Universidade de Aveiro Departamento de Biologia
2016

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Tese 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 Ricardo Beiras García-Sabell, Professor Catedrático em Ecologia da Facultade de Ciencias do Mar da Universidade de Vigo.

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Para o meu pai…

Está tudo bem… a Luísa vai casar, tento ser doutor!

o júri

presidente

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“I've nothing much to offer
There's nothing much to take
I'm an absolute beginner…”

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palavras-chave

Tributilestanho, *Imposex*, *Intersex*, Disrupção Endócrina, Biomarcador, Bioindicador, Directiva Quadro de Água, OSPAR, Estado Ecológico, Receptores Nucleares.

resumo

A Directiva Quadro da Água (DQA) foi introduzida pela União Europeia (UE) em 2000 com o objetivo de proteger as águas de superfície (interiores, de transição e costeiras) e subterrâneas. Esta ambiciosa directiva lançou novos desafios, entre os quais a necessidade de desenvolver novas ferramentas ecotoxicológicas para a avaliação do estado ecológico das massas de água. O tributilestanho (TBT), um biocida amplamente utilizado em tintas anti-incrustantes e identificado como agente causador do fenómeno de imposex / intersex em gastrópodes, está incluído na lista de substâncias prioritárias da DQA. No entanto, esta directiva não considera qualquer ferramenta de biomonitorização específica para avaliar os efeitos nocivos do TBT nos ecossistemas. O único objetivo concreto para esta substância é referido nas Normas de Qualidade Ambiental (NQA) e atingido a 0,2 ng TBT / L (NQA - Média Anual). Mais tarde, em 2008, a Directiva Quadro da Estratégia Marinha (DQEM) foi introduzida na UE com objectivos semelhantes à DQA, mas direcionada para águas marinhas. No entanto, uma vez mais, não foi proposta nenhuma ferramenta ecotoxicológica para a monitorização dos efeitos biológicos provocados por este poluente. Assim, de forma a integrar a biomonitorização da poluição por TBT ao abrigo destas directivas, esta tese tem como objetivo desenvolver e testar, sob diferentes cenários, ferramentas baseadas nos biomarcadores imposex/intersex para avaliar o estado ecológico de massas de água.

Assim, é proposto um sistema de classificação baseado nos níveis de imposex/intersex de populações de gastrópodes para avaliar o estado ecológico de águas costeiras e de transição, relativamente à poluição por TBT. Utilizando a Ria de Aveiro (NW Portugal) como estudo de caso, três bioindicadores - os gastrópodes *Nucella lapillus*, *Nassarius reticulatus* e *Littorina littorea* - foram utilizados tendo em conta o elemento de qualidade biológica para invertebrados bentónicos da DQA, de forma a classificar o estado das massas de água desta

região. O índice de sequência do vaso deferente (VDSI) e o índice de intersex (ISI) foram os parâmetros de imposex/intersex selecionados para avaliar o estado ecológico, relativamente à poluição por TBT. Os limites definidos pelos Rácios de Qualidade Ecológica (EQR) foram obtidos para cada espécie, a fim de definir as cinco classes de estado ecológico (Excelente, Bom, Razoável, Medíocre e Mau). A espécie *N. lapillus* é proposta como bioindicador chave na monitorização da poluição por TBT, no entanto, o uso combinado das outras espécies revela-se muito útil porque permite monitorizar uma área maior e uma maior diversidade de habitats. Esta ferramenta de monitorização multiespecífica demonstrou ser bastante útil para avaliar a evolução temporal do estado ecológico das massas de água da Ria de Aveiro entre 1998 e 2013, que demonstrou uma clara melhoria, alcançando um bom estado ecológico em 2013.

Este trabalho propõe também uma outra ferramenta destinada a avaliar a qualidade dos sedimentos. Esta ferramenta consiste num bioensaio em que se expõem fêmeas de *N. reticulatus* a sedimentos recolhidos no ambiente para determinar se ocorre um aumento de imposex. Este bioensaio provou ser uma ferramenta complementar e com relevância ecológica na monitorização da poluição por TBT na medida em que fornece informação sobre a fracção biodisponível de TBT nos sedimentos e permite avaliar locais onde não existem bioindicadores disponíveis.

Para testar o uso de ambas ferramentas em programas de monitorização da DQA, estas foram aplicadas em dois sistemas estuarinos - Minho e Lima - localizados no Norte de Portugal. A monitorização de imposex evidenciou um bom estado ecológico no Minho mas o estuário do Lima não conseguiu alcançar o objetivo de bom estado ecológico, apresentando locais com um estado "moderado" ou de qualidade inferior. Em conformidade, o bioensaio com sedimentos confirmou a presença de TBT biodisponível apenas nos sedimentos do estuário do Lima, já que se observou o aumento de alguns parâmetros de imposex com estes sedimentos. O uso combinado destas ferramentas permitiu aumentar a área de monitorização nestes sistemas estuarinos, uma vez que não foi possível encontrar bioindicadores em todas as estações de amostragem, sendo

resumo (cont.)

nesse caso obtidas apenas amostras de sedimento.

