1} as MBT^{3+}) were found to be in accordance with the certified value reported (1.3±0.1 µg.g^{-1} as TBTCl) for NIES CRM No. 11 and also for ERM-CE 477 (2.2±0.19 µg.g^{-1} as TBT^{+}, 1.5±0.12 µg.g^{-1} as DBT^{2+}, 1.5±0.28 µg.g^{-1} as MBT^{3+}), although some difference was found for MBT and therefore those levels should be considered as reference ones. In addition, a procedural blank was included, with each analytical batch, to check for interfering compounds and to correct sample values, if necessary. Methods gave a tin detection limit (in terms of ng Sn.g^{-1} dw) of 5.0 for MBT; 0.5 for DBT; 0.5 for TBT; 0.5 for DPT; 0.5 for TPT; 5.0 for MOT and 5.0 for DOT.

All chemicals (for ultra trace analysis) were purchased from Wako Pure Chemical Industries (Japan), except hydrobromic acid (Kanto Chemical Co., Inc., Japan) and sodium tetraethylborate (Hayashi Pure Chemical Ind., Co., Ltd., Japan). Standard solutions were purchased from Hayashi Pure Chemical Ind., Co., Ltd., Japan.

5.2.5. Statistical analysis

All statistical analysis was performed using SigmaStat software v3.5. Correlation analysis refers to the non-parametric Spearman rank order correlation. Comparison of imposex levels (VDSI and FPL) and TBT concentrations between 2003 and 2008 at each site was performed using the non-parametric Mann-Whitney U-test. For laboratory data the non-parametric Kruskal Wallis test (Anova on ranks) was used followed by the post-hoc Student Newman Keuls. The adopted critical significance level was 5%.

5.3. RESULTS

5.3.1. Current status of imposex levels along the coast

Of the 1145 studied animals, females represented 58% and males represented 42%. Imposex affected females were found at all sampling sites [Table 5.2]. Imposex

parameters across sampling stations varied between 6.3 and 100% for %I, 0.2-4.4 for VDSI, 0.1-7.8 mm for FPL, 0.3-88.9% for RPLI and 0.0-1.1 for AOS. Highly significant correlations were obtained between VDSI *vs* FPL (r=0.944, p<0.001) and VDSI *vs* RPLI (r=0.944, p<0.001). The degree of oviduct convolution expressed by AOS also exhibited a highly significant correlation with VDSI (r=0.787, p<0.001) and only females with VDSI>2 exhibited a convoluted oviduct. Sterile females were found at two locations: St. 4 (7.1%) and St. 6 (2.9%). Those females exhibited an obstructed vulva and aborted egg capsules inside the capsule gland. Although this condition was observed only in three of all the analyzed females in the current campaign (two at St. 4 and one at St. 6), other females were close to sterilization with partial obstruction of the vulva (three at St. 4; one at St. 6 and four at St. 19) but no aborted egg capsules were found inside the capsule gland.

Spatial distribution of imposex levels confirms harbors as hotspots of TBT pollution, in particular V. Castelo (Sts. 2-4), Leixões (St. 6) and Setúbal harbors (St. 19) where VDSI attained values between 3.1 and 4.4. High levels of imposex were also detected in Nazaré and Peniche fishing ports (St. 14 and St. 15), with VDSI values of 3.8 and 4.1, respectively. The lowest imposex levels were found in sites located away from harbors in the south coast, namely St. 20, St. 21 and St. 23 with VDSI values of 0.7, 0.4 and 0.9, respectively; and surprisingly (see discussion) inside Aveiro harbor (St. 12, VDSI=0.3) [Table 5.2]. The other imposex parameters followed the same spatial trend.

5.3.2. Organotin concentrations along the coast

Organotin compounds were quantified in 11 samples collected during the 2008 campaign [Table 5.3]. Butyltins (BuTs) accounted for 98.6% of total organotins (∑OTs). TBT levels varied between 3.5 and 380 ng Sn.g^{-1} dw, representing an average proportion of 50.4% of total BuTs (∑BuTs= MBT+DBT+TBT); DBT levels varied between 3.1 and 220 ng.Sn g^{-1} dw, accounting for 26.7% of ∑BuTs; MBT levels varied between <5.0 and 180 ng Sn.g^{-1} dw, representing 22.9% of ∑BuTs. Phenyltins represented only 1.4% of ∑OTs, and were detected at three locations: St. 4, St. 16 and St. 18. Octyltins were always below the detection limit (<5.0 ng Sn.g^{-1} dw). Significant correlations between VDSI *vs* TBT (r=0.700;

p<0.05) and between FPL *vs* TBT (*r*=0.609; *p*<0.05) were observed. The highest TBT level was found in St. 4 (380 ng Sn.g^{-1} dw) that also registered the highest VDSI level in the present study (VDSI=4.4). The lowest TBT concentrations were detected at St. 8 (3.5 ng Sn.g^{-1} dw) and St. 20 (4.9 ng Sn.g^{-1} dw). In order to investigate the predominance of TBT over its metabolites, and thus the occurrence of fresh TBT inputs in the environment, the butyltin degradation index (BDI=([MBT]+[DBT])/[TBT]) proposed by Díez et al. (2002) for sediments and later used in molluscs (Kim et al., 2008; Ruiz et al., 2008; Sousa et al., 2009) was calculated. BDI levels varied between 0.4 and 1.4, with an average value of 0.9±0.30, indicating that fresh inputs still occur in the Portuguese coast (BDI<1 corresponds to recent inputs) at least in some locations.

The results from the 2003 samples are shown in Table 5.3. Similarly, butyltins represented by far the highest fraction with 99.6% of total butyltins. Phenyltins represented only 0.4% and octyltins were always bellow detection limit (<5.0 ng Sn.g^{-1} dw). TBT levels varied between 67 and 203 ng Sn.g^{-1} dw, representing 58% of ∑BuTs; DBT levels varied between 13 and 83 ng Sn.g^{-1} dw, representing 20% of ∑BuTs and MBT levels varied between 15 and 86 ng Sn.g^{-1} dw accounting for 22% of ∑BuTs. BDI values varied between 0.42 and 0.94, with an average value of 0.7±0.22.

5.3.3. Temporal trends of imposex and organotin levels

In order to assess temporal variation of imposex and organotin concentrations in *N. reticulatus* females, results from the present campaign were compared to the ones reported for the year 2003 (Sousa et al., 2005). Since at some of the sampling sites no animals could be found, and at St. 19 the collection was not performed at the same place as in 2003, only 16 locations were used in the temporal trend analysis. Temporal variation of VDSI levels between 2003 and 2008 is shown in Figure 5.2. Significant decreases were noticed at most of the common locations. Mann-Whitney U-test demonstrates highly significant decreases (*p*<0.001) at 11 sites and significant decreases (*p*<0.05) at three sites (St. 6, 8, 20). Only St. 4 and St. 21 did not exhibit any significant difference between 2003 and 2008.

Table 5.2. Description of sampling locations with the indication of: number of males (N♂) and females (N♀) with respective mean shell heights (SH) ± StDev; male penis length (MPL); female penis length (FPL); relative penis length index (RPLI), *vas deferens* sequence index (VDSI); female oviduct convolution (AOS) and percentage of affected females (%I). Geographical coordinates refers to WGS84 Datum. B: beach; CM: Commercial Port; E: Estuary Entrance; FB: Ferry Boat; FP: Fishing port; M: Marina; S: Shipyard.

Station code - name	Type	Geographical Coordinates	N♂	♂SH (mm)	MPL (mm)	N♀	♀SH (mm)	FPL (mm)	RPLI (%)	VDSI	$VDSI_{(4)}$	AOS	%I
1 – Praia do Norte	B	41º41’43.39’’N/8º51’08.13’’W	9	20.7±2.0	10.0±2.1	18	24.9±2.2	0.1±0.2	1.4	1.1±0.6	1.1±0.6	0.0±0.0	88.9
2 – V. Castelo: Cais	FP	41º41’17.59’’N/8º50’04.30’’W	39	24.1±2.3	10.2±2.1	27	25.4±1.8	7.7±2.4	74.8	3.6±1.1	3.4±0.9	0.7±0.8	96.3
3 – V. Castelo: Marina	M	41º41’39.08’’N/8º49’15.90’’W	19	23.0±1.9	11.1±1.4	38	25.4±2.3	1.4±1.0	12.3	3.1±1.0	3.0±0.8	0.0±0.0	100
4 – V. Castelo: Estaleiro	S	41º41’15.73’’N/8º50’20.50’’W	29	23.2±2.1	11.2±1.9	28	24.4±2.9	7.4±1.6	65.7	4.4±0.8	3.8±0.4	0.9±0.7	100
5 – Praia da Amorosa	B	41º38’30.55’’N/8º49’26.06’’W	14	22.9±1.9	11.5±1.3	31	24.8±1.9	0.1±0.2	0.9	1.1±1.1	1.1±1.1	0.0±0.0	61.3
6 – Leixões: Marina	M	41º11’11.89’’N/8º42’18.31’’W	22	21.8±2.1	10.9±1.5	35	22.8±2.7	7.2±2.4	66.7	3.8±0.9	3.6±0.8	0.6±0.7	97.1
7 – Leixões: Pl.2	CP	41º11’19.12’’N/8º41’37.04’’W	30	24.5±2.4	12.1±1.3	35	24.8±2.2	1.5±1.1	12.1	2.8±0.6	2.8±0.6	0.1±0.4	100
8 – Praia da Foz	B	41º09’36.32’’N/8º41’11.18’’W	22	23.3±1.8	14.1±3.0	35	25.7±2.0	0.1±0.1	0.6	0.6±0.9	0.6±0.9	0.0±0.0	42.9
9 – Aveiro: Barra	E	40º30’39.26’’N/8º44’55.64’’W	22	24.2±2.3	13.4±1.9	43	26.1±2.5	0.6±1.0	4.2	1.1±1.3	1.1±1.3	0.0±0.0	55.6
10 – Aveiro: M. Mira	FP	40º38’34.65’’N/8º44’06.80’’W	20	23.0±3.3	11.0±2.8	31	24.5±3.2	0.6±0.7	5.7	2.2±1.1	2.2±1.1	0.0±0.0	93.6
11 – Aveiro: Forte Barra	FB	40º38’52.94’’N/8º44’03.66’’W	19	23.5±2.6	13.1±2.7	37	24.7±2.0	0.4±0.4	3.4	1.7±1.4	1.7±1.4	0.0±0.0	67.6
12 – Aveiro: P. Com. Norte	CP	40º38’56.65’’N/8º43’46.82’’W	10	21.8±0.8	9.5±3.82	22	23.4±1.6	0.1±0.0	0.3	0.5±0.6	0.5±0.6	0.0±0.0	40.9
13 – Figueira Foz	M	40º08’50.65’’N/8º51’42.50’’W	13	24.9±1.5	9.7±2.61	26	25.5±1.2	0.1±0.1	0.7	0.6±0.8	0.6±0.8	0.0±0.0	42.3
14 – Nazaré	FP	39º35’00.03’’N/9º04’26.02’’W	15	21.1±2.0	9.9±1.57	17	21.1±2.5	3.6±0.7	36.6	3.8±0.7	3.8±0.4	0.7±0.6	100
15 – Peniche	FP	39º21’20.25’’N/9º22’28.69’’W	22	21.9±1.3	10.6±1.7	30	23.9±1.7	3.6±0.6	33.8	4.1±0.5	3.9±0.2	0.9±0.8	100
16 – Praia do Guincho	B	38º43’40.55’’N/9º28’35.81’’W	20	22.7±2.9	12.3±2.2	16	25.2±1.9	0.6±0.5	4.5	2.1±1.0	2.1±1.0	0.0±0.0	100
17 – Praia das Avencas	B	38º41’08.31’’N/9º21’19.94’’W	19	20.3±1.7	9.5±2.39	35	21.6±1.2	0.1±0.2	1.6	0.9±0.9	0.9±0.9	0.0±0.0	68.6
18 – Lisboa: Trafaria	CP	38º40’26.43’’N/9º14’06.67’’W	6	23.4±1.6	12.8±2.5	13	23.2±1.7	0.9±0.7	6.7	2.2±1.0	2.2±1.0	0.0±0.0	100
19 – Setúbal: Lota	FP	38º31’11.28’’N/8º53’58.24’’W	47	21.3±2.2	8.8±1.90	27	21.6±2.5	7.8±3.8	88.9	3.7±1.6	3.4±1.3	1.1±0.8	88.9
20 – V. N. Mil Fontes	B	37º43’12.31’’N/8º47’17.53’’W	23	21.9±1.7	12.7±2.0	48	22.3±1.5	0.1±0.5	0.7	0.2±0.8	0.2±0.7	0.0±0.0	6.3
21 – Zambujeira do Mar	B	37º33’05.56’’N/8º47’35.98’’W	22	18.7±2.1	11.5±2.0	19	22.0±2.7	0.1±0.1	0.4	0.4±0.5	0.4±0.5	0.0±0.0	36.8
22 - Lagos	E	37º05’59.13’’N/8º40’07.11’’W	15	20.4±1.1	8.7±1.84	25	22.3±1.9	1.0±0.9	11.9	2.3±1.2	2.3±1.2	0.0±0.0	88.0
23 - Alvor	E	37º07’21.96’’N/8º37’19.82’’W	21	23.1±2.1	8.9±1.49	31	25.1±1.8	0.1±0.4	0.9	0.2±0.5	0.2±0.5	0.0±0.0	9.7

Table 5.3. Organotin concentrations (ng Sn.g^{-1} dw) quantified by GC-MSD in soft tissues of *N. reticulatus* females at selected sampling locations. MBT: monobutyltin, DBT: dibutyltin, TBT: tributyltin, DPhT: diphenyltin, TPhT: triphenyltin, MOcT: monooctyltin, DOcT: dioctyltin.

Station code - name	YEAR	MBT	DBT	TBT	DPhT	TPhT	MOcT	DOcT
1 – Praia do Norte	2008	<5.0	3.5	10	<0.5	<0.5	<5.0	<5.0
4 – Viana Castelo: Estaleiro	2008	180	220	380	1.2	9.3	<5.0	<5.0
8 – Praia da Foz	2008	2.1	19	3.5	<0.5	<0.5	<5.0	<5.0
9 – Aveiro: Barra	2008	8.6	9.5	19	<0.5	<0.5	<5.0	<5.0
10 – Aveiro: M. Mira	2008	12	9.0	31	<0.5	<0.5	<5.0	<5.0
11 – Aveiro: Forte Barra	2008	7.5	7.6	21	<0.5	<0.5	<5.0	<5.0
12 – Aveiro: P. Com. Norte	2008	6.4	8.7	23	<0.5	<0.5	<5.0	<5.0
16 – Praia Guincho	2008	12	9.1	15	<0.5	0.7	<5.0	<5.0
18 – Lisboa: Trafaria	2008	14	11	24	<0.5	4.1	<5.0	<5.0
20 – V. N. Mil Fontes	2008	<0.5	3.1	4.9	<0.5	<0.5	<5.0	<5.0
21 – Zambujeira do Mar	2008	9.5	8.5	17	<0.5	<0.5	<5.0	<5.0
1 – Praia do Norte	2003	27	28	100	<0.5	<0.5	<5.0	<5.0
9 – Aveiro: Barra	2003	38	32	74	<0.5	<0.5	<5.0	<5.0
11 – Aveiro: Forte Barra	2003	31	22	69	<0.5	<0.5	<5.0	<5.0
12 – Aveiro: P. Com. Norte	2003	86	83	200	<0.5	<0.5	<5.0	<5.0
20 – V. N. Mil Fontes	2003	15	13	67	<0.5	2.5	<5.0	<5.0

When considering female penis length, similar results were obtained (statistical tests not shown) with significant declines in 10 of the 16 locations. However, FPL may be affected by female shell height (FSH) and at seven locations FSH varied significantly (p<0.05) between both campaigns possibly affecting the FPL results. RPLI showed a marked decrease in all locations. The generalized decrease in imposex parameters was accompanied by a decrease in butyltin levels in female's soft tissues. The comparison between butyltin levels in samples from the two campaigns discloses a significant (p<0.01) reduction in TBT, DBT and MBT. Considering the five common locations presented in Table 5.3 the average decrease was 83±10% for TBT, 78±10% for DBT and 89±12% for MBT. A good agreement is observed between the decrease of TBT and

imposex levels at those locations: 83% average decrease in the values of TBT concentrations was accompanied by a decrease of 68% and 83% in VDSI and FPL levels, respectively.

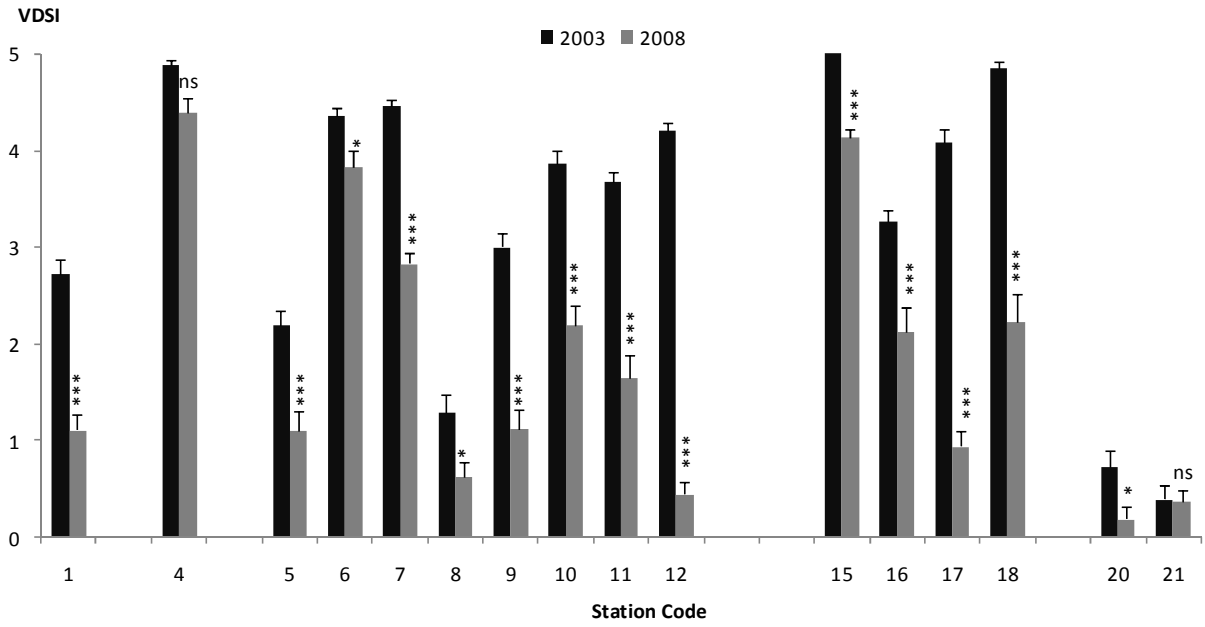

Figure 5.2. Temporal comparisons of VDSI values between 2003 and 2008. Error bars denote standard error. Significant differences are marked. ns: not significant; *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$.