O critério proposto para a monitorização de imposex na DQA foi também testado ao longo da costa portuguesa de forma a avaliar a evolução do estado ecológico de 2000 a 2014. Em 2014, as fêmeas de *N. lapillus* colhidas em praias ao longo da costa exibiram baixos níveis de imposex, exceto numa estação situada na Zambujeira do Mar (SW Portugal) onde foram encontradas fêmeas estéreis. Já as fêmeas de *N. reticulatus* mostraram baixos níveis de imposex na maioria das estações amostradas, porém elevados níveis foram observados em áreas sujeitas a um intenso tráfego naval. Comparando estes valores com os observados em anos anteriores, é possível perceber que ambas as espécies recuperaram de forma significativa ao longo dos últimos 15 anos. *N. lapillus* apresentou uma redução dos níveis de imposex mais pronunciada do que *N. reticulatus*, porém esta diminuição parece ter abrandado nos últimos anos (2011-2014), prevendo-se uma estabilização por algum tempo em níveis muito baixos. *N. reticulatus* também mostrou uma diminuição gradual, mas os valores de imposex indicam uma recuperação mais lenta em portos de pesca e marinas. Isto representa uma mudança das principais fontes de poluição por TBT, já que os níveis de imposex em grandes portos comerciais, outrora locais altamente poluídos, caíram rapidamente. Assim, na costa Portuguesa, o bom estado ecológico foi alcançado na maior parte das estações localizadas em águas costeiras, no entanto uma qualidade ecológica inferior foi observada em massas de água de transição, especialmente onde estão localizados portos de pesca e marinas. A classificação da qualidade ecológica da costa portuguesa segundo a DQA (proposta apresentada nesta tese) e a OSPAR, relativamente à poluição por TBT, é analisada no presente trabalho. Esta comparação é da maior importância, pois tanto as premissas da DQA, como as ferramentas de monitorização utilizadas pelas convenções regionais marinhas (por exemplo a OSPAR) devem ser integradas na aplicação da DQEM. As classificações obtidas segundo a DQA e a OSPAR apresentam um resultado global muito semelhante para 2014, e ambas denotam uma apreciável recuperação do estado ecológico ao longo dos últimos anos. A integração da monitorização de imposex/intersex na DQEM é aqui recomendada e discutida, tendo em conta

possíveis divergências nos objetivos definidos por cada legislação e os meios para alcançá-los.

Mesmo que a utilidade das ferramentas empregadas para monitorizar a poluição por TBT tenham sido comprovadas, a monitorização de imposex/intersex a ser aplicada na DQA e DQEM só será eficaz se: (i) houver uma boa escolha de bioindicadores; (ii) o imposex/intersex constituírem uma resposta específica para a poluição por TBT porque se outros contaminantes tiverem a capacidade de induzir imposex ou intersex, estes biomarcadores perdem grande parte do seu valor operacional. Por estas razões, esta tese apresenta ainda alguns resultados que devem ser considerados para a validação da utilização do imposex/intersex na monitorização da poluição por TBT.

A Ria de Aveiro é uma área que tem sido intensamente estudada em relação à poluição por TBT e, conforme descrito anteriormente, apresentou uma importante melhoria do estado ecológico em relação a este poluente prioritário. No entanto, uma tendência contrária foi observada na evolução dos níveis de imposex no gastrópode *Peringia ulvae*. Esta espécie - considerada por vários autores como um bom bioindicador da poluição por TBT - não demonstrou uma recuperação dos níveis de imposex, apesar dos níveis de TBT e de imposex/intersex noutras espécies terem vindo consistentemente a diminuir nesta área. Em vez disso, houve um aumento global na percentagem de fêmeas afetadas por imposex e nos níveis de VDSI até 2012, sendo que os níveis de imposex em 2015 são ainda semelhantes aos encontrados em 1998. As possíveis razões para o padrão contracorrente observado nesta espécie podem estar relacionados com a interferência de outros factores que influenciam a expressão do imposex; por exemplo, a indução de imposex por parasitas - que são difíceis de controlar quando a monitorização é realizada com esta espécie - pode enviesar a interpretação dos resultados. É, portanto, necessária uma escolha criteriosa dos bioindicadores a utilizar no âmbito das directivas DQA e DQEM.

O imposex não é apenas um efeito exclusivo do TBT, uma vez que já foi descrito que o trifenilestanho (TPT) pode também causar este fenómeno em algumas espécies de gastrópodes. Aqui, é descrita, pela primeira vez, a

resumo (cont.)

indução de imposex por TPT em *N. lapillus*, a espécie que foi proposta como bioindicador chave na DQA. Curiosamente, o desenvolvimento do imposex em fêmeas de *N. lapillus* injetadas com TPT é diferente das injectadas com TBT, uma vez que as primeiras desenvolvem uma via de imposex principalmente afálica (sem desenvolvimento do pénis). Estes resultados sugerem que o TPT e o TBT podem agir de forma diferente no processo de masculinização de fêmeas de gastrópodes, lançando novas perspectivas sobre as hipotéticas vias subjacentes ao desenvolvimento de imposex.

É importante saber se existem outros contaminantes, para além dos organoestânicos, capazes de induzir (ou interferir com) o desenvolvimento de imposex ou intersex, com vista à validação das ferramentas de monitorização propostas nesta tese. Para tal, um ensaio com um gene repórter foi realizado em células transfectadas com recetores humanos, cujos ortólogos foram anteriormente identificados em gastrópodes (RXR, PPAR, ER e RAR). Sabendo que o imposex é causado pela ativação dos recetores nucleares RXR e PPARy por parte do TBT e TPT, é importante perceber se outros contaminantes são capazes de ativar estes recetores. Testaram-se vários contaminantes ambientais e mesmo que algumas substâncias tenham tido a capacidade de ativar ligeiramente o RXR e PPARy, nenhuma foi capaz de o fazer com uma potência semelhante ao TBT e TPT, sugerindo que estas são as únicas substâncias testadas capazes de induzir imposex para concentrações ambientalmente relevantes. Não obstante, todas as substâncias que foram capazes de ativar os recetores foram identificadas e o seu potencial para causar disrupção endócrina nos gastrópodes é discutido.