5.3.4. TBT degradation under laboratory conditions

The evolution of imposex, TBT and its metabolites in *N. reticulatus* females after a 60 day depuration period is shown in Figure 5.3. Female shell height between sampling periods did not exhibit any significant change, thus not influencing FPL values. Imposex levels did not change significantly over time as demonstrated by the results of Kruskal Wallis test for VDSI and FPL in St. 4 (H=2.404, p=0.301 and H=0.528, p=0.768, respectively) and for VDSI and FPL in St. 10 (H=0.0129, p=0.994 and H=3.539, p=0.170, respectively). Despite such maintenance in imposex levels, a decrease in TBT concentrations over time is evident in the organisms collected at both locations.

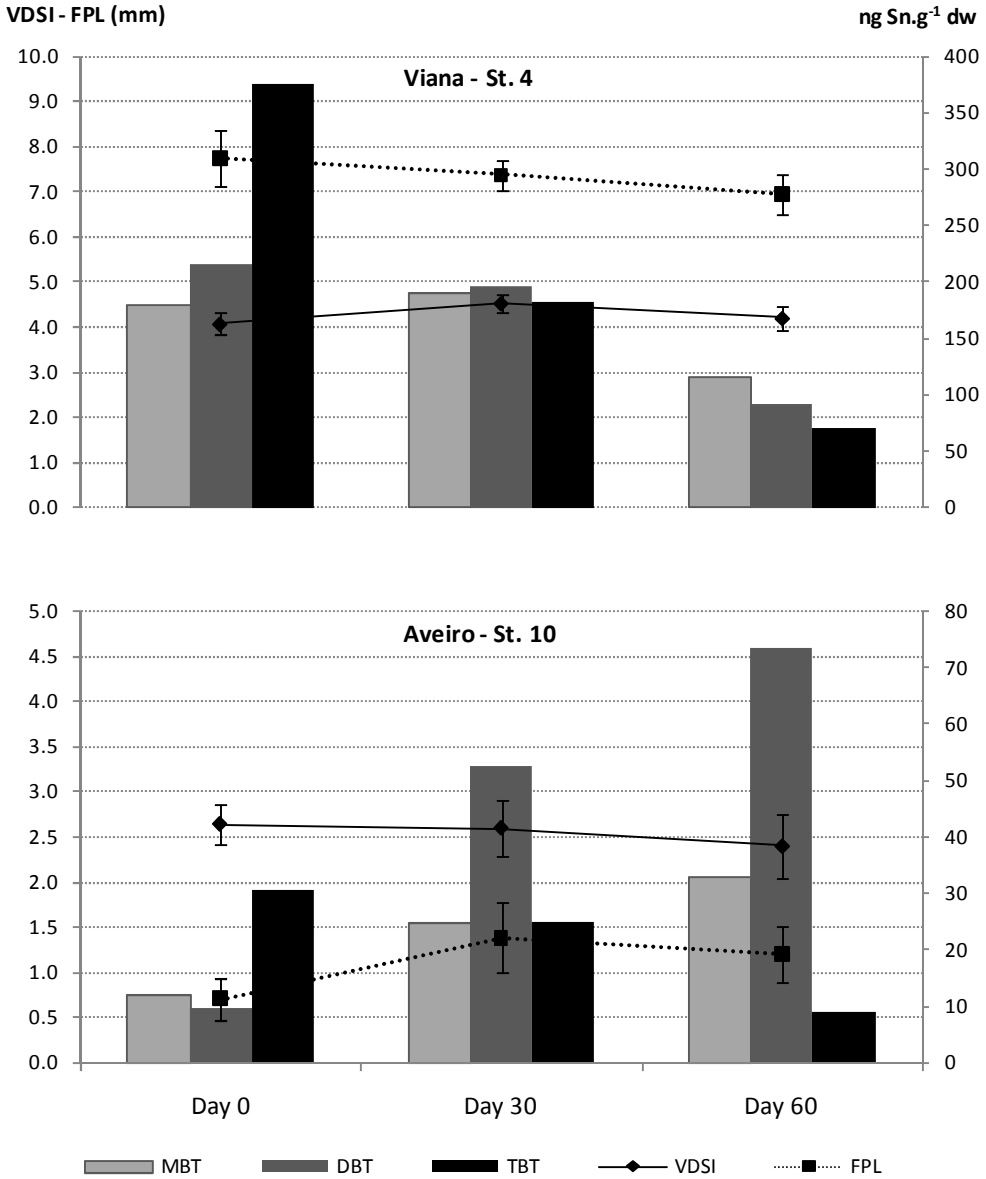

Figure 5.3. Evolution of TBT, DBT and MBT tissue concentrations and imposex levels (VDSI and FPL) in *N. reticulatus* females collected at St. 4 and St. 10 over a 60 days depuration period. Error bars denote standard error. No significant differences in both imposex indices (VDSI and FPL) over time were detected.

In order to have an idea on the half life of TBT we estimated the degradation rate constant assuming that the organisms behave as a single compartment. In such case, the kinetic of TBT loss can be described as a first order kinetics, and the equation $C_t = C_0.e^{-kt}$ (where C_t is the concentration at time t; C_0 is the initial concentration and *K* is the

degradation rate constant) was applied (Walker et al., 2006). Depuration *K* values of 0.024 and 0.007 were obtained for the TBT levels in animals from St. 4 and St. 10 after 30 days, respectively. However, even after a longer depuration time (60 days) *K* values are almost similar (0.03 for St. 4 and 0.02 for St. 10). If the estimation of the half life of TBT in *N. reticulatus* tissues is performed using *K* values obtained after 60 days depuration, the results disclose a half life of 25 days for animals from St. 4 and 34 days for animals from St. 10.

5.4. Discussion

5.4.1. Spatial evolution of imposex and OT levels in *Nassarius reticulatus*

In the present work, imposex and organotin levels were evaluated in the Portuguese coast in order to track the spatial evolution of TBT pollution in this area. Females with imposex occurred in all sampling sites surveyed in 2008. Generally, imposex incidence was lower at sites away from harbors, e.g. St. 20 with 6.3% and St. 23 with 9.7% of affected females, whilst the most affected populations (with 100% of imposex incidence) were found inside the harbors of V. Castelo, Leixões and Setúbal and inside Nazaré and Peniche fishing ports. The levels of VDSI, FPL, RPLI and AOS followed the same spatial trend [Table 5.2] as they attained higher levels for sites closer or inside harbors, which corroborates previous findings indicating that harbors are 'hotspots' of TBT pollution in the Portuguese coast (Barroso et al., 2002a; Sousa et al., 2005). This makes sense since these facilities enclose commercial/fishing ports and marinas where many boats are gathered and are sources for TBT pollution. Besides, most of them contain dockyards where TBT old paints are removed contaminating the surrounding environment.

Significant correlations were noticed between imposex and TBT concentrations in *N. reticulatus* tissues collected along the coast (Section 6.3.2). These results are in good agreement with other works reporting high correlations between imposex and TBT tissue concentrations for this species in the field (Stroben et al., 1992a; Bryan et al., 1993; Oehlmann et al., 1998; Barroso et al., 2002a; Ruiz et al., 2005; Sousa et al., 2005; Sousa et

al., 2007; Wirzinger et al., 2007; Rodríguez et al., 2009). Triphenyltin was detected only at three locations (St. 4, St. 16 and St. 18) and in very low concentrations. Previous surveys conducted along the Portuguese coast with gastropods and mussels also shows that TPhT levels are much lower when compared to TBT ones (Barroso et al., 2002a; Barroso et al., 2004; Sousa et al., 2005; Sousa et al., 2007; Sousa et al., 2009). TPhT can also induce imposex in *N. reticulatus* under laboratory conditions; however the low concentration of this compound in the environment probably plays only a negligible role in promoting the development of imposex in natural populations (Barroso et al., 2002b).

Sterile females were detected at two locations: St. 4 and St. 6. The mechanism underlying sterility in *N. reticulatus* is still poorly understood. So far sterility in *N. reticulatus* females was never demonstrated in the laboratory; nevertheless field data suggest a strong relationship between TBT pollution, imposex and sterility, since the latter occurs only in females with advanced stages of imposex and at highly polluted locations (Huet et al., 1995; Barreiro et al., 2001; Barroso et al., 2002a; Sousa et al., 2005; Rodríguez et al., 2009). This might be the case in the present work where sterile females were found in locations with high TBT levels (e.g. [TBT]=300 ng $Sn.g^{-1}$ dw at St. 4). Shi et al. (2005) suggested that in this species two major sterilizing mechanisms can occur: (i) the blockage of the vaginal opening by the vas deferens, and/or (ii) the blockage of the pallial oviduct without closure of the vaginal opening. In the Portuguese coast both sterilizing mechanisms seem to occur in *N. reticulatus*. In the current campaign the three sterile females found exhibited visible signs of vulva obstruction and aborted egg capsules inside the capsule gland. Barroso et al. (2002a) detected 22 sterile females in a survey conducted in 2000: 22.7% exhibiting a blocked vulva whilst in 77.3% no external sign of vulva blockage by vas deferens proliferation was noticed. In 2003, four sterile females were found (Sousa et al., 2005). Sterility in *N. reticulatus* was also reported from highly polluted areas in Spain (Barreiro et al., 2001; Rodríguez et al., 2009) and France (Huet et al., 1995). However, either in Portugal or elsewhere this phenomenon seems to pose limited risk to the populations since the frequency of sterile females is generally low and also because this species exhibits a planktonic larval development and thus the larval drift

from less polluted sites will ensure recruitment of new cohorts at the most polluted locations.

5.4.2. Temporal trends of imposex and OT levels in *Nassarius reticulatus*

The results from our depuration experiments disclose approximately 30 days of half life for TBT. Our estimated values indicate faster TBT degradations when compared to the ones previously reported by Stroben et al. (1992b) for *N. reticulatus* females (65 days) and for gastropods in general (60-80 days) as reported by the OSPAR Commission (OSPAR, 2008). Nevertheless it should be noted that our values were estimated using a first order kinetics model and observations were obtained from a limited number of samples and shorter depuration time experiments. Furthermore, other factors such as differences in TBT initial concentrations, different degradation kinetics between butyltins and different metabolic abilities of the organisms might affect TBT degradation and can explain the differences between our results and the ones previously reported and also why the animals from two different sites exhibited different TBT degradation results.

Results from the depuration experiments also disclose that no significant decreases in imposex (VDSI and FPL) occurred after a depuration period of 60 days despite the big reductions in TBT concentrations in female tissues [Figure 5.3]. Similar results were obtained under laboratory conditions by Stroben et al. (1992b) that showed the irreversibility of imposex in *N. reticulatus* over 18 months. However, our results from temporal comparisons on VDS levels between 2003 and 2008 [Figure 5.2] clearly demonstrate a decrease in all sampling stations. Such a decrease can be the result of a limited reversibility of imposex as already noticed in previous studies (Bryan et al., 1993; Sousa et al., 2005; Sousa et al., 2007) or a consequence of cohorts turnover with older and more affected females being substituted by younger and less affected ones. As female sexual maturity is achieved at the 4th year of age (Barroso et al., 2005), new born animals and juveniles that grew after the 2003 ban would have been sampled in 2008 as adult animals. Therefore, even though *N. reticulatus* can live for 10 years or more (Barroso et al., 2005), our results suggest that the adult cohorts sampled in 2003 were replaced by

new ones that grew under decreasing levels of TBT in the environment leading to a notorious imposex recovery. The relation between imposex recovery and TBT decline is obvious as the average decrease in TBT concentration in the snail tissues in the five common locations [Table 5.3] was 83±10%. Particularly interesting are the results obtained for the commercial port at Aveiro (St. 12), where VDSI and FPL declined 89% and 99% and TBT levels reduced 89% despite the increase of 22% in naval traffic (in terms of gross tonnage) [Table 5.1]. Lisbon harbor also showed the same tendency: a reduction in imposex and TBT levels (74% for VDSI, 67% for FPL and 93% for TBT) in spite of the increase in naval traffic. If we compare 2003 TBT levels in female tissues obtained at other sampling sites by a different analytical technique (Sousa et al., 2005) with those obtained in 2008, the same decreasing tendency is observed: reductions in TBT concentrations to the extent of 54%, 86%, 62% and 93% at St. 4, St. 10, St. 16 and St. 18, respectively, accompanied by respective VDSI declines of 10%, 43%, 35% and FPL declines of 1.7%, 88%, 64% and 67%. The reduction in butyltin levels is most probably a consequence of a decline in TBT inputs enforced by EC Regulation 782/2003. Some studies already suggested the effectiveness of this regulation on reducing imposex levels in Portugal between 2003 and 2005 (Sousa et al., 2007) and between 2003 and 2006 (Rato et al., 2009).

Despite this amelioration, TBT is still a matter of concern. Analysis of butyltin biodegradation index (BDI) demonstrated that values lower than 1 were still recorded in 2008 indicating that, although to a lesser extent, TBT fresh inputs still occur. These inputs might be derived from: (i) release from ships hulls that were using this compound until 2008; (ii) continuous release from dockyard facilities; (iii) illegal use of banned antifouling paints; (iv) continuous release from sediments; and (v) other sources of TBT not derived from AF paints, such as preservative or disinfecting agents.

As previously stated, not all the locations exhibited significant decreases in imposex levels between 2003 and 2008. At St. 4 and St. 21 no significant differences in VDSI or FPL values were noticed. At St. 21 very low levels of imposex were noticed in both campaigns making it difficult to notice any change, whereas St. 4 is located inside a shipyard, where continuous TBT inputs are expected since old TBT coatings are being removed and

substituted by alternative ones. Dry dock operations taking place at shipyards have been recently considered as important sources of TBT after the introduction of legislative restrictions (Kotrikla, 2009). For instance, Gibbs (2009) conducted a survey over a period of 20 years with *Ocenebra erinacea* populations collected close to a shipyard at Falmouth (SW England) and demonstrated that over the elapsed time little improvement in the reproductive capacity of the population was observed and in 2006 75% of females were found to be sterile. The author suggested that, in recent years much of the TBT pollution in the study area was probably a result of discharges from the shipyard. Furthermore, release of TBT from sediments also constitutes an important source of this pollutant. In fact Ruiz et al. (2008) recently demonstrated that desorption from sediments contributes to an increase in TBT and its metabolites in the water column.

5.4.3. Biomonitoring and ecological impact

Imposex monitoring on a regular basis is mandatory under the OSPAR (Oslo and Paris Convention) Coordinated Environmental Monitoring Program (CEMP). Due to the recent introduction of global ban on TBT, the OSPAR commission also recommends that monitoring programs should frame this event. Hence, our results provide a unique dataset concerning imposex and organotin levels in the shores neighboring one of the busiest naval traffic lines in Europe on the year that the IMO ban entered into force. In order to interpret monitoring data OSPAR established an Assessment Class Criterion for Imposex (ACCI) in *Nucella lapillus* and other gastropods according to six classes (A-F) (OSPAR, 2004). For *N. reticulatus*, classes A and B (VDSI<0.3) indicate exposure to TBT concentrations below the Environmental Assessment Criteria (EAC), i.e., concentrations for which there is no concern that negative impacts might be observed in marine organisms. Class C (0.3<VDSI<2.0) indicates exposure to TBT concentrations higher than the EAC, for class D (02.0<VDSI<3.5) the reproductive capacity of the populations of the most sensitive species (such as *N. lapillus*) is affected as a result of the presence of sterile females, and for class E (VDSI>3.5) populations of the more sensitive gastropod species are unable to reproduce and the majority, if not all females within the population are

sterile. During the present campaign two locations fell into category A, three into category C, six into category D and six into category E. Considering 2003 data for the same locations (n=16), it was found that the majority of sites (56%) fell into category E, and no sites fell into category A or B. Such results demonstrate that, although a reduction in imposex levels can be noticed, it is clearly not enough to achieve the EcoQO (Ecological Quality Objective) set for imposex in *N. reticulatus* (VDSI<0.3) (OSPAR, 2007). In fact, with the exception of St. 20 and St. 23, all locations had VDSI values higher than the EcoQO, indicating that at those sites negative ecological impacts are likely to occur.

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CHAPTER 6

CHANGES IN *NASSARIUS RETICULATUS* IMPOSEX EXPRESSION BETWEEN 2000 AND 2008

CHAPTER 6. CHANGES IN *N. RETICULATUS* IMPOSEX EXPRESSION BETWEEN 2000 AND 2008

Abstract

Nassarius reticulatus imposex levels were surveyed in the Portuguese coast in 2000, 2003 and 2008. The occurrence of imposex affected females not bearing a penis (b-type females) was studied in detail and the obtained results disclosed increasing levels since 2000. The percentage of b-type females in relation to the total number of imposex affected ones (%b) was 3.5% in 2000, 11% in 2003 and 24% in 2008. An increase in the number of sites where this phenomenon was recorded was also evident: two sites in 2000, seven sites in 2003 and thirteen sites in 2008. The proportion of b-type females with VDS stage 1 also exhibited the same increasing pattern with 37.5% of VDS 1 b-type females in 2000 and 65% in 2008. The emergence of this phenomenon seems to be associated with TBT pollution decline and a shift toward b-type females is expected in the future due to the TBT global ban.

Keywords:

Nassarius reticulatus; imposex; b-type; TBT

6.1. INTRODUCTION

The marine netted whelk *Nassarius reticulatus* (Linnaeus, 1758) is a ubiquitous nassarid gastropod in European coastal areas from the Canaries and Azores to Norway and throughout the Mediterranean and Black Seas (Fretter & Graham, 1994). It was proposed as a bioindicator of tributyltin (TBT) pollution in the early 90's by Stroben et al. (1992a). Since then, organotin pollution has been assessed using this species in several European countries: England (Bryan et al., 1993), Germany (Duft et al., 2007 and references therein), France (Stroben et al., 1992a, 1992b; Oehlmann et al., 1993; Huet et al., 1995; Wirzinger et al., 2007), Spain (Barreiro et al., 2001; Ruiz et al., 2005; Quintela et al., 2006; Ruiz et al., 2008; Couceiro et al., 2009; Rodríguez et al., 2009) and Portugal (Gibbs et al., 1997; Barroso et al., 2000; Pessoa et al., 2001; Barroso et al., 2002a; Santos et al., 2004; Barroso et al., 2005; Sousa et al., 2005; Rato et al., 2006; Sousa et al., 2007; Rato et al., 2008; Rato et al., 2009; Sousa et al., in press).

The assessment of TBT pollution using *N. reticulatus* relies on the measurement of imposex levels in natural populations that are well correlated with the environmental concentrations of this pollutant (see e.g. Barroso et al., 2002a; Sousa et al., 2005; Wirzinger et al., 2007; Sousa et al., in press). The causal relationship between imposex development and TBT exposure has also been confirmed in laboratory experiments (Stroben et al., 1992b; Bettin et al., 1996; Barroso et al., 2002b).

Several indices are used to assess the degree of imposex in a population among which are the percentage of affected females (%I), the relative penis length index (RPLI) defined as the proportion of female penis length (FPL) over male penis length (MPL) and the *vas deferens* sequence index (VDSI). The VDSI classifies the degree of female's masculinization with 0 corresponding to unaffected females and 4+ (or 5 according to Barroso et al. (2002a)) corresponding to severely affected ones (at this stage the *vas deferens* reaches the vulva and sterility can eventually occur). Intermediate stages correspond to the development of a penis rudiment (Stage 1), a penial duct (Stage 2) and a *vas deferens* (Stage 3). However, this is not the only possible scheme for this species. When describing VDS development in *N. reticulatus*, Stroben et al. (1992a) also reported an alternative way denominated as b-type, in which no penis is observed, with affected females in initial stages exhibiting only a *vas deferens*. Those authors examined 2983 imposex affected females between 1988 and 1991 and 45 (representing 1.5%) exhibited b-type VDS stages. Other species also display this alternative b-type, such as, *Hydrobia ulvae*, *Buccinum undatum* and *Neptunea antiqua* (Schulte-Oehlmann et al., 1998; OSPAR, 2003), among others.

Regular monitoring surveys of *N. reticulatus* imposex along the Portuguese coast have been performed between 2000 and 2008 but data concerning b-type females was never analyzed in detail. Thus the b-type female's data set obtained in the above surveys is gathered and addressed in the present work in order to characterize the spatial and temporal distribution of this phenomenon and to better understand its expression in this gastropod.

6.2. Materials and Methods

N. reticulatus specimens were collected along the Portuguese Continental shoreline between May and August of 2000, 2003 and 2008; details concerning sampling can be found in Barroso et al. (2002a); Sousa et al. (2005) and Sousa et al. (in press). For each site 60 adult animals were selected and shell heights (distance from shell apex to lip of siphonal canal) measured with vernier calipers to the nearest 0.1 mm. After narcotization with magnesium chloride (7% $MgCl_2$ in distilled water) shells were cracked open with a bench vice and the animals gently removed, sexed and dissected under a stereo microscope. The VDSI classification scheme adopted was the one developed by Stroben et al. (1992a) with slightly modifications as proposed by Barroso at al. (2002a) and the occurrence of b-type females, i.e. those affected by imposex but not bearing a penis, was carefully analyzed. The percentage of b-type females (%b) was calculated in relation to the total number of females with imposex at each site. However, as some studies refer to the total number of females (with or without imposex) per site ($\%b_{total}$), we also included values of $\%b_{total}$ in Table 6.1 to allow comparisons with those works. Data regarding imposex levels (percentage of affected females (%I), female penis length (FPL), relative penis length index (RPLI), *vas deferens* sequence index (VDSI)) are published elsewhere: data concerning 2000 survey is described in Barroso et al. (2002a), whereas 2003 results are provided by Sousa et al. (2005) and 2008 ones by Sousa et al. (in press). Statistical analyses were performed using SigmaStat v3.5 software. Correlation analysis refers to the non-parametric Spearman rank order correlation. The adopted critical significance level was 5%.

6.3. Results and Discussion

The spatial distribution of b-type females along the Portuguese coast in 2008 (23 sites) is illustrated in Figure 6.1. Details on each sampling site are provided elsewhere (Sousa et al., in press). Only at four locations (Sts. 4, 6, 14, 15) no b-type females could be found [Figure 6.1] and these corresponded to the ones with the highest VDSI levels (Sousa et al., in press) being located inside a shipyard (St. 4), a marina (St. 6) or inside fishing ports (Sts.

14 and 15). On the contrary, the highest incidence of b-type females was recorded in sites exhibiting VDSI ≤ 1 (Sts. 1, 5, 12, 17, 21). Highly significant negative correlations (p<0.001) are found between the %b registered in the current work and the imposex levels reported by Sousa et al. (in press) across stations: r=-0.651 for %I; r=-0.846 for FPL; r=-0.842 for RPLI and r=-0.847 for VDSI. A significant correlation (p<0.05) is also found between %b and TBT whole tissue concentrations reported by the same authors (r=-0.624).

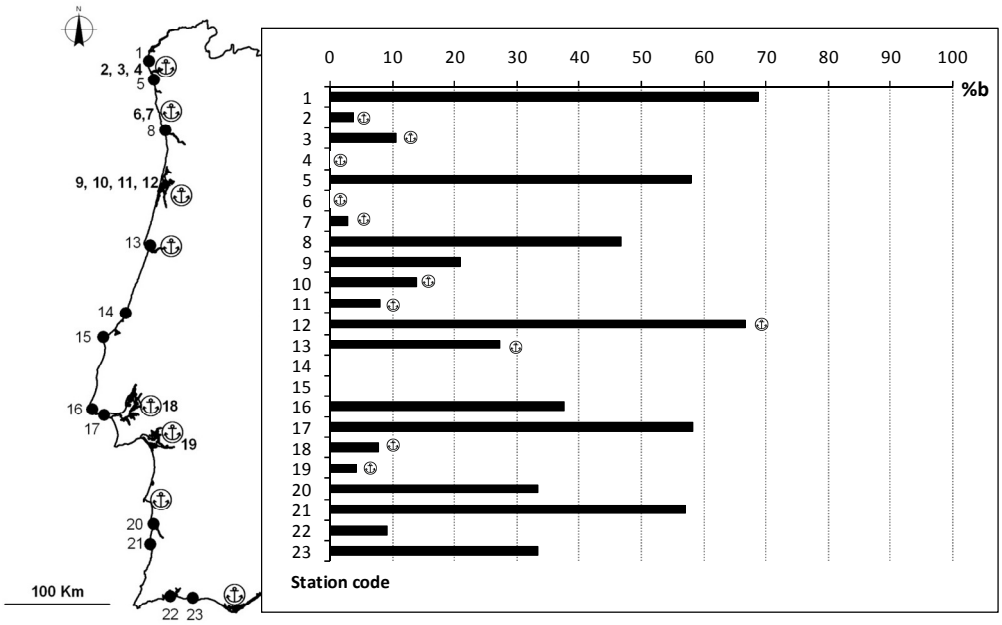

Figure 6.1. Spatial distribution of b-type females incidence across sampling locations, in 2008 survey, with the indication of main harbors location (⚓). For further details on sampling sites name and description see Sousa et al. (in press).

The same general trend was observed in 2003 and 2000 but the number of sites where b-type females occurred was lower in the past. In 2003 survey, 10 of the 23 sampling sites exhibited b-type females and VDSI was always bellow 3 (excluding one location where only one b-type female was found) and TBT levels were always <100 ng Sn.g^{-1} dw. Highly significant negative correlations (p<0.001) are found between %b and the imposex levels reported by Sousa et al. (2005): r=-0.652 for %I; r=-0.819 for FPL; r=-0.835 for RPLI and r=-0.846 for VDSI. A significant negative correlation is also found between %b and TBT

female whole tissue levels reported elsewhere (Sousa et al., 2005; Sousa et al., in press): r=-0.657; p=0.01. In 2000, only 6 locations of the 40 analyzed exhibited b-type females and at those sites imposex and TBT levels were low (VDSI<2; [TBT]<100 ng Sn.g^{-1} dw). Significant negative correlations are observed between the %b and imposex or TBT levels reported by Barroso et al. (2002a): %I (r=-0.543, p<0.001), FPL (r=-0.535, p<0.001); RPLI (r=-0.538, p<0.001), VDSI (r=-0.520, p<0.001) and TBT levels (r=-0.519, p<0.05).

Previous surveys performed in the continental shelf off the Portuguese coast have shown that these areas exhibited low levels of TBT pollution and there is a widespread occurrence of *N. reticulatus* b-type females (Rato et al., 2006; Rato et al., 2008), denoting again that this type of imposex expression is linked to low levels of imposex. The spatial trend observed is not surprising as b-type is, by definition, restricted to initial stages of *vas deferens* development (in the majority VDS=1 ou more rarely VDS=3) which occur at low polluted sites.

In order to study the temporal evolution of b-type females between 2000 and 2008 only common locations to the three surveys are considered (n=15) [Table 6.1; Figure 6.2]. Our results indicate an increasing occurrence of b-type females in the Portuguese coast since 2000 [Figure 6.3]. The percentage of b-type females in relation to the total number of imposex affected ones (%b) in the 2000 survey was 3.5%. In 2003 that percentage rose to 11% and in 2008 to 24%. Besides the rise on b-type females, a consistent increase in the number of sampling locations where this phenomenon was recorded is also evident: two sites in 2000, seven sites in 2003 and thirteen sites in 2008 [Table 6.1; Figure 6.3], indicating that this phenomenon is emerging in the Portuguese coast.

Two distinct periods have been recognized concerning temporal trends of TBT pollution levels in the Portuguese coast: one before the introduction of the European Union regulation No. 782/2003 (banning the use of TBT based antifouling paints in July 2003) and the other following the implementation of this regulation (Barroso et al., 2002a; Sousa et al., 2005; Rato et al., 2009; Sousa et al., in press).

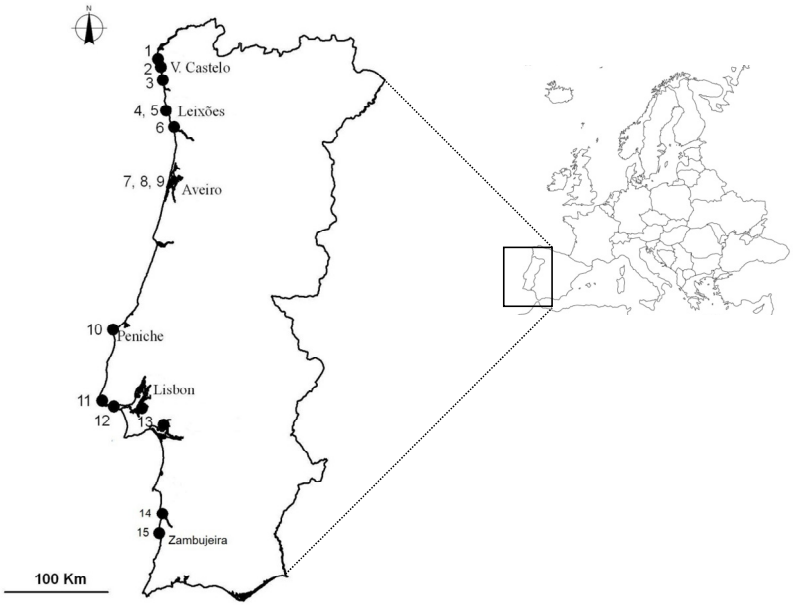

Figure 6.2. Map of the Portuguese coast indicating the sampling stations common to the three surveys.

Monitoring surveys performed in the Portuguese coast before 2000 disclose high levels of TBT concentration in female tissues and imposex with the exclusive occurrence of a-type females (Barroso et al., 2000; Barroso et al., 2005). Between 2000 and 2003 there was no global trend in the evolution of TBT pollution along the coast, nevertheless *N. reticulatus* imposex and TBT tissue levels exhibited some rises or declines at specific sites due to local variation of ship traffic or closure of shipyards (Sousa et al., 2005).

The increase in b-type females between 2000 and 2003 occurred only at those locations where TBT pollution was already low. The following period, from 2003 to 2008, is characterized by a general decrease in imposex levels and TBT tissue concentrations as a consequence of the TBT ban (Sousa et al., 2007; Rato et al., 2009; Sousa et al., in press), and this was accompanied by an increase in the occurrence of b-type females [Figure 6.3].

Only at one location (St. 15) the incidence of b-type females decreased since 2000 (from 91% to 78% in 2003 and then to 57% in 2008). Exceptionally at this site, TBT concentrations were very low in 2000 and an increase in TBT tissue concentrations and

imposex levels was recorded between 2000 and 2008 possibly as a consequence of an increase of ship traffic in a local fishing port [Table 6.1].

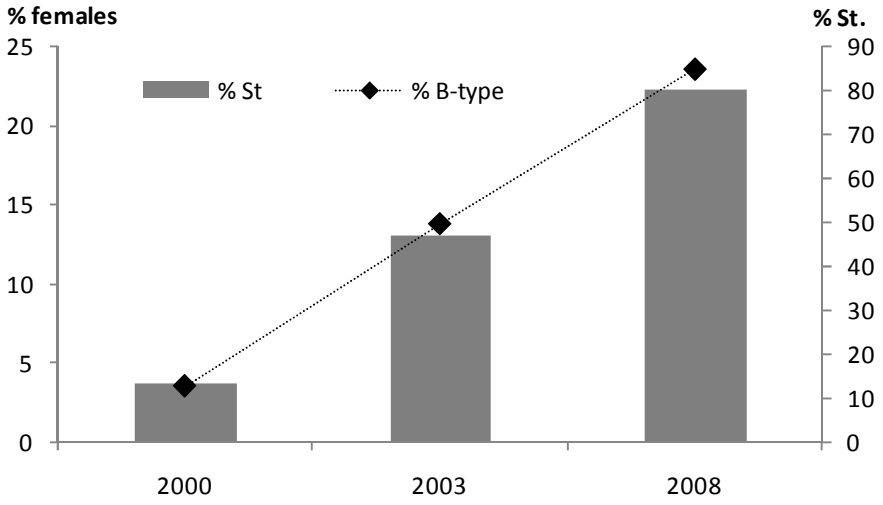

Figure 6.3. Temporal evolution of b-type females observed at 15 common stations surveyed in 2000, 2003 and 2008. Vertical bars represent the % of common stations with b-type females and the dots correspond to the percentage of b-type females in relation to the total affected females registered along the 15 common locations.

The general evolution described above is well illustrated in Figure 6.4, which shows the relative proportion of each VDS stage in the different surveys. A temporal decreasing tendency of higher VDS stages followed by an increase in lower ones in notorious and, as explained, this is accompanied by an increase of b-type females.

Our results indicate that the decrease of TBT levels may be the cause for the emergence of b-type females along the Portuguese coast, as most of b-types belong to VDS stage 1. Hence, whilst in the past imposex levels were generally high, b-type females were rare as they are typically associated to low levels of VDS. Presently, the number of females with low VDS levels is rising due to pollution decline, either belonging to b-type or a-type [Figures 6.3, 6.4]. Particularly relevant is the fact that the proportion of VDS 1 b-type and VDS 1 a-type females seems to be changing with time [Table 6.2].

Table 6.1. Temporal comparisons of the percentage of b-type females in relation to the total number of affected females (%b) and percentage of b-type females in relation to the total number of females in the sample (%b_{total}). TBT concentrations in female's tissues are also presented. Data cited from: [(a)] Barroso et al. (2002a); [(b)] Sousa et al. (2005); [(c)] Sousa et al. (in press). (-) Data not available.

Station name	St. Code	%b			%b_{Total}			TBT		
		2000	2003	2008	2000	2003	2008	2000[(a)]	2003[(b, c)]	2008[(c)]
Praia Norte	1	0	33	69	0	33	61	77	100[(c)]	10
V. Castelo:	2	0	0	0	0	0	0	1368	832[(b)]	380
Praia da	3	6.7	49	58	5.4	49	35	37	-	-
Leixões:	4	0	0	0	0	0	0	602	-	-
Leixões: Pl.2	5	0	0	2.9	0	0	2.9	481	330[(b)]	-
Praia da Foz	6	0	50	47	0	34	20	55	-	3.5
Aveiro:	7	0	4.0	21	0	4.0	12	60	74[(c)]	19
Aveiro: M.	8	0	0	14	0	0	13	132	218[(b)]	31
Aveiro: P.	9	0	0	67	0	0	27	262	200[(c)]	23
Peniche	10	0	0	0	0	0	0	912	1679[(b)]	-
Guincho	11	0	20	38	0	20	38	90	39[(b)]	15
Avencas	12	0	0	58	0	0	40	128	48[(b)]	-
Trafaria	13	0	0	7.7	0	0	7.7	488	400[(b)]	24
V. N. Mil	14	0	63	33	0	23	2.1	23	-	4.9
Zambujeira	15	91	78	57	50	21	21	<20	67[(c)]	17

This pattern is clearly noticed in Sts. 1, 3, 14 as an obvious increase in female b-type proportion occurred over time and it is partly noticed at St. 6 where the rise in b-type proportion occurred only between 2000 and 2003 and almost maintaining afterwards. The exceptions from this tendency are St. 7 due to bias caused by low sample size and St. 15 in which a distinct TBT pollution scenario evolution has been happening as previously explained. Globally, in 2000, the percentage b-type female's in VDS stage 1 was 37.5% and in 2008 that percentage rose to 65%. It seems that in a less polluted scenario the development of imposex starts with the *vas deferens* and not with the penis, as it was usual in the past. Regarding VDS 3, fewer females were found in the entire study and there was a different evolution pattern as VDS=3 tend to decline when TBT pollution is decreasing.