Em conclusão, as ferramentas propostas nesta tese podem ser extremamente úteis na monitorização do ambiente aquático ao abrigo das recentes directivas da União Europeia. Em Portugal existem três peças legislativas que se sobrepõem espacialmente nas águas costeiras (DQA, DQEM e OSPAR) e águas de transição (DQA e OSPAR), e podem diferir nos seus objetivos específicos relativamente à poluição por TBT. Logo, existe uma necessidade urgente de encontrar objetivos comuns na UE, tanto químicos e biológicos, para este poluente. Para além disto, e num momento em que a busca

de modelos biológicos não vertebrados está a aumentar em ecotoxicologia, esta tese realça o uso de gastrópodes como uma possível opção. O trabalho desenvolvido nesta tese mostra que a poluição por TBT tem vindo a diminuir em Portugal mas ainda existem alguns locais onde os níveis são altos e não atingem os objectivos definidos pela OSPAR e pela DQA (segundo a proposta aqui apresentada). As ferramentas propostas nesta tese podem ser úteis para acompanhar a evolução da situação em Portugal e na UE nos próximos anos e identificar os locais onde será necessário melhorar a qualidade ecológica das massas de água relativamente a esta substância prioritária, de acordo com as metas estabelecidas pela legislação vigente.

keywords

Tributyltin, *Imposex*, *Intersex*, Endocrine Disruption, Biomarker, Bioindicator, Water Framework Directive, OSPAR, Ecological Status, Nuclear Receptors.

abstract

The Water Framework Directive (WFD) was introduced in the European Union (EU) in 2000 with the objective to protect the EU surface waters (inland, transitional and coastal) and ground waters. This ambitious directive raised new challenges, as the need to develop quick and low-cost effect-based monitoring tools, which are increasingly being recommended to perform a proper environmental assessment under this directive. Tributyltin (TBT), a biocide largely used in antifouling paints and identified as a causative agent of imposex/intersex in gastropods, is listed as a priority substance in WFD. However, this directive does not consider any particular biomonitoring tool to assess TBT deleterious effects on the ecosystems. The only explicit objective for TBT regards the Environmental Quality Standard (EQS – Annual Average = 0.2 ng TBT/L). Later on, in 2008, the Marine Strategy Framework Directive (MSFD) was introduced in the EU with similar objectives but specifically related to marine waters that again failed to propose an effect based tool to monitor this pollutant. Therefore, to integrate TBT pollution monitoring within these legislative frameworks, it is the aim of this thesis to develop effect based tools centred on the imposex/intersex biomarkers in order to assess the ecological quality status of EU water bodies regarding TBT pollution.

This work proposes a scoring system based on imposex/intersex levels to assess the WFD ecological quality status regarding TBT pollution of transitional and coastal waters. Taking Ria de Aveiro (NW Portugal) as a case study, three bioindicators – the gastropods *Nucella lapillus* (dog-whelk), *Nassarius reticulatus* (netted-whelk) and *Littorina littorea* (periwinkle) - were used under the general WFD benthic invertebrate quality element to classify the ecological status of this area. The vas deferens sequence index (VDSI) and the intersex index (ISI) were selected as biomarkers to assess the condition of this quality element regarding the impact of TBT pollution. EQR boundaries were set for each species in order to define the five ecological status classes (High, Good,

Moderate, Poor and Bad). *N. lapillus* is proposed as a key bioindicator species, however the combined use of further species is very useful to cover a wider monitoring area and a higher diversity of habitats. This multi-species monitoring tool was useful to assess the temporal evolution of the ecological status of Ria de Aveiro water bodies between 1998 and 2013, showing that all the surveyed area improved and reached a good ecological status in 2013.

This work also proposes other imposex based tool that allows to evaluate the sediment quality, regarding TBT pollution. The use of this bioassay has proven to be a practical and ecological relevant tool as (i) it can give information for sites with no native populations of snails, (ii) it provides early identification of polluted sites anticipating future imposex levels or early identification of recovering, and (iii) it yields information on the bioavailable fraction of the TBT in the sediment. Therefore, this tool can also be of extreme usefulness under the scope of recent European legislative frameworks.

To confirm the suitability of the above mentioned tools in WFD monitoring programs, they were both applied in two estuarine systems - Minho and Lima - located in NW Portugal. The imposex field monitoring evidenced a good ecological status in Minho while the Lima estuary fail to reach the WFD good ecological status objective by presenting sites with “Moderate” and “At Best Poor” ecological status. Accordingly, the sediment bioassay confirmed the presence of bioavailable TBT only in the Lima sediments. The combined use of these tools allowed to increase the monitoring area in these estuarine systems and enhanced the robustness of the assessment, as the bioindicators fail to exist at many sites of the study area.

The proposed criteria for imposex monitoring under the WFD was tested along the Portuguese coast to assess the evolution of the ecological status from 2000 to 2014, using *N. lapillus* and *N. reticulatus* as bioindicators. Lately, the dog-whelks collected in coastal shores exhibited low imposex levels, except at one station in Zambujeira do Mar (SW Portugal) where sterile females were still found. Accordingly, the netted-whelk showed low levels of imposex at the majority of the sampled stations but high levels were still observed at areas subjected to intense naval traffic. When comparing these data regarding the most recent years with past imposex levels, it is perceived that both species recovered significantly over the

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last 15 years. *N. lapillus* presented a more pronounced decrease in imposex levels than *N. reticulatus*, however this declining trend decelerated from 2011 to 2014. *N. reticulatus* also showed a gradual decrease but the imposex values indicate a slower recovery at fishing harbours and marinas, which represents an apparent shift in the TBT hotspots as imposex levels in large commercial ports fell rapidly. Consequently, in the Portuguese coast a good ecological status was achieved in most of the stations located in coastal waters while a worse ecological quality was observed in transitional water bodies where fishing ports and marinas are located. A comparison between the proposed WFD imposex tool and the OSPAR Assessment Criteria for imposex shows that both classifications schemes present a general similar result for 2014 and point to a clear recovery of the quality status over the years. This comparison between two legislations is of foremost importance since both WFD premises and Regional Sea Conventions (e.g OSPAR) knowledge and monitoring tools should be integrated by member states on the application of the MSFD. Therefore the imposex/intersex monitoring integration within MSFD is discussed taking into account possible divergences between the objectives defined by each piece of legislation and the means to achieve it.

Even if the usefulness of the employed tools to monitor TBT pollution has been proved, the WFD's monitoring proposal will only be effective if: i) there is a good choice of the bioindicators species; and ii) imposex/intersex are specific responses to TBT pollution, i.e., if other contaminants induce imposex or intersex, then these biomarkers lose much of their operational value. For that reason this thesis presents novel information regarding these aspects in order to validate this methodology and better interpret the obtained results.