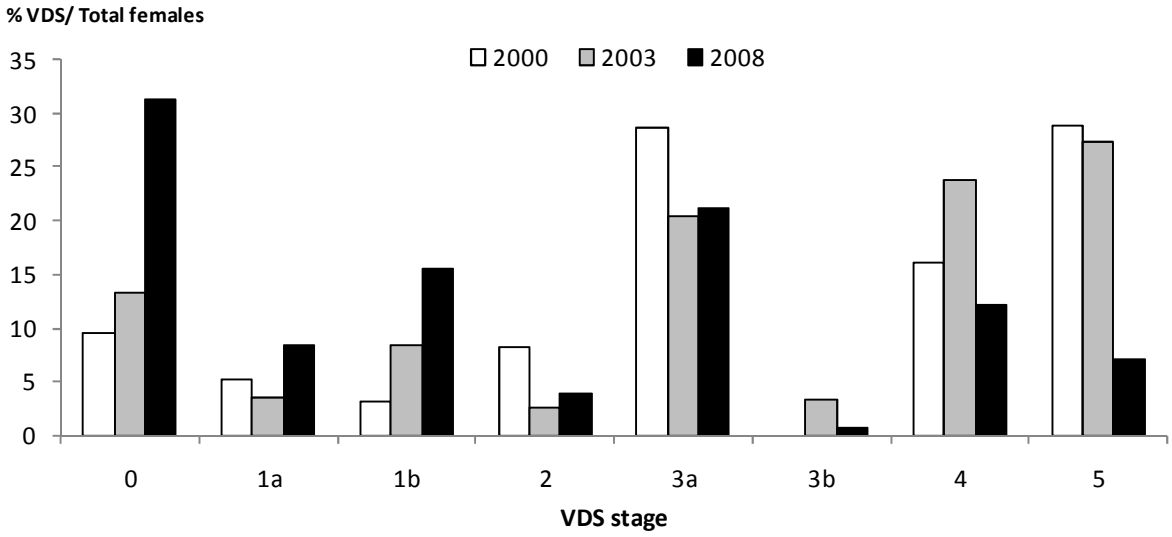

Figure 6.4. Relative percentage of each VDSI type in relation to the total number of females analyzed in each survey. The VDS was classified according to the scoring system proposed by Stroben et al. (1992b) except that the VDSI stage 4+ was converted to stage 5 as proposed by Barroso et al. (2002a).

Table 6.2. Relative percentage of females with b-type imposex expression in each survey, comparing to a-type. The % VDS 1b refers to the percentage of females with VDS=1 not bearing a penis whilst %3b refers to the percentage of females with VDS=3 not bearing a penis. (-) no females with VDS=1 or VDS=3 were found. (*) This value corresponds to one single female that presented VDS stage 1. Zero values imply that no b-type females where observed and all were a-type.

		%VDS 1b			%VDS 3b		
Station name	St. Code	2000	2003	2008	2000	2003	2008
Praia Norte	1	0	50	85	0	18	0
V. Castelo - Estaleiro	2	-	-	-	0	-	0
Praia Amorosa	3	22	58	73	0	17	43
Leixões - Marina	4	-	-	-	0	-	0
Leixões - Pl. 2	5	-	-	100	0	-	0
Praia Foz	6	0	76	64	0	20	0
Aveiro - Barra	7	0	100*	38	0	0	0
Aveiro - MM	8	-	-	44	0	0	0
Aveiro - PCN	9	-	-	75	0	0	-
Peniche - P. Pesca	10	-	-	-	-	-	0
Guincho	11	-	-	86	0	27	0
Avencas	12	-	-	74	-	0	0
Trafaria	13	-	-	20	-	-	0
V. N. Mil Fontes	14	0	73	100	0	33	0
Zambujeira	15	100	100	57	-	0	-

Although the rise of VDS 1 b-type females appears to be a natural consequence of imposex levels decline, the reason why the proportion of b-type females are rising in comparison to a-type remains to be explained and has to be addressed in future research. It is possible that distinct mechanisms or pathways are involved in the formation and development of the penis and the *vas deferens* but to the authors best knowledge such mechanisms were never investigated in detail.

Even if our results suggest that the spreading out of b-type females is only related with the decrease of TBT levels other hypothesis should also be considered. In *Nucella lapillus*, the occurrence of a genetically based resistance leads to the occurrence of imposex "aphalic" females (Gibbs, 1993; Barreiro et al., 1999; Quintela et al., 2002; Huet et al., 2008). This genetic deficiency known as Dumpton syndrome (DS) is responsible for the underdevelopment or non-development (aphally) of the penis and it seems to give some advantage to imposex affected females since it limits the development of the *vas deferens* preventing their sterilization (Gibbs, 1993; Quintela et al., 2002). This is particularly important since *N. lapillus* lacks a planktonic stage in their life cycle and thus at highly polluted locations where most females are sterile and no population renewal occurs, extinction becomes inevitable. Dumpton syndrome was until recently only registered in highly polluted locations (Gibbs, 1993; Barreiro et al., 1999; Quintela et al., 2002). However, Huet et al. (2008) recently reported its presence in lower polluted areas. In populations affected by DS aphallic males are usually (but not always) present and male penises are usually smaller (Gibbs, 1993; Barreiro et al., 1999). In *N. reticulatus*, this phenomenon was never described since no aphalic males were ever reported in locations sampled along the coast and a reduction in male penis was not observed (data not shown); on the other hand there is no apparent advantage for the imposex affected females because sterilization is rare and, besides, recruitment of juveniles can be assured by planktonic larvae coming from less polluted places. Such facts stand against the hypothesis of a genetic anomaly in *N. reticulatus* to explain b-type females. However, during an offshore survey conducted near the effluent discharge point of a submarine outfall at Aveiro (NW Portugal), one aphalic *N. reticulatus* male was found (Sousa, unpublished) and another one during a survey conducted in harbor areas in Spain

(Laranjeiro, *per com*). The existence of such males can hardly provide any support of the genetic anomaly hypothesis as it can be an extreme case of penis seasonal reduction associated with the reproductive cycle (see Barroso & Moreira, 1998), or a consequence of the presence of xenoestrogenic contaminants as already described for other species (Oehlmann et al., 2000; Santos et al., 2008). Nevertheless, aphally in *N. reticulatus* males is an extremely rare event and we cannot see an obvious analogy to what happens with *N. lapillus.*

Our data suggests that the decline in TBT pollution levels over the last years lead to the widespread occurrence of VDS 1 b-type females in the Portuguese coast. Following the implementation of the TBT global ban by IMO in September 2008 a progressive decrease of this pollution is expected in the near future; therefore, and in view of our results, a swift towards VDS b-type expression will likely occur in natural populations of *N. reticulatus*. Hence, in future monitoring surveys using this species, a sharper reduction in FPL and RPLI indices is expected to occur as b-type females don't exhibit penises.

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CHAPTER 7

CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF ESTROGENICITY IN EFFLUENTS FROM WWTPS IN RIA DE AVEIRO (NW PORTUGAL)

Archives of Environmental Contamination and Toxicology (in press)

CHAPTER 7. CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF ESTROGENICITY IN EFFLUENTS FROM WWTPS IN RIA DE AVEIRO (NW PORTUGAL)

Abstract

Effluents from wastewater treatment plants (WWTPs) are responsible for the input of estrogenic contaminants into aquatic ecosystems, leading to widespread effects in wildlife. In the present work levels of estrone (E1), 17α- and 17ß-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA) and nonylphenol (NP) were quantified in effluents from WWTPs located in Ria de Aveiro (NW Portugal), as well as in the final effluent discharged into the Atlantic Ocean through the S. Jacinto submarine outfall. Reference sites, located at the entrance of the estuarine system and at the sea side, were also included. Samples were collected under summer (June 2005) and winter (February 2006) conditions. For the summer survey sample's estrogenicity and androgenicity were evaluated using the yeast estrogen screen (YES) and the yeast androgen screen (YAS) assay. Estrone levels varied from 0.5 to 85 $ng.L^{-1}$ in the summer survey and between <LOD and 43 $ng.L^{-1}$ in winter; estradiol levels ranged from <LOD to 9.2 $ng.L^{-1}$ in summer and were always <LOD in the winter survey; EE2 levels were always <LOD for both surveys. NP concentrations ranged from 75 $ng.L^{-1}$ up to 2350 $ng.L^{-1}$ in summer and 10 to 2410 $ng.L^{-1}$ in winter; BPA levels varied from 2.8 to 897 $ng.L^{-1}$ in summer, and from 2.6 up to 316 $ng.L^{-1}$ in winter. Biological assays disclosed estrogenic levels at reference sites lower than the ones reported to pose risk for wildlife. However, the S. Jacinto outfall effluent released high concentrations of NP and BPA into the marine environment.

Keywords

Steroid hormones; Nonylphenol; Bisphenol A; Submarine outfall; YES/YAS assays

7.1. INTRODUCTION

Over the last years many studies have reported the occurrence of endocrine disrupting chemicals (EDCs) in the environment. These substances can interact with the endocrine system, leading to widespread effects in wildlife. In some cases it may lead to disturbances at the community level and even population declines. Two of the most notorious examples are: the feminization of fish downstream of the discharge point of

wastewater treatment plants (WWTPs) (Jobling et al., 1998) and the masculinization of female gastropods (imposex) after exposure to tributyltin (TBT) (Smith, 1981). Imposex is a remarkable example of chemical induced endocrine disruption in wildlife and has been regularly monitored in Portugal over the last two decades. In Ria de Aveiro, a NW Portuguese estuarine system, imposex and organotin contamination have been intensively studied since 1997. Levels of the androgenic compound TBT in water, sediments and biota are available in the literature (Barroso et al., 2000; Sousa et al., 2007). However, there is no information available on the levels of estrogenic chemicals. In order to bridge this gap, levels of steroid hormones and phenolic EDCs were assessed under summer and winter conditions with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Androgenic and estrogenic activities were also evaluated for the 2005 survey using the yeast estrogen screen (YES) and yeast androgen screen (YAS) assay.

In the present survey effluent samples from all the WWTP facilities as well as the final one discharged by S. Jacinto outfall were analysed. Furthermore two seawater samples, referred to as reference samples throughout the paper, were collected at Ria de Aveiro and at the seaside. Ria sample was collected at Barra (entrance of the lagoon) and Sea sample was collected at a beach located to the North of the lagoon inlet.

7.2. Material and Methods

7.2.1. Study area

Ria de Aveiro is a coastal lagoon located in the NW Portuguese continental coast, formed by a complex net of channels and wide intertidal areas (mudflats and salt marshes). It has three main branches radiating from the sea entrance: Mira, S. Jacinto and Ílhavo channels [Figure 7.1].

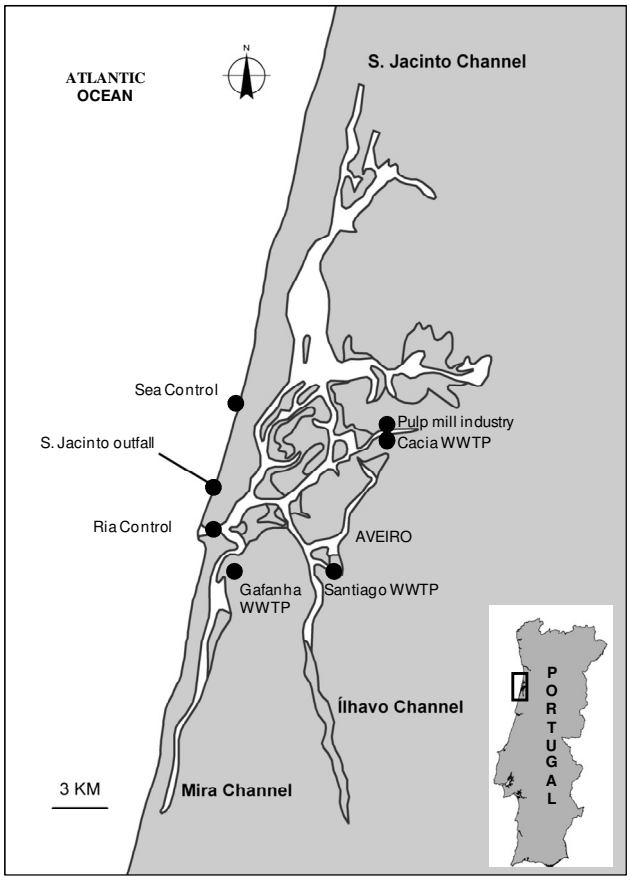

Figure 7.1. Map of Ria de Aveiro and adjacent coastal area indicating location of sampling sites.

The water exchange between the lagoon and the Atlantic Ocean goes through a single inlet at the western boundary, locally known as Barra de Aveiro. The water mass dynamics inside the system is controlled by tides and freshwater inflow from river runoff. Aside from urban pressure derived from human settlement with associated recreational amenities, a set of industrial and harbor structures, benefiting from the regions morphology and resources, have an important impact. Effluents from municipalities bordering Ria de Aveiro are collected and treated at three main WWTPs: Gafanha, Santiago and Cacia. The treated effluent is then redirected to a submarine outfall (S. Jacinto outfall) and discharged into the ocean. This outfall discharges the effluents 3 km offshore at ~16 m water depth, 5 km to the north of the inlet to the Ria de Aveiro (Figueiredo da Silva et al., 2002). The discharged effluent is a sum of domestic effluents

from the WWTP (30%) plus the effluent of a pulp and paper mill (70%). The pulp mill effluent has been considered one of the worst polluting sources of the Aveiro lagoon during the last 40 years, before the implementation of a secondary treatment with activated sludge (Maria et al., 2003). This industrial unit produces bleached kraft pulp and paper using *Eucalyptus globulus* and *Pinus pinaster* (75 and 25%, respectively) as wood supply (Maria et al., 2003). Effluent samples from WWTPs are mainly domestic and undergo secondary treatment with the exception of Santiago WWTP that only undergoes primary treatment. Effluents of this WWTP were discharged directly into Ria de Aveiro until January 1st 2006 and afterwards redirected to Gafanha WWTP.

7.2.2. Sampling procedure

Samples from WWTP effluents as well as reference sites [Figure 7.1] were collected in June 2005 (summer survey) and in February 2006 (winter survey). Reference surface samples were collected at the entrance of Ria de Aveiro (Ria) and at sea (Sea). The Sea sample was collected during a high tide and Ria is a combination of two sub samples (1:1 v/v) collected at high and low tide from the same tidal cycle. For the summer survey, effluent samples were collected in three WWTPs (Gafanha WWTP, Cacia WWTP and Santiago WWTP) and at the submarine outfall (S. Jacinto).

For the winter survey, samples from Ria, Sea, Cacia WWTP and Gafanha WWTP as well as the effluent from the S. Jacinto outfall, were collected following the same procedure. Because the effluent from Santiago WWTP was redirected to Gafanha WWTP in January 2006, no sample was collected at this site. For the winter survey, an additional sample of the pulp mill effluent was collected.

All effluent samples are 24h composite samples (250 mL.hour^{-1}), collected with an automatic sampler. After sample collection, 2L sub-samples were stored in Schott bottles with Teflon stoppers previously rinsed with acetone. For steroid analysis, samples were maintained at -20°C until treatment; for alkylphenols, samples were preserved by adding

30 mL of 36% formaldehyde solution per litre and stored at 4°C for the summer survey, whereas for the winter survey, samples were frozen at -20°C until treatment.

7.2.3. Enrichment for steroid analysis and in vitro testing

Solid-phase extraction (SPE) was based on the procedure published by Aerni et al. (2003). In short, 1 L sample was filtered through a glass filter frit (GF/F), the pH adjusted to 3 and the internal standards added. The SPE cartridge (LiChrolut EN RP18) was conditioned using 6 mL hexane, followed by 2 mL acetone, 6 mL methanol and 10 mL nanopure water (pH 3). The sample was then enriched using a slight vacuum, the cartridge then washed with 8 mL methanol:water (7:3 v/v), followed by acetonitrile: water (3:7 v/v), and finally dried under nitrogen for 30-45 min. The enriched sample components were then eluted using 4 mL acetone and the eluate dried down to 100 µL under a gentle stream of nitrogen. The sample was then cleaned by passing it through a silica gel column (1 g) that had been conditioned with 10 mL hexane:acetone (6:4 v/v), using 7.1 mL of this eluent. The solvent was then evaporated with nitrogen and reconstituted in methanol:water:acetone (5:4:1 v/v) to a final volume of 200µl. Analytical grade solvents were used for extraction and cleaning; for LC-MS/MS analysis HPLC grade solvents were used.