Ria de Aveiro is an area that has been intensively studied regarding TBT pollution and, as previously reported, presented a major improvement of the ecological status regarding this priority pollutant. An opposite trend has been however observed in the imposex evolution in the gastropod *Peringia ulvae*. This species - regarded by some authors as a good bioindicator of TBT pollution - did not show an imposex recovery despite the fact that TBT levels have been consistently decreasing in this area. Instead, there was a global increase in the

percentage of females affected by imposex and VDSI levels until 2012, and the imposex levels in 2015 were similar to those found in 1998. Possible reasons for this counter current pattern could be related with other factors that can influence imposex expression, such as parasitism, that probably cannot be totally controlled when monitoring is performed with this species. Hence, there is the need to carefully choose the right bioindicators to implement TBT monitoring assessments under WFD or MSFD, and *P. ulvae* is not recommended for this purpose.

Imposex is not an exclusive response to TBT since triphenyltin (TPT), a related organotin, is already known to cause the same phenomenon in some gastropod species. This thesis describes, for the first time, that TPT also induces the development of imposex in *N. lapillus*, the species considered the key bioindicator in the WFD monitoring proposal. However, imposex development in TPT-injected females followed mostly an aphallic route (no penis development). These results suggest that TPT and TBT may act differently in the sequential process of female masculinization casting new insights about the hypothetical pathways underlying imposex development. Besides TBT and TPT, it is important to know if other environmental contaminants can induce or interfere with the imposex/intersex development to ensure that the proposed tools are specific to organotin pollution. With this purpose, a reporter gene assay was performed using the GAL4/UAS system in cells transfected with human receptors, which were already identified in gastropods: Retinoid X Receptor (RXR), Peroxisome Proliferator-Activated Receptor gamma (PPARγ), Retinoic Acid receptor (RAR) and Estrogenic Receptor (ER). It has been reported in the literature that imposex is caused by the activation of the nuclear receptors RXR and/or PPARγ by TBT or TPT, and therefore if other contaminants can bind these receptors they could have the potential to induce imposex/intersex as well. Even if there were other substances that slightly activated RXR and PPARγ, none was able to do it with similar potency than the organotins (TBT and TPT), suggesting so far that these are the only tested substances capable to induce imposex at realistic environmental concentrations. Nevertheless, all substances that were able to activate the reporter gene were identified and their potential to cause endocrine disruption in gastropods is discussed.

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Concluding, the tools proposed in this thesis can be of extreme utility to monitor the aquatic environment under the frame of the recent EU directives. Moreover, in Portugal there are three legislative pieces that are spatially overlapped regarding coastal waters (WFD, MSFD and OSPAR) and transitional waters (WFD and OSPAR), and they may differ in their specific objectives regarding TBT pollution. Therefore there is a need to find common EU objectives, both chemical and biological, for this pollutant. Furthermore, in a time where the search of non-vertebrate biological models in ecotoxicology is increasing, this thesis enhances the use of gastropods as an option. Also the work here developed shows that TBT pollution has been decreasing in Portugal but there are some sites where levels are still high. In this scenario, the tools here proposed can be useful to track the evolution of TBT pollution during the next years and to identify waters bodies that will require an action to improve their ecological status.

“How many roads must a man walk down
Before you can call him a man?”

Bob Dylan

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CHAPTER 1 - INTRODUCTION

CHAPTER 1- GENERAL INTRODUCTION

"Don't it always seem to go
That you don't know what you've got
'Till it's gone
They paved paradise
And put up a parking lot"

Joni Mitchell, 1970

The impact of humans on planet Earth have been so extensive that it is now suggested that we live in an geological epoch driven by human pressures, the Anthropocene (Crutzen, 2002). Environmental pollution arose mainly with the industrial revolution advent in 18th/19th century. The first reports of deleterious effects on wildlife, caused by metal pollution, came by that time but it was only in the first decades of the 20th century, with the generalized use of organic chemicals, that environmental pollution became an worldwide issue (Rattner, 2009). The publication of Rachel Carson's *Silent Spring* (1962) reflected the scientific community concerns of that time, reporting the indiscriminating use of pesticides and their effects to non-target organisms. This book allowed environmental pollution to gain visibility to a wider audience, from governments to industry, as well as to the general society, changing consequently the paradigms in pollution monitoring. Hence, the constant evolution of the chemical industry raises new challenges for scientific community and governments that are requested to give adequate solutions to pollution events.

Taking this into account, the present thesis intends to shows innovative work regarding the environmental monitoring of tributyltin (TBT) under the new European Union (EU) directives, namely Water Framework Directive (WFD) and Marine Strategy Framework Directive (MSFD). TBT is an historical case of a biocide which was thought to be non-toxic to non-target organisms but was rapidly considered by Goldberg (1986) as the most toxic compound ever deliberately released into the environment. TBT is known to be the causative agent of imposex (imposition of male sexual characters onto the reproductive

tract of gastropod females) and intersex (a related phenomenon that occurs in gastropods) (see section 1.2.1). These are among the best examples of specific and dose-dependent biomarkers that have been use in pollution biomonitoring and therefore this thesis proposes the use of imposex/intersex as a tool to access the ecological quality status of EU water bodies under the WFD and MSFD.

1.1. Organotin compounds: uses and adverse effects

Organotins (OTs) are chemical compounds defined by an Sn atom covalently bound to organic substituents as denoted in the general formula $R_n\mathrm{Sn}X_{4-n}$, where *R* represents an alkyl or aryl group (e.g. methyl, butyl, phenyl), *X* the anionic species (e.g. oxide, hydroxide) and *n* can assume a positive numerical number up to 4 (Hoch, 2001; de Carvalho Oliveira and Santelli, 2010). It is the number of bonds between Sn and organic substituents, as well as their length, which are responsible for the OTs chemical and physical properties. For example, water solubility is affected by the number and length of organic substitutes decreasing in more complex forms. Moreover, these substances are stable in the presence of water, atmospheric oxygen and heat but rapidly cleave in the presence of UV radiation, strong acids and electrophilic agents (Hoch, 2001). All OTs are of anthropogenic origin except methyltins that can also be produced by environmental biomethylation (Fent, 1996). OTs were firstly synthetized in 19th century, however a large scale production only began after 1940's when the first economical applications for these substances started to appear. Some OTs compounds were, and still are, widely used in the plastic industry, predominantly in PVC industry as stabilizers but also as catalysts in the production of polyurethane foams and silicone. Later in the 50’s, the discover of biocidal properties of the tri-substituted OT compounds prompted its extensive use in antifouling (AF) paints formulation, specially Tributyltin (TBT) and Triphenyltin (TPT) (Sousa et al., 2014). Beside the referred applications, OTs are also widely used with less prominence in diverse industrial areas as seen in Table 1.1.