7.2.4. LC-MS/MS analysis of steroid hormones

LC-MS/MS was done using an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Rotkreuz, Switzerland) and an Agilent HP1100 HPLC system with a Waters X Terra MS C18 (2.1 x 100 mm, 3.5 µm) column. Eluent A was water:acetonitrile (9:1 v/v), eluent B water:acetonitrile (1:9 v/v), the flow rate was 250 µL.min^{-1}, and the injection volume was 10 µL. The gradient run was as follows: 2 min 100% A, then in 19 min to 100% B that was maintained for 3 min, and back to initial conditions in 1 min and reconditioning for 10 min, giving a total run time of 35 min. A post-column addition of 2.5% ammonium acetate at 10 µL.min^{-1} increased ionization efficiency. Multiple reaction monitoring was

done using negative mode electrospray (needle voltage -4.5 kV, collision energy typically -50 V, declustering potential -100 V). Two transitions were monitored per analyte and standard. Deuterated estrogens were used as internal standards. Limits of detection (LOD) are matrix dependent and ranged between: 0.1-1.6 $ng.L^{-1}$ in 2005 and 0.2-4.4 $ng.L^{-1}$ in 2006 for E1; 0.2-4.2 $ng.L^{-1}$ in 2005 and 1.4-4.8 $ng.L^{-1}$ in 2006 for both α- and β-E2; 0.2-6.4 $ng.L^{-1}$ in 2005 and 0.4-1.2 $ng.L^{-1}$ for EE2.

7.2.5. Analysis of NP and BPA

The analytical procedure was based on the work published by Benijts et al. (2004). In brief, 500 mL of each sample (250 mL for the S. Jacinto outfall sample) were filtered using GF/F filters and then spiked with internal standard [*n*-NP (nonylphenol) for the samples from 2005 and a mix of $^{13}C_6$-NP, $^{13}C_6$-NPEO, $^{13}C_{12}$-BPA and $nNPE_2C$ for the samples from 2006]. Extraction was performed at neutral pH using Oasis HLB SPE-cartridges (200 mg). The cartridges were eluted with 2x3 mL of MTBE:propanol (1:1 v/v) for the samples from 2005, and with 1x3 mL MTBE:propanol (1:1 v/v) and 1x3 mL hexane for the samples from 2006. Analyses were performed with LC-MS/MS, with a Lichrospher C18ec LC column and electrospray in negative ionization mode (see above). All solvents used were HPLC grade. NP and BPA LODs were 29 and 1.1 $ng.L^{-1}$, respectively.

7.2.6. *In vitro* assays (YES/YAS)

In vitro assays were carried out only for the summer survey samples. Yeast cell culturing and exposure were performed as described by Routledge and Sumpter (1996), using yeast cells kindly provided by J. Sumpter (Brunel University, Uxbridge, UK). Dilution series of 17β-E2, dihydrotestosterone (DHT) and sample effluent extracts (1/1, 1/10, 1/100) were prepared in ethanol (for detailed description of effluent extract preparation see Section 4.2.2). 10 µL of each standard compound dilution and 20 µL of sample dilutions were added to 96-well microtiter plates. The ethanol was evaporated and yeast cells were added in medium growth. At least three replicate wells per dilution were used.

To test the occurrence of antiestrogenic and antiandrogenic activity, E2 and DHT EC_{50} (median effective concentration) were added to samples effluent extracts. As previously, three sample extracts dilutions were tested (1/1, 1/10, 1/100) with at least three replicate wells per dilution.

Estrogenic and androgenic activity, expressed as estradiol equivalency (EEQ) and dihydrotestosterone equivalency (DHTEQ), were determined by interpolation from E2 and DHT standard curves, respectively (Rutishauser et al., 2004). Furthermore, we calculated estradiol equivalency (EEQ_{calc}) considering the concentration addition model and the analytical data for E1, E2, EE2, NP and BPA in 2005. Relative potencies of each compound used were determined elsewhere and were calculated as the ratio of EC_{50} for E2 to the EC_{50} for each estrogenic substance tested (Rutishauser et al., 2004). If the concentration of a compound was below LOD it was considered as zero. Limits of detection for YES assays varied between 0.12-9.95 $ng.L^{-1}$ for Ria, Sea and Santiago WWTP samples; and between 0.12-12.0 $ng.L^{-1}$ for Cacia, Gafanha and S. Jacinto outfall samples. For YAS assay they varied between 2-182 $ng.L^{-1}$ for Ria, Sea and Santiago WWTP samples; and between 1-126 $ng.L^{-1}$ for Cacia, Gafanha and S. Jacinto outfall samples.

7.3. Results and Discussion

7.3.1. Chemical analysis

Table 7.1 summarizes the levels of estrogenic compounds found in the study area. The levels of natural estrogens varied from <LOD to 85 $ng.L^{-1}$ for estrone (E1), and to 9.2 $ng.L^{-1}$ for 17β-estradiol (β-E2), and are similar to those obtained by other studies in Europe and Canada (Ternes et al., 1999; Aerni et al., 2003; Johnson et al., 2005; Pojana et al., 2007), Australia (Tan et al., 2007) and Japan (Isobe et al., 2003). As expected, our reference samples had low levels of natural estrogens not exceeding 0.5 $ng.L^{-1}$ for E1 and 1.1 $ng.L^{-1}$ for β-E2. Effluent samples from WWTPs with secondary treatment (Gafanha and Cacia WWTP) also displayed low levels of natural estrogens in both surveys (<5.6 $ng.L^{-1}$ for E1,

<LOD for α-E2 and <2.3 ng.L^{-1} for β-E2) suggesting high efficiency of these units for estrogen removal from the initial influent.

The highest estrone levels observed in the present study are the ones from Santiago WWTP and from the S. Jacinto outfall in 2005. The high levels detected in Santiago WWTP (50 ng.L^{-1}) are probably due to the limited treatment before the upgrade. The high variation in estrone levels in S. Jacinto outfall between 2005 (85 ng.L^{-1}) and 2006 (5.8 ng.L^{-1}) might be explained by the high daily, and even hourly, fluctuations in the concentrations of estrogenic compounds in WWTP effluents (Tan et al., 2007).

Table 7.1. Chemical data for estrone (E1), 17α-estradiol (α-E2), 17β-estradiol (β-E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA) and nonylphenol (NP) at the various sampling sites, in summer (June 2005) and winter (February 2006) surveys. All values expressed as ng.L^{-1}.

	E1		α–E2		β–E2		EE2		BPA		NP	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2	2005	2006
Sea	0.5	<LOD	-	<LOD	1.1	<LOD	<LOD	<LOD	13	2.6	98	15
Ria	0.5	<LOD	-	<LOD	0.8	<LOD	<LOD	<LOD	897	7.7	75	10
Santiago WWTP	50.0		-	-	9.2	-	<LOD	-	2.8	-	164	-
Gafanha WWTP	0.2	5.6	-	<LOD	<LOD	<LOD	<LOD	<LOD	6.2	316	695	770
Cacia WWTP	1.8	2.6	-	<LOD	2.3	<LOD	<LOD	<LOD	14	158	923	2410
Pulp Mill	-	43.2	-	<LOD	-	<LOD	<LOD	<LOD	-	207	-	922
S. Jacinto outfall	85.3	5.8	-	<LOD	<LOD	<LOD	<LOD	<LOD	275	117	2350	2090

The concentrations of β-E2 ranged from <LOD to 9.2 ng.L^{-1} in the 2005 survey, whereas for the 2006 survey all the samples exhibited levels below the LOD. Concentrations of α-E2 were only quantified in 2006 and were always <LOD. β-E2 originates mainly in the human endocrine system whilst α-E2 is the predominant estrogen in cattle faeces and urine (Isobe et al., 2006) which can explain the levels below LOD obtained in our study since, as far as we are aware, no relevant livestock waste is discharged into the study area. The synthetic estrogen 17α-ethinylestradiol (EE2) was always below the LOD. These results are in agreement with the ones published previously for effluent and river water

samples in Europe and Canada (Ternes et al., 1999; Petrovic et al., 2002; Aerni et al., 2003) where EE2 is usually <LOD. Some studies, however, reported detectable concentrations of this compound in WWTP effluents. Aerni et al. (2003) found concentrations up to 2.8 $ng.L^{-1}$ (only at one of 17 European WWTPs analysed) and Vethaak and co-workers (2005) found mean concentrations of 2.6 $ng.L^{-1}$ in Dutch effluents from 2 WWTPs with combined physical and biological treatment with additional nitrogen and (chemical) phosphate removal. In surface waters high concentrations of EE2 were also reported for the Venice lagoon, with concentrations up to 75 $ng.L^{-1}$ (Pojana et al., 2004) and for the Douro estuary (NW Portugal) with values up to 56 $ng.L^{-1}$ (Ribeiro et al., 2007).

Nonylphenol (NP) and Bisphenol A (BPA) concentrations varied greatly along the sampled stations [Table 7.1] and within and between the sampling periods. For NP, concentrations ranged from 75 to 2350 $ng.L^{-1}$ in the summer survey, and for the winter survey they ranged from 10 to 2410 $ng.L^{-1}$. BPA levels varied between 2.8 and 897 $ng.L^{-1}$ in summer and 2.6 and 316 $ng.L^{-1}$ under winter conditions. Concentrations decreased from summer to winter in both reference sites for NP and BPA. A surprisingly high concentration of BPA was detected in Ria in 2005 (897 $ng.L^{-1}$). This unexpected concentration contrasts with the one detected in February 2006 at the same site. It is also much higher than the levels found in another survey in August 2006 (Jonkers et al., unpublished). This high value is expected to be an occasional BPA input, and is therefore considered an isolated event. For effluent samples the concentrations exhibit an opposite trend, with marked increases from summer 2005 to winter 2006 (with β-E2 levels at Cacia WWTP being the only exception). Differences in total hydraulic retention times (HRT) at each WWTP might explain such variation: lower HRT corresponds to lower degradation of EDC's and therefore higher concentrations in the final effluent (Johnson et al., 2005). During winter lower HRT are expected mainly due to the higher volume of influent received, for example in Cacia WWTP the volume of effluent was 468 252 m^3 in February 2006 *vs* 349 169 m^3 in June 2005. Another possible explanation is that a reduction in temperature may cause reduced WWTP treatment efficiency since the metabolic rate of microorganisms present in the various treatment steps slows down, resulting in higher

EDC's concentrations in winter than in summer (Tan et al., 2007). The highest levels of NP and BPA were consistently detected at the final effluent discharged by S. Jacinto submarine outfall. Such high levels were also observed in another survey conducted in August 2006 (Jonkers et al., unpublished) and are most likely a consequence of the effluent from the pulp mill.

Despite seasonal variations, our values are in the same order of magnitude as the ones reported for Europe (Petrovic et al., 2002; Aerni et al., 2003; Johnson et al., 2005) and for Portuguese surface waters (Azevedo et al., 2001) but much lower than those reported for the most polluted rivers in Portugal (Azevedo et al., 2001; Céspedes et al., 2004; Quirós et al., 2005) [Table 7.2].

Table 7.2. Concentration range (ng.L^{-1}) of NP and BPA in European waters. <LOD: below the limit of detection of the applied analytical procedure.

Location	Sample	BPA	NP	Reference
NE Spain	River water	-	1500	Petrovic et al., 2002
Switzerland	River and lake water	-	<LOD-490	Aerni et al., 2003
Glatt River, Switzerland	River water	9-76	68-326	Voutsa et al., 2006
Portugal	River and coastal water	200-4000	200-30000	Azevedo et al., 2001
Portugal	River water	-	100-4400	Céspedes et al., 2004
Portugal	River water	<LOD-5030	290-25530	Quirós et al., 2005
Douro Estuary, Portugal	River and coastal water	5100	-	Ribeiro et al., 2007
Switzerland	WWTP effluent	-	110-1740	Aerni et al., 2003
Several European	WWTP effluent	-	5-1310	Johnson et al., 2005
The Netherlands	WWTP effluent	<LOD-4090	<LOD-1500	Vethaak et al., 2005

Due to the introduction of regulatory measures such as the Water Framework Directive in which NP is identified as a priority hazardous substance (Annex 10 of Directive 2000/60/EC) and Directive 2003/53/EC that restricts both the marketing and use of nonylphenols in Europe from January 2005 onwards, a tendency of decreasing environmental levels has already been observed at some locations in Europe (Quednow

and Püttmann 2008). Future monitoring surveys are required in order to check this tendency and evaluate the legislation effectiveness.

7.3.2. Estrogenicity and androgenicity

Results from the YAS and YES assays are shown in Table 7.3. Estrogenicity was detected in Santiago WWTP and S. Jacinto outfall samples, and only weak estrogenicity could be detected at other locations. The estrogenicity expressed as EEQ ranged from <LOD to 25 $ng.L^{-1}$. The highest EEQ was detected in Santiago WWTP, followed by the S. Jacinto outfall. These locations also showed the highest estrone levels in the present survey as previously described. Calculated EEQ (EEQ_{calc}) varied between 0.09 and 32.5 $ng.L^{-1}$. For reference samples the major contributor to the total estrogenicity was estradiol (with contributions of 85.0 and 73.4% for Sea and Ria samples, respectively) followed by estrone (with contributions of 14.7 and 17.4% for Sea and Ria samples, respectively). In effluent samples, estrone was the major contributor (with contributions of 67.4% in Santiago, 80.8% in Gafanha and 99.7% in S. Jacinto) except in Cacia WWTP were estradiol contributed with 76% against the 23% of estrone. Contributions of NP and BPA to the calculated EEQs are almost negligible due to the small relative potencies of these compounds.

Table 7.3. Estrogenicity values measured with the YES assay (EEQ) and calculated (EEQ_{calc}), and androgenicity measured with the YAS assay (DHTEQ) for the summer survey. All values expressed as $ng.L^{-1}$.

Site	EEQ	EEQ_{calc}	DHTEQ
Sea	<LOD-0.23	1.29	<LOD
Ria	<LOD-0.18	1.09	<LOD
Santiago WWTP	<LOD-25.3	28.2	<LOD-311
Gafanha WWTP	<LOD-0.30	0.09	<LOD
Cacia WWTP	<LOD-1.29	3.01	<LOD
S. Jacinto outfall	<LOD-5.95	32.5	<LOD

When comparing the measured EEQs from the YES-assay with the calculated EEQs, the values are generally lower for the YES assay (with the Gafanha WWTP exception). Körner and colleagues (2001) suggested that calculated EEQs are always higher (by a factor of 2-4) than those determined by biological analysis, mainly due to detoxification mechanisms taking place at the cellular level. Another explanation could be the presence of some antiestrogenic substances that are not detected by the chemical target analysis, but this is unlikely to have occurred since the only effluent that exhibited antiestrogenic activity was Santiago WWTP and for this sample there was no considerable difference between the observed and the calculated EEQ.

Androgenic activity was only detected at the Santiago WWTP (311 $ng.L^{-1}$). In the present survey we did not quantify any environmental androgens but other studies pointed out that in WWTP effluents the concentration of androgens is generally higher than that of estrogens (Kirk et al., 2002; Thomas et al., 2002; Tan et al., 2007), mainly because androgens have greater synthesis and excretion rates in humans. Therefore, it seems reasonable to assume that androgenicity in this sample is due to the presence of androgens in the domestic effluent that only undergoes primary treatment. In reference samples, androgenic activity was not detected. From our previous data on TBT and imposex levels, some androgenicity was expected at Ria sample, since TBT is considered a potent androgen (Sumpter, 2005). The lack of androgenic activity might be explained by the fact that the analysis was conducted in a grab sample and TBT levels exhibit large temporal variability, or by the fact that TBT probably does not act through the androgen receptor. Antiandrogenic activity was noticed at Sea, Gafanha WWTP, Cacia WWTP and S. Jacinto outfall. The androgenic and antiandrogenic activity of several compounds has been reported in many studies [see for example Andreas (2002)]. However, several substances usually described as estrogens also exhibit androgenic or antiandrogenic activity (Sohoni and Sumpter, 1998): BPA, for instance, has been described both as an estrogenic and antiandrogenic compound. Furthermore, and considering that there are several substances that can act as antiandrogens (e.g. DDE, vinclozolin and benomyl (Andreas 2002), not included in the present study); it is impossible to ascertain which compounds are causing antiandrogenic activity in our samples.

7.3.3. Environmental impact

The effects of effluent exposure in humans and wildlife are well documented in the literature (for further details see reviews by Langston et al., 2005; Sumpter, 2005). Environmental levels of estrogenic substances are known to affect fish: EEQ levels between 0.1 and 5 $ng.L^{-1}$ for vitellogenin induction have been reported (Solé et al., 2000; Körner et al., 2001; Seki et al., 2002). Molluscs are also very sensitive to estrogenic chemicals: in the mussel *Mytilus galloprovincialis,* a common species in the Portuguese coast, mixtures of E2, EE2, NP, BPA, MES (mestranol) and BP (benzophenone) affect haemocyte parameters at concentrations comparable to environmental exposure levels (Canesi et al., 2007). For the oyster *Crassostrea gigas*, NP concentrations as low as 100 $ng.L^{-1}$ can affect the development of larvae and increase their mortality rate (Nice et al., 2000). Some studies also reported adverse effects to natural gastropod populations. Oehlmann and co-workers (2006) found that BPA induces super-feminization in the ramshorn snail *Marisa cornuarietis* at environmentally relevant concentrations and they also proposed a NOEC value of 1 $ng.L^{-1}$ for EE2 for this species (Schulte-Oehlmann et al., 2004). The same team reported alterations in the reproductive system of *Nucella lapillus* after laboratory exposure to 1000 $ng.L^{-1}$ of BPA (Oehlmann et al., 2000). Our study discloses estrogenic levels at reference sites lower than the ones reported above; however, it should be considered that S. Jacinto outfall releases effluent with high levels of E1, BPA and NP into the marine environment. The NP levels are much higher than the Environmental Quality Standard (EQS) set by the European Water Framework Directive (300 $ng.L^{-1}$). Although some dilution is expected, the concentrations found in the water column near the effluent discharge point may pose a risk for sensitive species and therefore further studies on environmental levels of estrogens around the S. Jacinto outfall are required. Moreover, estrogens associated with the particulate fraction aggregate in contact with seawater and settle on the seafloor after discharge through deep ocean outfalls (Braga et al., 2005), thus communities around the outfall are susceptible to be affected and further investigations on target species should be performed.