Table 1.1 - Organotin compounds applications, adapted from Sousa et al. (2014).

Organotin classes	Applications	References
Monosubstituted	Stablizers in PVC films	WHO (1990)
	Glass treatment	ATSDR (2005)
Disubstituted	Stabilizers in plastics industry (particularly PVC)	WHO (1990)
	Catalysts in the production of polyurethane foams and in room temperature vulcanization of silicones	Hoch (2001)
	Glass treatment processes as precursors for SnO2 film	de Carvalho Oliveira and Santelli (2010)
	Dewormers in poultry farming	Antizar-Ladislao (2008)
	Water-proofing agents for cellulosic materials (e.g., cotton textiles, paper and wood)	
	Flame retardants for wool fabrics	
	Binder in water-based varnishes	
Trisubstituted	Biocides in antifouling paint formulations	WHO (1990)
	Fungicides, insecticides, miticides, and antifeedants in agrochemical industry	Hoch (2001)
	Pesticides for ornamental plants	RPA (2005)
	Miticides in citrus fruits	
	Acaricides in vineyards	
	Insecticides and fungicides in wood preservation	
	Biocides in construction materials	
	Disinfectants and biocides for cooling systems in power stations, pulp and paper mills, textile mills, breweries, tanneries	
	Insecticides and antifeedants in textiles	
	Biocides in allergic pillows	
	Biocides in insoles for shoes	
	Biocides in cycling shorts padding	
	Biocides in sprays for athlete's foot treatment	
Tetrasubstituted	Intermediates in the preparation of other organotin compounds	WHO (1990)
	Oil stabilizers	de Carvalho Oliveira and Santelli (2010)

Inorganic tin is considered to be a non-toxic metal but, on the other hand, organic forms of this metal can cause severe toxicological effects. Again, the nature and number of organic substitutes influences the toxicity of these compounds. The tri-substituted ones, in general, have shown to be the most hazardous towards organisms at very low concentrations (Fent 1996; Hoch 2001). For example, TBT has effects on the growth of several bivalve species, like *Crassostrea gigas* (LOEC=5 ng TBT/L) and *Saccostrea commercialis* (LOEC=5 ng TBT/L) (Nell and Chvojka 1992), but also in phyto- and zooplankton (1 ng TBT/L) (Alzieu, 2000a). Significant TBT effects are also observed on the larvae development of the crustacean *Acartia tonsa* (EC_{10}= 0.7 ng TBT/L) (Kusk and Petersen, 1997). The high toxicity of this compound to non-target organisms was primarily perceived when shell malformations in oysters were related with TBT pollution in Arcachon bay, France (Alzieu et al., 1986). It was shown that TBT caused the thickening of the shell, which reduced significantly the body meat, leading to extensive economic losses in this area (Alzieu, 2000a). By that time, first evidences pointed out that imposex phenomenon was also related with TBT pollution (Smith, 1981c; Smith, 1981a). Therefore due to these deleterious effects, first restrictions on TBT use were applied (described in chapter 1.1.1). Also, hazardous effects of TPT in environment, including imposex, were widely described with some works pointing out a comparable toxicity with TBT (Fent and Meier, 1994; Schulte-Oehlmann et al., 2000). An extended review of organotin's deleterious effects in wildlife, and specially TBT, can be found in several scientific reviews (Alzieu, 2000b; Hoch, 2001; Antizar-Ladislao, 2008). More recently, large attention has been given to the effects of these compounds in humans, since several studies reported adverse reproductive and metabolic effects in mammals, as well as their capacity to accumulate in human tissues (Graceli et al., 2013; Sousa et al., 2014). Moreover, it was found that some OTs are potent disruptors of human nuclear receptors (Nishikawa et al., 2004; Grün and Blumberg, 2006) (to be described afterwards).

1.1.1. Regulatory actions on organotins

Regulatory actions on TBT were firstly adopted in France in 1982 after shell thickening effects observed in oysters being related with TBT pollution, as previously

referred. A partial ban was implemented, forbidding the application of antifouling paints containing more than 3% of TBT in boats smaller than 25m (Alzieu, 1998; Champ, 2000). Similar measures were subsequently adopted during the 80's in other countries as United Kingdom, United States of America, Australia and New Zealand, being followed in the 90´s decade by Japan, Austria and Switzerland (Alzieu, 1998; Champ, 2000). In line with this trend, in 1989 the European Commission introduced the directive (89/677/CEE) that forbad the application of TBT and TPT based antifouling paints in vessels smaller than 25m and other aquatic structures, full or partially submerged, such as cages or nets. This directive was transposed into Portuguese legislation in 1993 (Decreto Lei 54/93). Despite these efforts, and the fact that some studies reported a recovery in oyster farming, as well as a decrease in imposex levels and TBT environmental concentrations (Evans et al., 1995; Evans et al., 1996; Harding et al., 1997; Alzieu, 2000a), it was found that the partial ban was globally ineffective, with high imposex levels still being registered at sites characterized by the presence of larger vessels (>25m), like commercial and fishing ports (Minchin et al., 1995; Morgan et al., 1998; Barroso et al., 2002b; Santos et al., 2002a). Therefore, in 2001 the International Maritime Organization (IMO) through the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention) banned the application of paints containing TBT and TPT in all vessels. This convention only entered into force in September of 2008 after 25 states, that represented not less than 25% of the gross tonnage of the world's merchant shipping, signed it. Foreseeing some delays in this process, in 2003 the EU, through the Directive 2002/62/EC and Regulation 782/2003, also adopted a total ban of OTs based antifouling paints. Moreover, from January of 2008 all boats sailing in EU waters could not use any paint containing these compounds in their hulls. These restrictions were also applied to non-European vessels entering EU harbours. In addition, organotin compounds were also banned from consumer products in EU by the directive 2009/425/EC, and are regulated under REACH (Regulation 1907/2006) (Sousa et al., 2014).