7.4. CONCLUSIONS

In the present study steroid hormones and phenolic endocrine disrupting chemicals were assessed for Ria de Aveiro effluents and reference sites. Generally the levels of steroids and phenolic EDCs are low in reference samples. Of the steroid hormones estrone presented the highest levels, whereas the synthetic hormone EE2 was always below the detection limit. The highest levels of steroids were associated with an urban WWTP with no secondary treatment (Santiago) whereas the highest levels of phenolic compounds were detected in industrial effluents, namely the effluent from a paper and pulp mill facility. Biological assays disclose higher estrogenic activities in effluents from Santiago WWTP and S. Jacinto outfall. The main compound responsible for the total estrogenicity of the effluent samples was estrone. Androgenic activity was only detected in the effluent sample from Santiago WWTP, probably a result of the low treatment efficiency of this facility. Although the present study discloses levels of EDCs at reference sites lower than the ones reported to pose risk for wildlife, the concentrations of E1, NP and BPA in the final effluent released by S. Jacinto outfall are very high and deserve further attention. Additional studies on environmental levels of EDCs around the S. Jacinto outfall and possible effects on target species are required.

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CHAPTER 8

INVOLVEMENT OF RETINOID X RECEPTOR IN IMPOSEX DEVELOPMENT IN *NUCELLA LAPILLUS* AND *NASSARIUS RETICULATUS*: PRELIMINARY RESULTS

CHAPTER 8. INVOLVEMENT OF RETINOID X RECEPTOR IN IMPOSEX DEVELOPMENT IN *NUCELLA LAPILLUS* AND *NASSARIUS RETICULATUS* - PRELIMINARY RESULTS

Abstract

The imposex phenomenon provides one of the best examples of endocrine disruption in wildlife and has been studied for about three decades. However, the exact biochemical mechanism by which it develops is still unclear. Several pathways have been proposed: neuroendocrine, steroid and retinoic. In the present work the possible role of Retinoic X Receptor (RXR) in the development of imposex in *N. lapillus* and *N. reticulatus* was investigated: snails of both species were injected with ethanol containing tributyltin (TBT) or with Fetal Bovine Serum (FBS) containing 9-*cis*-retinoic acid (9CRA: the natural ligand for humans RXRs) and maintained in a flow-through system with artificial seawater for 30 days (temperature: 18.4±0.44 ^{o}C; salinity: 33.0±1.06‰).

Both TBT and 9CRA induced imposex development in *N. lapillus* and *N. reticulatus*. Significant (p<0.05) increases in the *vas deferens* sequence index (VDSI) and female penis length (FPL) were registered between the ethanol control and the TBT treatment and between the FBS control and the 9CRA treatment. The obtained results provide further evidence of the involvement of RXR signalling pathway on imposex development in both species.

Keywords: Imposex; *Nassarius reticulatus*; *Nucella lapillus*; Retinoid X Receptor; Tributyltin (TBT); 9-cis-Retinoic Acid (9CRA)

8.1. INTRODUCTION

Imposex is considered the best documented example of endocrine disruption in wildlife (Matthiessen & Gibbs, 1998). About 200 gastropod species worldwide are affected by this phenomenon (Shi et al., 2005) and some are routinely used as bioindicators of TBT pollution. Although the causal relationship between TBT and imposex is more than consensual in the scientific literature, the exact mechanism of TBT action on imposex induction still remains under debate. Several hypotheses have been postulated in order to explain the mechanisms underlying imposex induction and three possible

pathways have been identified: the neuroendocrine, the steroid and the retinoic. A detailed description of each pathway can be found in Chapter 1 of the present thesis. Succinctly, the neuroendocrine pathway suggests that the homeostasis of certain neuroendocrine factors can be disrupted by TBT, leading to the formation of accessory sex organs in females (Féral & Le Gall, 1983; Oberdörster & McClellan-Green, 2000; Oberdörster & McClellan-Green, 2002). The steroid pathway postulates that TBT causes an imbalance in steroid hormones characterized by higher testosterone levels in females which leads to the imposex development; this disruption in steroid homeostasis can be promoted either trough interferences with the steroid biosynthesis (see Spooner et al., 1991; Bettin et al., 1996) or with steroid excretion (see Ronis & Mason, 1996; Gooding et al., 2003). The retinoic pathway proposes that TBT and TPhT mimic the endogenous ligand of retinoid X receptor (RXR) and thus activate the signaling cascades which are retinoic acid dependent (Nishikawa et al., 2004; Castro et al., 2007; Horiguchi et al., 2007a; Horiguchi et al., 2007b). So far, both neuroendocrine and steroidal theories could not provide unequivocal experimental results as they were unable to promote the dincrease in female penis lenght to the same extent as TBT whilst in the RXR theory a single injection of 9-*cis*-retinoic acid (9CRA), the natural ligand for human RXRs, induced the development of imposex (percentage of affected females) as well as the substantial growth in female penises to the same extent as TBT or TPhT, when administrated at similar concentrations (Horiguchi, 2006).

The present work aims to understand if 9CRA is able to induce imposex in *Nucella lapillus* (Linnaeus, 1758) and in *Nassarius reticulatus* (Linnaeus, 1758) and therefore to provide insights on imposex mechanism in these species.

8.2. Materials and Methods

8.2.1. Sampling and animal's selection

N. lapillus and *N. reticulatus* collection was performed in March 2008, at two locations in the North Western Portuguese coast. The dogwhelks were collected by hand at the

open coast near Aveiro (Poço da Cruz, 40º29'22.91''N/8º47'37.06''W, Portugal) whereas the netted whelk specimens were captured with baited hoop nets inside the Ria de Aveiro estuarine system (Magalhães Mira, 40º38'34.65''N/8º44'06.80''W, Portugal). Once in the laboratory, the animals were narcotized with magnesium chloride so that females could be selected: for *N. lapillus* only the ones without penis were chosen whereas for *N. reticulatus* only those with small penis (<1mm) were considered since imposex-free snails could not be found.

8.2.2. Specimens maintenance, transportation and acclimatization

The selected females were maintained in the laboratory with artificial seawater (Crystal Sea® Bioassay Formulation, Marine Enterprises International; salinity of 33‰) for two weeks and then transported to the National Institute for Environmental Studies (NIES, Japan) in refrigerated conditions. Once arrived at NIES, the animals were maintained in an aquarium with artificial seawater (Tomita Pharmaceutical Co., Tokushima, Japan) under the same salinity and allowed to acclimate for ten days before the experiments started. During the acclimatization period, animals were fed with mussels (*Septifer virgatus*), which were collected at Hiraiso, Japan, a less contaminated site by organotins and therefore usually used with this purpose (Nishikawa et al., 2004; Horiguchi et al., 2007a).

8.2.3. *In vivo* experiments

Females of both species were narcotized in 72 $g.L^{-1}$ magnesium chloride hexahydrate (Nacalai Tesque, Japan) and then injected with the test solutions in the foot using a 10 µL Hamilton micro syringe (Hamilton 80300 - 10 µL syringe 701 N). For each species four groups of 20 animals each were injected with: fetal bovine serum (designated as "FBS control"); 9-*cis*-retinoic acid ("9CRA"); ethanol ("EtOH control") and tributyltin chloride ("TBTCl"). The 9CRA (Wako Pure Chemicals Industries, Japan) solution was prepared in FBS (Flow Laboratories, USA) whilst the TBTCl (95% pure, Tokyo Kasei Kogyo Co., Japan)

solution was prepared in EtOH (Wako Pure Chemicals Industries, Japan). Each animal in solvent control (EtOH and FBS) was injected with 1 µL per gram of soft tissue (in a wet weight basis). The same volume was injected in the other treatments: 9CRA in FBS was injected at a concentration of 1 $\mu g.g^{-1}$ ww in *N. lapillus* and *N. reticulatus* whereas TBTCl was injected in EtOH at a concentration of 1 $\mu g.g^{-1}$ ww in *N. lapillus* and 2 $\mu g.g^{-1}$ ww in *N. reticulatus*.

The animals of each experimental group where kept separately in 2-L beakers immersed in a temperature controlled water bath (TK 60; Takara Japan). Each beaker was provided with oxygen saturated artificial seawater and was connected to a flow-through system (Eyela Mp, Tokyo Rikakikai Co., Japan) at a constant rate (20 $L.beaker^{-1}.day^{-1}$). *N. lapillus* containing beakers were covered with plastic film to prevent animals to escape. Water parameters were monitored daily and presented mean values (± St. Dev) of: 8.0±0.10 for pH; 33.0±1.06 for salinity and 18.4±0.44 ^{o}C for temperature. Animals where fed *ad libitum* three times a week. For *N. lapillus* live mussels (*Septifer virgatus*) were provided whereas for *N. reticulatus* open dead mussels were offered.

8.2.4. Imposex analysis

At the end of the experimental period animals were morphologically examined for imposex development. Imposex parameters were assessed after narcotization: percentage of females affected by imposex (%I), *vas deferens* sequence index (VDSI), and mean female penis length (FPL). The penis length was measured with a digital vernier calliper to the nearest 0.01 mm. The *vas deferens* sequence in *N. lapillus* was classified according to the scoring system proposed by Gibbs et al. (1987) and in *N. reticulatus* according to the system proposed by Stroben et al. (1992a). Soft tissues of each animal were preserved for future histological studies and for chemical analysis, except the 9CRA group where no specimens for chemical analysis were reserved.

8.2.5. Statistical analysis

SigmaStat software v3.5 was used to perform all statistical analysis. Differences in VDSI between groups were tested trough the non-parametric Kruskal Wallis test (Anova on ranks) followed by the post-hoc Dunn's test whereas differences in FPL between groups were tested trough one way ANOVA after confirming data normality and homocedasticity. The adopted critical significance level was 5%.

8.3. Results and Discussion

8.3.1. Imposex induction in *Nucella lapillus*

N. lapillus mortality rates varied between 5% in EtOH control and 75% in 9CRA. In FBS control and TBTCl group mortality rates were 15 and 20%, respectively. Despite the high mortality rate registered in the 9CRA treatment it was decided to include *N. lapillus* results in the thesis as they still provide useful information to achieve the objective of the present study.

Highly significant VDSI increases ($p<0.001$) were observed between the EtOH control (VDSI=0.0) and the TBTCl treatment (VDSI=2.0) and between the FBS control (VDSI=0.0) and the 9CRA treatment (VDSI=2.4), [Figure 8.1A]. Female penises also increased significantly from the EtOH control (0.0 mm) to the TBTCl (0.6 mm) and from the FBS control (0.0 mm) to the 9CRA (0.7 mm), [Figure 8.1B]. No significant differences in VDSI or FPL were observed between both controls or between the TBTCl and the 9CRA groups [Figure 8.1A, 8.1B].

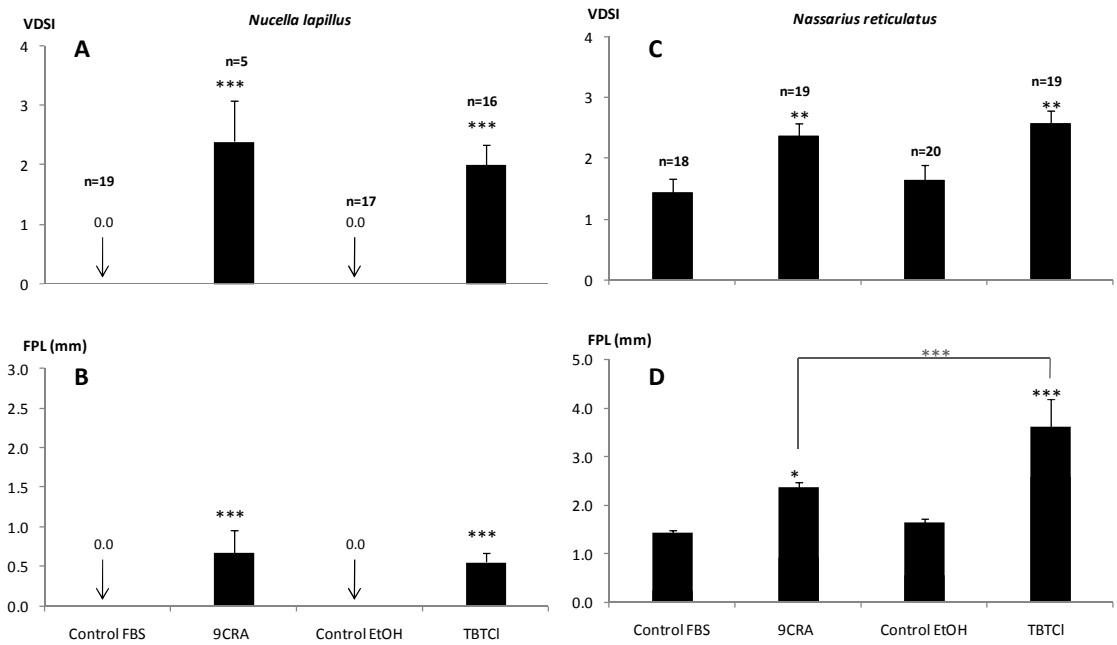

Figure 8.1. Variation of *vas deferens* sequence index (VDSI) and female penis length (FPL) in *N. lapillus* (A and B) and *N. reticulatus* females (C and D) after 30 days of exposure. Error bars denote standard error. Significant differences in relation to control groups (FBS for 9CRA and EtOH for TBTCl) are marked: * $p<0.05$; ** $p<0.01$; *** $p<0.01$. Significant differences between 9CRA and TBTCl are also indicated.

Our results indicate that 9CRA induces imposex in *N. lapillus* with the same severity as the positive control TBTCl, suggesting that both compounds may act through the same signalling pathway. To the author's best knowledge, only two studies on RXR and imposex in *N. lapillus* were performed (Castro et al., 2007; Oehlmann et al., 2007) and they disclose contradictory results. Oehlmann and co-workers injected 9CRA in *N. lapillus* females and reported that injection of 9CRA did not cause any effect on the development of imposex (i.e., percentage of affected females, increase in penis length and the development of *vas deferens* in females) after a 56 days period, even exposed at the highest concentration tested (2.5 $\mu g.g^{-1}$). Castro et al. (2007) injected *N. lapillus* females with the 1 $\mu g.g^{-1}$ of 9CRA and demonstrated that it induces imposex to the same degree as TBT, when administered at similar doses (1 $\mu g.g^{-1}$). These authors suggested that the use of different carriers (FBS in both Castro et al. (2007) and this study and vitamin A-free peanut oil in Oehlmann et al. (2007)) might explain the observed differences since dosing

9CRA in peanut oil can affect its biodisponibility during the experiment course, rendering it less bioactive. It should also be noticed that 9CRA is easily photo decomposed. Even knowing that the number of females in the 9CRA group at the end of the experiment was extremely low (n=5), our results corroborate the findings of Castro et al. (2007) reinforcing the hypothesis that imposex in *N. lapillus* is mediated trough the RXR pathway.

Figure 8.2. *N. lapillus* female with visible signs of imposex (VDS stage=4; FPL=1.4 mm) 30 days after a single injection with 9CRA (1 µg.g^{-1} ww). P: penis.

8.3.2. Imposex development in *Nassarius reticulatus*

N. reticulatus mortality rates varied between 0% and 10% (0% in EtOH, 5% in TBTCl and 9CRA; 10% in FBS). As already referred, *N. reticulatus* females used in the present study were slightly affected by imposex (mean VDSI and FPL of 1.6 and 0.4 mm, respectively) in the beginning of the experiment.

VDSI levels in *N. reticulatus* females increased significantly (p<0.01) from the EtOH control (VDSI=1.7) to the TBTCl (VDSI=2.6) and from the FBS control (VDSI=1.4) to the 9CRA (VDSI=2.4), [Figure 8.1C]. Female penis length increased in a highly significant

manner ($p<0.001$) between the EtOH control (0.6 mm) and the TBTCl (3.6 mm) whereas between FBS control (0.3 mm) and 9CRA (0.9 mm) the increase was not so substantial, despite being significant ($p<0.05$), [Figure 8.1D]. Females injected with TBT developed longer penises than those injected with 9CRA ($p<0.001$) and, besides, no significant differences in FPL between EtOH and FBS controls were found [Figure 8D]. Such results might suggest that 9CRA does not promote imposex in the same way as TBT does, however it should be stressed that this difference is most probably a consequence of the different concentrations used: 1 $\mu g.g^{-1}$ ww for 9CRA and 2 $\mu g.g^{-1}$ ww for TBTCl. According to Stroben et al. (1992b) *N. reticulatus* is less sensitive than *N. lapillus* and for this reason a higher TBT concentration was used. For 9CRA the selected concentration was the same as the one used in other studies (Nishikawa et al., 2004; Castro et al., 2007; Oehlmann et al., 2007) so that comparisons could be performed.

As far as we are aware, the only study with *N. reticulatus* is the one by Oehlmann et al. (2007) and, as for *N. lapillus*, these authors did not detect any differences in the development of imposex between the control and the 9CRA groups. Our results point in the opposite direction, with significant differences in both female penis length and VDSI between the control and the 9CRA groups. The use of different carriers (FBS and vitamin A-free peanut oil) can be a possible reason to explain such differences, as suggested by Castro et al. (2007). Our results also demonstrate that RXR plays an important role in imposex development in *N. reticulatus* and reinforce the retinoid theory originally proposed by Nishikawa et al. (2004) for *T. clavigera* and already confirmed for *N. lapillus* (Castro et al., 2007).

8.3.3. The role of RXR in imposex development

Several theories have been proposed to explain the imposex phenomenon, but so far the exact mechanism by which imposex develops is still elusive. Until recently the aromatase inhibition and the neuroendocrine theories were the most consistent ones but recently a new pathway was proposed - the retinoic pathway. The retinoid X receptor (RXR) is a member of the nuclear receptor super-family which is highly conserved

throughout metazoans (Nishikawa, 2006; Castro et al., 2007; Sternberg et al., 2008). Retinoid signaling is involved in the regulation of male reproductive differentiation and development (Sternberg et al., 2008). Recently, it was proposed that imposex in gastropods is mediated trough the RXR signaling pathway (Nishikawa et al., 2004). Organotins (tributyltin and triphenyltin) apparently mimic the role of the natural ligand, binding RXR with high affinity (Nishikawa et al., 2004). This theory was initially proposed for the rock shell *Thais clavigera* (Nishikawa et al. 2004), and was confirmed for the same species through a series of laboratory experiments (Horiguchi et al., 2007a) and also through the assessment of RXR gene expression and protein content in wild animals (Horiguchi et al., 2007b). The involvement of RXR in imposex induction was further investigated by Oehlmann et al. (2007) in *N. lapillus* and *N. reticulatus*. However, these authors showed that for both species 9CRA had no significant effects on imposex parameters after almost 2 months following injection. Such contradictory results lead Castro et al. (2007) to perform a series of experiments with *N. lapillus* in order to test if the RXR is or not involved in the imposex induction and the obtained results indicate that RXR is the primary target for TBT in *N. lapillus.*

In order to provide further evidences of the involvement of RXR in imposex mechanism we injected 9CRA in *N. lapillus* and in *N. reticulatus.* Our results confirm the findings of Castro et al. (2007) for *N. lapillus* and provide evidences that imposex is also mediated trough the RXR signaling pathway in *N. reticulatus*. Recent studies also seem to provide further evidences of RXR involvement, with the cloning of RXR in imposex susceptible gastropods as the mud-snail *Ilyanassa obsoleta* (Sternberg et al., 2008) and the above mentioned *N. lapillus* (Castro et al., 2007) and *T. clavigera* (Nishikawa et al., 2004). Additional reports confirmed that TBT activates the RXR-PPAR[8] heterodimer trough a covalent interaction with Cys432 residue in the RXR-α (le Maire et al., 2009). Despite the increasing amount of evidences linking imposex and the RXR signaling pathway many questions remain unsolved (as for example the interplay between pathways) and further research is mandatory.

[8] RXR: Retinoid X Receptor; PPAR: Peroxisome Proliferator Activated Receptor

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CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9. GENERAL DISCUSSION

9.1. SPATIAL TRENDS OF ORGANOTIN POLLUTION IN THE PORTUGUESE COAST AND ASSOCIATED IMPACTS

Previous reports regarding tributyltin (TBT) pollution in the Portuguese coast indicate a widespread presence of this compound in inshore as well as offshore areas with harbors considered as hotspots of pollution (see Chapter 2 and references therein). The present thesis aims to investigate the current status of organotin pollution in the Portuguese coast and seeks to confirm if the above spatial patterns are still maintained. Moreover, it intends to assess the spatial patterns of a variety of OTs compounds that are predicted to occur in the environment, namely butyltins, phenyltins and octyltins, so that the relative importance of TBT may be rigorously determined regarding the overall OT pollution scenario. Hence, the environmental levels of OTs were quantified in the entire breatdh of the Portuguese coast from Viana do Castelo to Faro. Two key bioindicators were used in a complementary approach: the mussel - *Mytillus galloprovincialis* – provided information regarding pollution levels in the water column whereas the netted whelk - *Nassarius reticulatus* – provided information mainly related to the levels in sediments. Imposex in the nassarid was additionally used as a biomarker of TBT pollution.

The levels of total tin (Sn_T) in mussels collected along the coast during 2006 were quantified and related to the levels of total organic tin compounds including monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), diphenyltin (DPhT), triphenyltin (TPhT), monoctyltin (MOcT) and dioctyltin (DOcT). The obtained results indicate that the organic forms of tin (OTs) largely contribute to the total amount of tin detected in mussel's tissues and that butyltins (MBT, DBT and TBT) represent the major organotin contaminants. The highest levels of contamination were observed inside harbors, corroborating the established idea that they represent the hotspots of OTs pollution in the Portuguese coast. The contribution of OTs to Sn_T is also higher at these sites where they can reach up to 55% of the Sn_T. Such results demonstrate the influence that human

activities have in the natural environment: the levels of a natural element such as tin are increased several times due to anthropogenic inputs.

TBT concentrations in mussels varied between 0.9 and 720 ng Sn.g^{-1} dw. Such values are, in 69% of the sampling sites, higher than the lower-EAC[9] (4.9 ng TBT-Sn.g^{-1}) proposed by OSPAR (2004), which indicates that at those sites adverse effects are possible to occur. The assessment of TBT levels in mussel's soft tissues also enabled a rough estimation of TBT seawater concentrations. Figure 9.1 discloses the estimated seawater levels along 29 sites in the Portuguese coast estimated using the quantified TBT levels in mussel's tissues and the bioconcentration factor described for this species by Takahashi et al. (1999).

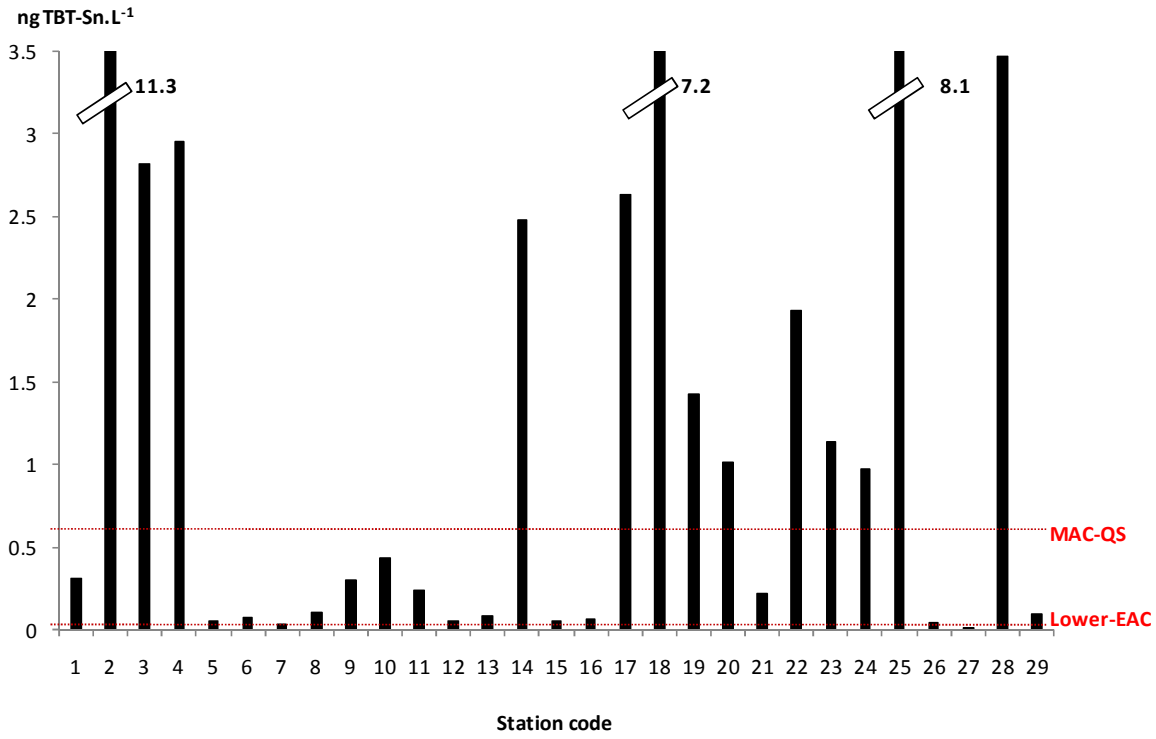

Figure 9.1. Estimated TBT seawater concentrations in 29 sampling sites located between V. Castelo and Faro, calculated using TBT concentration in soft tissues of *M. galloprovincialis* and the bioconcentration factor described for this species (Takahashi et al., 1999). Details concerning each sampling site are provided in Chapter 3. The dot lines refers to: MAC-QS: the maximum acceptable concentrations not to be exceeded any time proposed under the Water Framework Directive for the protection against acute effects exerted by transient concentration peaks (SCTEE, 2005); Lower-EAC: Lower Environmental Assessment Criteria set by OSPAR Commission (2004).

[9] EAC: Environmental Assessment Criteria

The estimated seawater concentrations varied between 0.014 and 11.3 ng TBT-Sn.L^{-1} and in 93% of the sampling sites they were higher than the lower-EAC set for TBT in water by OSPAR Commission (0.04 ng Sn.L^{-1}). Therefore, at those locations negative biological effects are likely to occur. For example, TBT seawater levels as low as 0.4 ng Sn.L^{-1} induce imposex development in *Nucella lapillus* (Bryan & Gibbs, 1991) and sterility in this gastropod may occur at 1-2 ng Sn.L^{-1}, whereas virtually all females in a population become sterilized if TBT in water reaches 3-4 ng Sn.L^{-1} (Gibbs et al., 1991). This later scenario is likely to occur at sampling stations 2, 18, 25 and 28. Other possible deleterious biological effects include impaired growth and reproduction in plankton, mollusks and fish. Table 9.1 provides a brief overview on toxicity data of most sensitive species.

Table 9.1. Overview on toxicity data of most sensitive species adapted from SCTEE (2005). NOEC: no observed effect concentration; LC_{50}: Median Lethal Concentration; EC_{50}: Median Effective Concentration.

Species	Taxonomic group	Duration	Effect	Endpoint	Value (ng TBT-Sn.L^{-1})
Dunaliella tertiolecta	Algae	18 days	growth	NOEC	20.1
Acartia tonsa	Crustacea	8 days	larvae survival	LC_{50}	0.5
Eurytemora affinis	Crustacea	13 days	reproduction	NOEC	4.1
Acartia tonsa	Crustacea	8 days	larvae development	EC_{50}	10.9
Palaemocetes pugio	Crustacea	21 days	mortality	NOEC	13.5
Nucella lapillus	Mollusca	320 days	imposex	NOEC	0.4
Saccostrea gigas	Mollusca	28 days	growth	NOEC	2.1
Mercenaria mercenaria	Mollusca	4 days	growth	NOEC	9.8
Crassostrea gigas	Mollusca	28 days	growth	NOEC	9.8
Crassostrea gigas	Mollusca	21 days	mortality	NOEC	10.2
Buccinum undatum	Mollusca	19 months	growth	NOEC	11.4

Our estimated values are also generally higher than the quality standards defined for all types of surface waters covered by the Water Framework Directive (2000/60/EC). In 45% of the sampling locations the levels are higher than the maximum acceptable concentrations (MAC-QS: 0.61 ng Sn.L^{-1}) [Figure 9.1]. If we consider the annual average quality standard (AA-QS: 0.082 ng Sn.L^{-1}) that proportion rises to 69% indicating that the quality standards are far from being reached.

The levels of organotin compounds were also assessed in the gastropod *N. reticulatus* during a survey performed along the Portuguese coast in 2008. As for *M. galloprovincialis* butyltins accounted for the majority of organotin compounds quantified and the highest levels were found inside or in the vicinity of harbors. Such results confirm the previous spatial pattern of OTs along the coast. TBT levels varied between 3.5 and 380 ng $Sn.g^{-1}$ dw, representing an average proportion of 50.4% of total butyltins (∑BuTs= MBT+DBT+TBT), and at some locations that proportion was higher, indicating that in 2008, inputs were still occurring. Imposex, wich was also used as a biomarker of TBT pollution, also exhibited similar spatial gradients. Females with imposex were present at all sampled sites and highly significant correlations were established between imposex indices and TBT concentrations in the whelk's soft tissues. The *vas deferens* sequence (VDS) varied between 0.2 and 4.4 across the sampling sites [Figure 9.2].

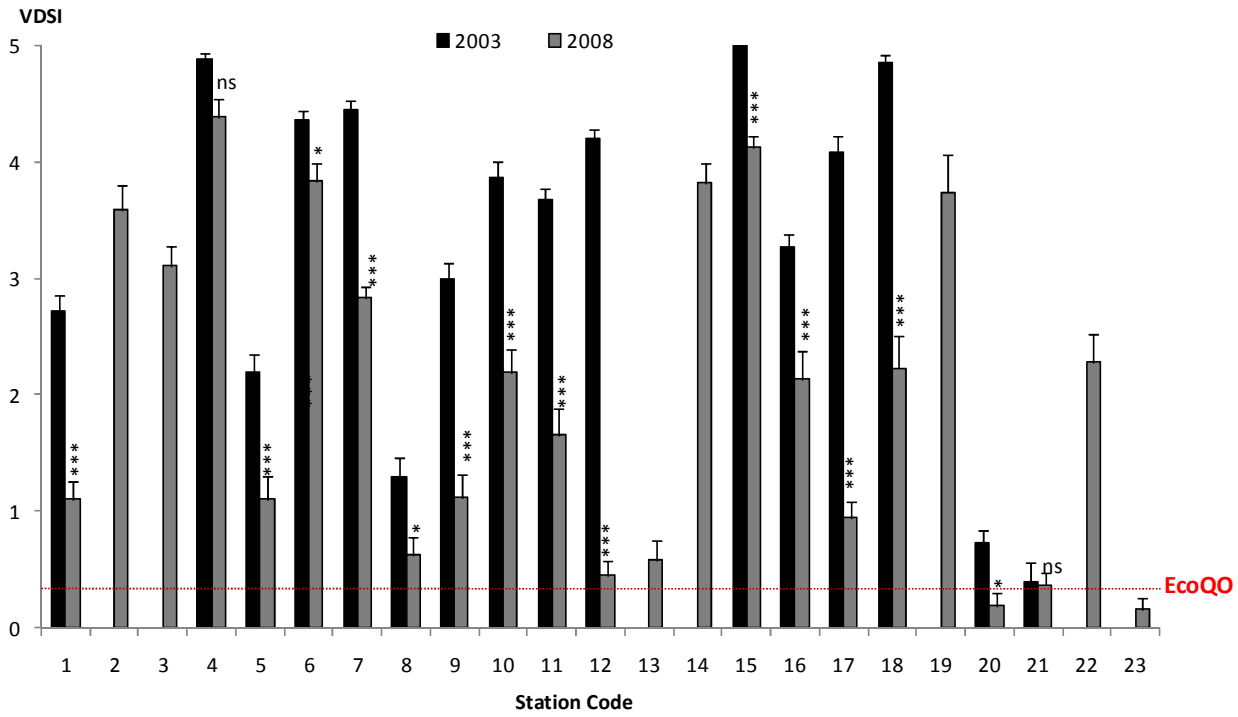

Figure 9.2. Temporal comparisons of VDSI levels between 2003 and 2008. Error bars denote standard error. Statistical differences are marked: ns: not significant; *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$. The line refers to the Ecological Quality Objective (EcoQO) proposed by OSPAR for imposex in *N. reticulatus* (OSPAR, 2007).

According to OSPAR (2007) the Ecological Quality Objective (EcoQO) for imposex in *N. reticulatus* is achieved for VDS levels below 0.3. In the 2008 survey, 91% of the surveyed locations exhibited levels above the EcoQO, indicating that for those sites there is risk of adverse effects, such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem. Such results corroborate the ones previously obtained using *M. galloprovincialis* as a bioindicator species (see above).

Organotins were also quantified in sediment samples collected in Ria de Aveiro during a survey conducted in 2005. As for biological samples butyltins represented the major group of OTs. Among the butyltins, TBT was dominant with 76% of total butyltins (∑BuTs), followed by DBT (12.6% of ∑BuTs) and MBT (11.8% of ∑BuTs). The predominance of TBT over its metabolites may reflect recent inputs of TBT and/or slow degradation rates characteristic of this compartment (Sarradin et al., 1995; Hoch, 2001). TBT concentrations varied between 2.7 and 1780 ng TBT-Sn.g^{-1} dw and were highly correlated with the sample's organic matter (OM) content. Considering the provisional lower (0.004 ng TBT-Sn.g^{-1} dw) and upper (0.06 ng TBT-Sn.g^{-1} dw) EAC proposed by OSPAR (2004) for sediments all the analyzed samples exhibit levels much higher than the proposed EACs. Taken into consideration the value set under the Water Framework Directive the reported concentrations are also much higher than the Quality Standard derived for TBT in sediments (0.008 ng TBT-Sn.g^{-1} dw). Overall our results clearly demonstrate that TBT pollution in still a matter of great concern along the Portuguese coast.

9.2. Temporal evolution of TBT pollution in the Portuguese coast

Initial reports on the temporal evolution of imposex levels along the Portuguese coast (Barroso & Moreira, 2002; Santos et al., 2002) exposed the inefficacy of the TBT partial ban (EEC Directive 89/677) whilst recent studies provide some evidences that TBT pollution is decreasing after the introduction of EC Regulation 782/2003 in localized areas such as Ria de Aveiro (Galante-Oliveira et al., 2009) or along the entire coast (Rato et al., 2009).

In this work we aimed to confirm this decreasing tendency along the entire coast not only using imposex as a biomarker of TBT pollution but also by quantifying the levels of organotin compounds in *N. reticulatus* soft tissues and, by this way to further validate this biomarker to monitor temporal trends of TBT pollution. OTs levels in *N. reticulatus* soft tissues collected during the 2008 and 2003 surveys were analysed in order to assess the change on TBT levels between this two years [Figure 9.3]. The comparison between butyltin levels in samples from the two campaigns discloses a significant ($p<0.01$) reduction in TBT, DBT and MBT. Considering the five common locations to both surveys the average decrease was 83±10% for TBT, 78±10% for DBT and 89±12% for MBT.