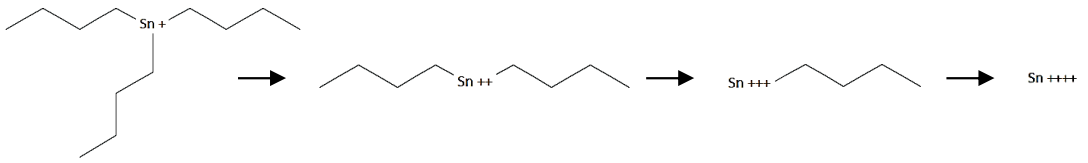
1.1.2. Tributyltin

Tributyltin (TBT) is at the present time the most infamous compound of all OTs, reflected by an extensive scientific bibliography available on its toxicity and deleterious effects. Since its biocide properties were found around 1950, TBT started to be used as an active ingredient in AF paints. These paints were used in vessels, or other aquatic structures, to prevent unwanted organisms incrustation obtaining obvious economic benefits from its use (Yebra et al., 2004). It is estimated that without the application of AF paints, a vessel hull may accumulate 150 $kg.m^{-2}$ of incrusted organisms at sea (IMO, 1999). Consequently, fuel consumption and time at dry dock (for organisms removal) will increase with consequent economic losses. Due to its efficiency, it was estimated that at some point during the last century 70% of the world fleet used TBT-based antifouling paints, which eventually contributed for a worldwide dispersal of this compound in the aquatic environment (Yebra et al., 2004). In addition, TBT was also used as a fungicide or as a wood preserver (Antizar-Ladislao, 2008).

TBT is characterized by 3 butyl groups covalently bound to a tin atom, as represented by the formula $(n\text{-}C_4H_9)_3Sn\text{-}X$, where X can be an anion or an anionic species, such as chloride or hydride (Yebra et al., 2004). Actually, chemical speciation is of extreme importance when describing TBT effects as it provide vital information for the toxicity and bioavailability of organotin compounds. In aquatic environment, TBT can be found in its ionic form (TBT^+) or in equilibrium (TBT-OH, TBT-Cl, etc.) being this distribution highly influenced by physicochemical factors. For instance TBT^+ is highly stable for pH values below the acid constant pK_a (6.51) (Alzieu, 1998; de Carvalho Oliveira and Santelli, 2010). The uptake of TBT by organisms will rise if dissolved organotin species are uncharged. Thus, TBTOH and TBTCl forms increase bioavailability of TBT. In seawater, under natural conditions of pH and salinity, TBT is usually found in the TBTOH form (Fent, 1996; Alzieu, 1998). In sediments, bioavailability depends of several parameters, such as pH and organic matter content. For example, it increases at a neutral or slightly basic pH or at low levels of organic matter. Once released into the environment, TBT is absorbed by bacteria, algae or suspended organic matter in the water column (Burton et al., 2004). Consequently, TBT is quickly integrated in filter-feeding or grazer organisms and reaches superior predators as

fishes, birds or mammals throughout the food chain (Takahashi et al., 1999b; Antizar-Ladislao, 2008), though they may also accumulate TBT through direct exposure to water and/or sediments, depending on the species. TBT can also accumulate in humans trough the ingestion of contaminated seafood (Takahashi et al., 1999a).

Persistence of TBT in environment is regulated by degradation processes. This degradation occurs due a progressive loss of butyl groups as shown below (Hoch, 2001).



Tributyltin (TBT) Bu_3Sn^+ | **Dibutyltin (DBT)** Bu_2Sn^{2+} | **Monobutyltin (MBT)** $BuSn^{3+}$ | **Tin (IV) cation** Sn^{4+}

Figure 1.1 - Tributyltin degradation process and the consequent formation of DBT, MBT and finally the tin (Sn) (IV) cation. Butyl represented by Bu.

Natural degradation of TBT produces Dibutyltin (DBT), Monobutyltin (MBT) and inorganic tin that are less toxic than TBT, being therefore an essential process in TBT remediation (Fent, 1996). TBT half-lives in water generally range from a few days to a few weeks while in sediment the degradation rates are lower, with estimated half-lives from days to decades (Dowson et al., 1996; Hoch, 2001). The predicted environmental TBT half-lives, which are reported in the literature, are mainly attained from laboratory experiments, and therefore should not be comparable to real environmental scenarios where breakdown rates depend on numerous biogeochemical factors. Thus, TBT persistence in natural sediments can be often underestimated and half-lives of TBT, estimated from in situ measurements, can reach more than 2 decades in anoxic sediments (normally in deeper sediments) (Dowson et al., 1993). Due to TBT low degradation rates and high persistence in sediments, this compartment appears to act as a sink for TBT representing a permanent threat for water contamination via desorption and remobilization processes or directly to benthic organisms trough the contact or particles ingestion (Hoch, 2001). This is one of the main reasons why TBT can still be an environmental issue even after having been banned globally.

1.2. Organotin compounds as endocrine disruptors

Increasing concerns among the scientific community have emerged during the second half of the last century on substances, or their degradation products, able to modulate or disrupt the endocrine system in organisms (Tyler et al., 1998). These manmade chemicals, defined as endocrine disrupting compounds (EDCs), can mimic, enhance (agonists) or inhibit (antagonists) the action of hormones at particularly low concentrations causing endocrine disruption effects (Colborn et al., 1993). The publication of Theo Colborn's *Our stolen future* brought to public discussion a major concern on chemicals and the necessity to screen their endocrine-disruption activity. Several definitions exist to describe an EDC, and here we transcribe the United States Environmental Protection Agency (EPA) definition:

"An endocrine disruptor is an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process."