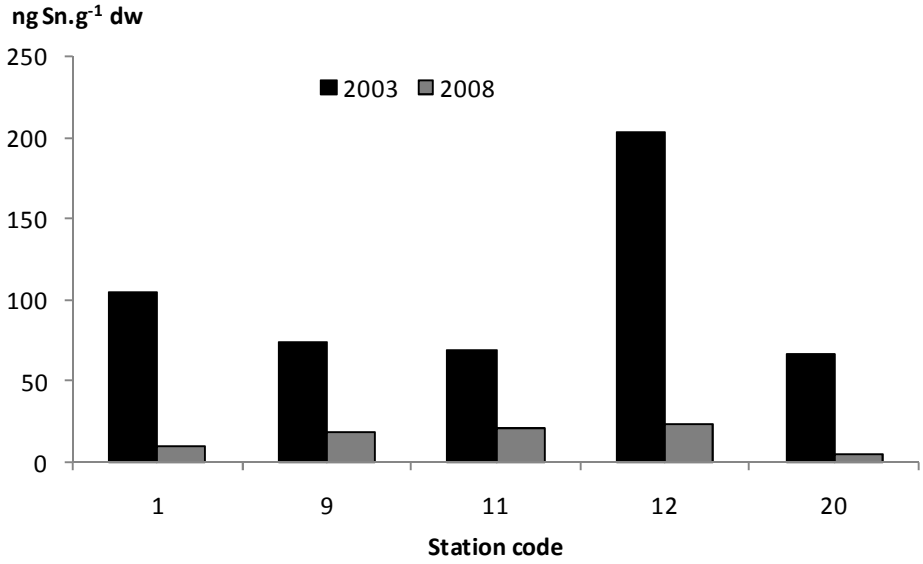

Figure 9.3. TBT concentrations in *N. reticulatus* soft tissues in the 2003 and 2008 surveys.

Figure 9.2 also discloses the temporal variation of VDSI levels between 2003 and 2008. Significant decreases ($p<0.05$) were noticed at 14 of the common locations. Only St. 4 and St. 21 did not exhibit any significant difference between 2003 and 2008. The imposex maintenance at those locations can be easily explained if we consider the specific characteristics of each site: at St. 21 low levels of imposex were noticed in both campaigns making it difficult to notice any change, whereas St. 4 is located inside a

shipyard, where continuous TBT inputs are expected to occur since old TBT coatings are being removed and substituted by alternative ones. A good agreement is observed between the decrease of TBT and imposex levels at those locations: 83% average decrease in the values of TBT concentrations was accompanied by a decrease of 68% and 83% in VDSI and FPL levels, respectively. As already mentioned, despite the general decreasing tendency in TBT pollution in the Portuguese coast, this pollutant is still a matter of concern as the EcoQO proposed by OSPAR was not achieved in the majority of sites (see Section 9.1 and Figure 9.2).

So far, the obtained results demonstrate that imposex in *N. reticulatus* is a useful biomarker to monitor spatial gradients of TBT pollution and it is also adequate to monitor temporal trends. The responsiveness of this biomarker over time periods of 3 to 5 years is probably associated with rapid cohort's turnover with older and more affected females being substituted by younger and less affected ones. As female sexual maturity is achieved at the 4th year of age (Barroso et al., 2005), new born animals and juveniles that grew after the 2003 ban would have been sampled in 2008 as adult animals. Therefore, even though *N. reticulatus* can live for 10 years or more (Barroso et al., 2005), our results suggest that the adult cohorts sampled in 2003 were replaced by new ones that grew under decreasing levels of TBT in the environment leading to a notorious imposex recovery. Nevertheless, it should not be excluded the limited degree of imposex reversibility, especially in what concerns to FPL index. Bryan et al. (1993), for instance, noticed that there was a slow reduction in the female penis length over time and Sousa et al. (2005) also suggested a reduction in FPL in some locations along the Portuguese coast.

Associated with the decreasing tendency of TBT pollution along the Portuguese coast a change in imposex expression in the gastropod *N. reticulatus* was detected with an increase proportion of females with initial stages of *vas deferens* development but without a penis (b-type females). This increase was notorious not only in the percentage of b-type females in relation to the total number of imposex affected ones (from 3.5% in 2000, to 11% in 2003 and to 24% in 2008) but also in the number of sites where this phenomenon was recorded: two sites in 2000, seven sites in 2003 and thirteen sites in

2008. By definition, b-type females are restricted to initial stages of *vas deferens* development (in the majority VDS=1 ou more rarely VDS=3) and therefore one might think that the swift towards b-type was merely a consequence of the higher prevalence of lower VDS stages associated with the decrease in TBT pollution. However, if we consider only females in VDS stage 1, the same increasing pattern with 37.5% of VDS 1 b-type females in 2000 and 65% in 2008 is observed. Hence, there seems to be a real change in imposex expression in this nassarid associated with lower levels of TBT pollution. The fact that the only sites presenting b-type females in 2000 where located in low polluted locations corroborates the theory that b-type females are associated with low TBT levels in the environment and therefore it is expected an increase of such females in the future due to the TBT global ban.

9.3. USEFULNESS OF *NASSARIUS RETICULATUS* AS A BIOINDICATOR OF TBT POLLUTION

The use of *N. reticulatus* as a bioindicator species was firstly proposed by Stroben and co-workers (1992) and later on by OSPAR commission to monitor TBT pollution (OSPAR, 2003). Recently an increasing number of studies around Europe elected this gastropod as the bioindicator species for monitoring spatial and temporal trends of TBT pollution (Wirzinger et al., 2007; Ruiz et al., 2008; Couceiro et al., 2009; Rato et al., 2009; Rodríguez et al., 2009). According to Barroso (2001) several characteristics accounts for its usefulness as bioindicator: (i) it is very abundant along the European coast including estuarine areas that enclose harbors as well as offshore locations; (ii) it presents a strong positive correlation between TBT body burden and the imposex indices; (iii) its moderate sensitivity to TBT enables the description of spatial gradients of pollution around point sources.

Despite all the evidences reinforcing the idea that imposex is the “best documented example of endocrine disruption in wildlife” (Matthiessen & Gibbs, 1998) several factors can affect its usage as a biomarker of TBT pollution and they need to be considered when performing monitoring surveys. Those factors include the following topics, among others: (i) particular aspects of the species biology like growth and reproduction; (ii) infestation

by parasites that can alter the endocrine system of the host; (iii) the occurrence of other EDC in the environment that can interfere with imposex expression.

Regarding the first topic, the growth, age and reproductive cycle of *N. reticulatus* were already fully characterized (Barroso & Moreira, 1998; Barroso et al., 2005a; Barroso et al., 2005b) and possible influences such as variations in penis length were carefully addressed in order to set adequate monitoring strategies as the one developed by Barroso (2001) for the Portuguese coast and followed in the present work. As for the second topic, since trematode parasites can disrupt the functioning of the endocrine system (Morley, 2006) it is reasonable to assume that the infestation by those parasites can alter the imposex expression. Several studies were performed in order to understand if parasitism had any effects on imposex development but some contradictory results were obtained: Curtis (1994) concluded that some species of trematode parasites could decrease the occurrence of imposex in *Ilyanassa obsoleta* whereas other studies point in an opposite direction, i.e., no relation between parasite infestation and imposex. Evans et al. (2000), for instance, investigated the role of parasites on imposex development in *Nucella lapillus* and concluded that there was no relation between the severity of imposex and the levels of parasitic infestation. Another study conducted in North Ireland confirmed that imposex severity in *N. lapillus* was not affected by the prevalence of parasites (Morley et al., 2003). Recently, Rato et al. (2008) studied the prevalence of digean parasitism *N. reticulatus* along the Portuguese coast and the authors didn't found any relation between imposex severity and parasite infestation, thus reinforcing the robustness of imposex in this species as a fairly specific biomarker of TBT pollution.

Regarding the third topic, several authors have proposed that some xenoestrogenic compounds such as the ones found in effluents from waste water treatment plants (WWTP) can counteract the masculinizing effect of TBT in fish (Santos et al., 2006) and gastropods (Oehlmann et al., 2000; Santos et al., 2008). In order to test the hypothesis that the presence of xenoestrogens in the aquatic environment can alter imposex in *N. reticulatus* we investigated the occurrence of such compounds in the effluents from Waste Water Treatment Plants (WWTP) located in Ria de Aveiro and also in the final

effluent discharged into the Atlantic Ocean trough S. Jacinto submarine outfall. Levels of xenoestrogenic compounds were also quantified in control seawater samples collected at the entrance of the Ria and at the seaside. Generally the levels of steroids and phenolic EDCs are low in reference sites. Of the steroid hormones estrone (E1) presented the highest levels (maximum value of 85.3 $ng.L^{-1}$), whereas the synthetic hormone EE2 was always below the detection limit. The highest levels of steroids were associated with an urban WWTP with no secondary treatment (Santiago) whereas the highest levels of phenolic compounds were detected in industrial effluents (maximum nonylphenol (NP) and bisphenol-A (BPA) values of 2410 $ng.L^{-1}$ and 897 $ng.L^{-1}$, respectively). The obtained results disclose lower levels of xenoestrogens at reference sites than the ones reported to pose risk for wildlife, however the concentrations of E1, NP and BPA in the final effluent released by S. Jacinto outfall are very high and deserve further attention.

After confirming the occurrence of steroid hormones and phenolic EDCs in the aquatic environment we performed a series of laboratory experiments in order to test the effects of xenoestrogens on imposex development of *N. reticulatus.* Females were exposed to TBT and several xenoestrogens such as E1, BPA and NP, single and in combination during 60 days. The single exposure to TBT induced imposex development in a dose dependent manner (results not shown) however results for the mixture exposures (repeated three times) were inconsistent as different responses were obtained for each experiment. For this reason the experiments were not included in this thesis, as already mentioned in Chapter 1. Such inconsistencies between exposures are probably a consequence of uncontrolled parameters during the experimental period. Such parameters can be as diverse as: different condition factors of the animals; differences in reproductive stages of the females between experiments since the three trials were performed in different times of the year (Winter, Spring and Summer); changes in water parameters (pH, Temperature, O2, salinity); and degradation of the test solutions during the experimental period. In order to better control such factors a flow-through system should be used and the concentrations in the test solutions as well as in female's soft tissues should be assessed. Therefore, if we attempt to prove that imposex might be affected by the presence of such

compounds in the environment a different approach is necessary and further experiments need to be conducted. Hence, we cannot infer about the possible influence of xenoestrogens in imposex, and the available literature (Oehlmann et al., 2000; Santos et al., 2008) is also not absolutely convincing, as their results were not confirmed by chemical analysis and/or the experiments were not repeated with females in different stages of their reproductive cycle.

Despite such possibilities, the highly significant correlations found between imposex levels and TBT concentrations in *N. reticulatus* tissues in the present thesis and previous works (see Chapter 1 and 2); the general decrease in imposex levels observed along the entire coast after the TBT ban and the fact that the decrease in imposex levels was always accompanied by a significant decrease in TBT body burdens reinforces the robustness of imposex as biomarker o TBT pollution. Nevertheless, we recommend the complementary use of imposex and chemical analysis in order to evaluate spatial and temporal distributions of TBT pollution.

9.4. Mechanisms underlying imposex development in *Nassarius reticulatus*

Several theories have been proposed to explain the imposex phenomenon, but so far the exact mechanism by which imposex develops is still elusive. Until recently the aromatase inhibition and the neuroendocrine theories were the most consistent ones but recently a new pathway was proposed - the retinoic pathway. In this work we tried to study this new hypothesis by performing a series of laboratory experiments with *N. reticulatus* females - the main bioindicator used in this thesis - and also with *Nucella lapillus*, in order to compare responses between species and provide a broader understanding of the importance of this pathway in imposex development among gastropods. Females of both species were injected with ethanol containing tributyltin (TBT) or with Fetal Bovine Serum (FBS) containing 9-*cis*-retinoic acid (9CRA: the natural ligand for humans RXRs) and imposex parameters assessed after 30 days. Significant ($p<0.05$) increases in the *vas deferens* sequence index (VDSI) and female penis length (FPL) were registered between the ethanol control and the TBT treatment and between

the FBS control and the 9CRA treatment. The obtained results confirm previous findings that imposex is mediated trough RXR signaling pathway in *N. lapillus* (Castro et al., 2007) and provide the same evidences for *N. reticulatus*. So far the involvement of RXR in imposex development has been confirmed for *Thais clavigera* (Nishikawa et al., 2004; Horiguchi et al., 2007a; Horiguchi et al., 2007b), *N. lapillus* (Castro et al., 2007) and *N. reticulatus* (Chapter 8) and further investigations are on the way with *Ilyanassa obsoleta* (Sternberg et al., 2008).

Even though the RXR is proven to be involved in the imposex induction mechanism it does not necessarily implies that this is the one pathway implicated. Most probably imposex induction in gastropods is an even more complex mechanism than previously considered with several different pathways involved. In our view, retinoic, steroid and neuroendocrine pathways are involved.

Recently, Sternberg and co-workers (in press) reviewed the available literature on the subject and proposed a tentative model for the imposex induction mechanism [Figure 9.4]. According to these authors, TBT activates RXR signaling pathway to initiate the transcription of genes necessary for male reproductive system development [Figure 9.4.a] directly, by binding to and activating RXR; or [Figure 9.4.b] indirectly, by inhibiting acyl coenzyme A:acyltransferase (AXAT), resulting in an increase in endogenous retinoid (and testosterone) levels. RXR is then activated by the endogenous free retinoid. RXR stimulates gene transcription through interaction with RXR response elements (RXRRE). Similarly, exogenously-administered testosterone [Figure 9.4.c] competitively inhibits retinoid esterification resulting in elevated free, endogenous retinoid levels that are capable of activating the RXR signaling pathway, leading to male reproductive organ development.

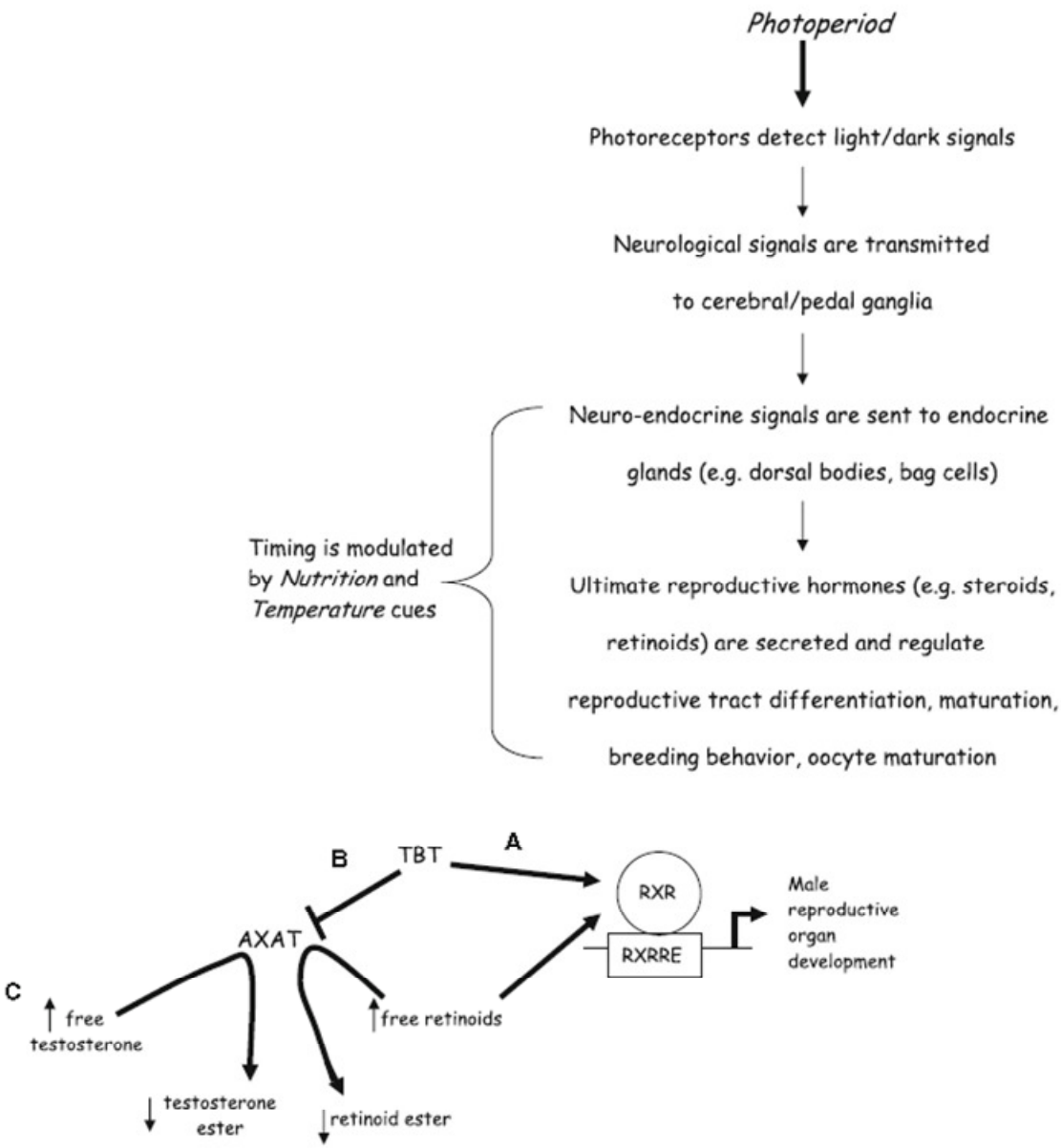

Figure 9.4. Proposed mechanism for TBT-induced imposex. RXR: retinoic X receptor; RXRRE: RXR response elements; AXAT: acyl coenzyme A:acyltransferase. Adapted from Sternberg et al. (in press).

However, several unsolved questions remain such as the clarification of exact role of steroid and neurohormones in the process and the identification of the natural ligand of RXRs in gastropods. As Sternberg et al. (in press) suggested only after all those questions solved can the TBT/imposex enigma be resolved. It remains therefore a challenge for the future the identification of all the intervenients in this bizarre and complex phenomenon,

which arguably became the best biomarker ever used to monitor a specific type of pollution. Expectations are that this global phenomenon will gradually disappear since the TBT introduction into the environment – considered as "the most toxic compound deliberately released into marine environment by man" – will ceasse.

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