The observation of endocrine disruption effects in wildlife are widely documented in scientific literature: I) the estrogenic, androgenic, antiandrogenic and antithyroid actions detected in fishes downstream of sewages or pulp and paper mills (Jobling and Tyler, 2003), II) the thinning of the eggshell in birds induced by DDT (Giesy et al., 2003) or III) alterations in steroidogenesis, hormone levels, and alterations in the morphology of endocrine organs (gonad, thyroid) of juvenile alligators when exposed to agricultural chemicals (Guillette and Iguchi, 2003). Still, the present work focuses over another classic endocrine disruption phenomena observed in wildlife, the imposex/intersex.

1.2.1. Imposex/intersex

Defined by Smith (1971), imposex is the superimposition of male characters onto gastropod females. This phenomenon was first described in the United Kingdom for *Nucella*

lapillus (Blaber, 1970). One year later, in the United States of America, the same phenomenon was reported in *Nassarius obsoletus* (Smith, 1971). One decade later, the same author related the phenomenon with antifouling paints containing organotin compounds (Smith, 1981a,b). Subsequently, other works confirmed the ability of TBT to induce imposex (Bryan et al., 1987; Gibbs et al., 1988). This phenomenon became an ecological issue as the most severe stages of imposex cause sterility in gastropod females leading to a consequent population decline or extinction (Bryan et al., 1986). Since then, imposex was reported in more than 260 species of gastropods (Titley-O'Neal et al., 2011) and it is now widely accepted as one of the best biomarkers in environmental pollution monitoring, since it is a highly specific response to TBT pollution (Axiak et al., 2003; Forbes et al., 2006), and, for some species, to TPT pollution as well. Moreover, the highly significant correlations found between imposex and TBT levels in tissues confirm imposex as an effective and low cost tool in environmental monitoring (Gibbs et al., 1987; Stroben et al., 1992a). *Nucella lapillus* was the first species proposed as a bioindicator of TBT pollution, with a specific scoring system being developed to classify imposex stages based on female morphological features that are developed after exposure to a given level of TBT (Gibbs et al., 1987). Afterwards, more species were proposed as bioindicators of TBT pollution with specific imposex scoring systems, since the morphological undergone changes may differ. In Europe, *Nassarius reticulatus* (Stroben et al., 1992b; Barroso et al., 2002a), *Peringia ulvae* (Schulte-Oehlmann et al., 1997) and *Buccinum undatum* (Ten Hallers-Tjabbes et al., 1994) are used as common bioindicators. Other commonly used bioindicator is *Littorina littorea*, however this species expresses a different response, yet caused by TBT pollution, named intersex (Bauer et al., 1995; Bauer et al., 1997). Intersex is defined as the disturbance of the phenotypic sex determination between gonad and genital tract which consists on malformations of the pallial genital tract that at advanced stages might inhibit a successful copulation and capsule formation (Bauer et al., 1997).

1.2.2. Mechanisms of imposex induction

After the imposex/intersex phenomenon emerged as an ecological issue, several hypotheses started to appear in order to explain how TBT or TPT could trigger this masculinisation phenomenon. Féral and Le Gall (1983) were the first to come up with an hypothesis where imposex could be associated with the action of neuroendocrine factors. They suggest that TBT inhibits the release of the Penis Regression Factor (PRF) that in unexposed females prevents the segregation of the Penis Morphogenic Factor (PMF), a substance responsible for the penis growth. Later on, it was hypothesized that neuropeptides, that control sexual differentiation in molluscs, could have the ability to induce imposex in gastropods (Oberdörster and McClellan-Green, 2000; Oberdörster and McClellan-Green, 2002) because it was found that the neuropeptide APGWamide was able to induce imposex and could be in fact the PMF, referred by Féral & LeGall (1983), released by neurotoxic action of TBT. Still, Horiguchi (2009) defended that laboratory results obtained with APGWamide injection retrieve much lower imposex incidence and penis growth than gastropod laboratory exposures to TBT or TPT, while Santos et al. (2006) showed that the injection of APGWamide in the gastropod *Bolinus brandaris* had no effect on imposex development.

Alternatively, it was also proposed that vertebrate-type steroids could be involved on imposex induction. Laboratory experiments demonstrated that the testosterone levels or testosterone/17-β estradiol ratio increased in gastropods after exposure to TBT (Spooner et al., 1991; Bettin et al., 1996). In these experiments, it was suggested that TBT has the capacity to inhibit cytochrome P450-dependent aromatase preventing the conversion of testosterone into 17-β-estradiol, and thus raising the levels of the androgen. This hypothesis was further supported by the fact that an aromatase inhibitor was able to induce imposex (Bettin et al., 1996) as well as the evidences of low aromatase activity in imposex affected gastropods (Santos et al., 2002b). Nevertheless, Santos et al. (2005) later considered that this mechanism would not be the primary pathway for imposex development. Another hypothesis raised by Ronis and Mason (1996) suggests that TBT inhibits the conjugation of testosterone with water soluble sulphur preventing its metabolization and consequently increasing free testosterone levels in the tissues and

preventing its normal excretion. However, this experiment was performed with extremely high TBT concentrations, which are not environmentally relevant (Horiguchi, 2009).

Even if all of these hypotheses might be to some extent involved in the imposex development, it seems now clear that there is one further hypothesis that may claim more attention, which imply nuclear receptors activation. Nishikawa et al. (2004) have shown that TBT and TPT efficiently bind the retinoid X receptor (RXR), and showed also that 9-cis retinoic acid, a RXR ligand, induces imposex in the gastropod species *Reishia clavigera*. Imposex induction by this ligand was also observed in other gastropod species, including *Nucella lapillus* (Castro et al., 2007; Sousa et al., 2010) confirming the strength of this hypothesis. It was also demonstrated that TBT activates RXR-PPARγ heterodimer (le Maire et al., 2009) and that rosiglitazone, a ligand for PPARγ, induces imposex in *Nucella lapillus* (Pascoal et al., 2013). Therefore, it seems evident that organotins interact with the nuclear receptors of gastropods, activating mechanisms that will regulate the sexual development in these organisms. Despite the latest advances in this area, there is still some lack of knowledge regarding the retinoid signaling pathway in gastropods (André et al., 2014; Sternberg et al., 2010) as well as possible interactions between the different imposex induction mechanisms proposed (neuroendocrine, steroid and retinoid).

1.2.3. Other endocrine disruption effects

Organotins were also identified as obesogenic compounds, which can be defined as chemicals that can have an influence on obesity development by inappropriately alter lipid homeostasis to promote adipogenesis and lipid accumulation (Grün and Blumberg, 2006; Grün and Blumberg, 2007; Grün and Blumberg, 2009a). This is caused by the activation of the nuclear receptor PPARγ which has a crucial role on the homeostatic control over energy, lipid and glucose metabolism (Grün and Blumberg, 2007). Moreover, when heterodimerized with RXR can act as a metabolic sensor that regulate adipocyte function, number and size, which can also be disrupted by organotins (Grün and Blumberg, 2009b). This obesogen action was already demonstrated in vertebrate models, as frogs and mice (Grün et al., 2006), but also in salmonids, snails and crustaceans (Janer et al., 2007; Meador et al., 2011; Jordão et al., 2015).

1.2.4. Imposex monitoring in Portugal

In Portugal, imposex monitoring was first performed in the late 80's in the north coast of the country (Peña et al., 1988) and in Azores (Spence et al., 1990). Then in the 90's, it was also assessed in some estuaries (Gibbs et al., 1997; Barroso et al., 2000; Pessoa et al., 2001) and then monitored along the country shoreline (Santos et al., 2000). During the first decade of this century the monitoring became more regular in estuaries (Vasconcelos et al., 2006; Sousa et al., 2007; Galante-Oliveira et al., 2009), along the coastal areas (Sousa et al., 2009; Galante-Oliveira et al., 2011) and also in offshore waters (Santos et al., 2004; Rato et al., 2008). Generally, the first studies evidenced the occurrence of very high levels of imposex/intersex as well as female sterility (Spence et al., 1990; Barroso et al., 2000; Santos et al., 2000). The inefficacy of the Directive 89/677/EEC (TBT/TPT ban on small boats) to reduce pollution was shown by Santos et al. (2000) and Barroso and Moreira (2002), while more recent studies registered a recovery in both imposex incidence and severity, as well as a gradual decrease of TBT environmental levels, as a consequence of the implementation of the Regulation (EC) 782/2003 that prohibited the application of organotin AF-paints on all vessels (Sousa et al., 2009; Vasconcelos et al., 2010; Galante-Oliveira et al., 2011).

1.3. Biomonitoring organotin pollution

Biomonitoring tools have been widely used to assess environmental pollution levels since the past century (Chapman and Long, 1983; Matthiessen et al., 1998; Beiras et al., 2003; Bellas et al., 2008). However in Europe, recent legislation on aquatic environmental quality (namely the Directives WFD and MSFD), reinforced the necessity to develop effective and low cost effect-based tools to be used in monitoring programs that would link the chemical and ecological status (Wernersson et al., 2015). Among those tools, biomarkers have been regarded as ecological relevant and early warning instruments directly measured from *in situ* organisms (Cajaraville et al., 2000; Forbes et al., 2006; Wernersson et al., 2015). A biomarker can be defined as a change in the biological status

of an organism related to contaminants exposure in the environment. This change can occur at a cellular, molecular or physiological level and can be perceived by the analysis of cell, tissues or even organs (Van der Oost et al., 2003). Biomarkers can be divided in three categories: biomarkers of exposure, of effect and of susceptibility (Council, 1987). However their definitions can easily overlap and they should be described based on the purpose of the study, since a biological effect occurs after the exposure to a pollutant (Van der Oost et al., 2003). Understanding the necessities imposed by the EU legislation, this work focus on the use of imposex/intersex as valuable biomarkers for pollution monitoring.

1.3.1. Imposex/intersex as biomonitoring tools

Imposex is considered one of the most reliable biomarkers as it reflects a dose-dependent and contaminant-specific effect (Axiak et al., 2003; Forbes et al., 2006), therefore it has been used worldwide to efficiently monitor organotin pollution (Gibson and Wilson, 2003; Bigatti et al., 2009; Castro et al., 2012; Lopes-dos-Santos et al., 2014). Several parameters are employed to assess the intensity of imposex in a given gastropod population, namely: i) percentage of imposex affected females (%I); (ii) percentage of sterile females (%STER); iii) mean female penis length (FPL); iv) relative penis length (RPL) (FPL × 100 / mean male penis length); v) relative penis size (RPSI) (female penis length^3 × 100 /male penis length^3) and vi) vas deferens sequence index (VDSI). This last parameter consists of a scoring system that measures the development of the vas deferens in females. This index is probably the most ecologically relevant since it gives information about the reproductive capacity of a given population. Since vas deferens development generally differs between species, this scoring system will be later described for each bioindicator that is used in the current work. The average oviduct stage (AOS) can be also used as proposed by Barreiro and co-workers (2001a) to measure the degree of oviduct convolution in *Nassarius reticulatus*, also representing a sign of masculinisation. Finally, the intersex index (ISI) is a scoring system based on a different yet imposex-related response - the intersex - developed for *Littorina littorea*.

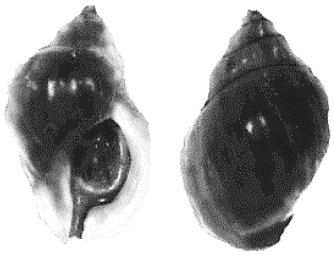
The following paragraphs describe the biology of the gastropod species taken as bioindicators for the present thesis and the VDS/ISI scoring scheme used to assess the levels of imposex/intersex in each case.

***Nucella lapillus* Linnaeus, 1758**

Commonly named dog-whelk, *N. lapillus* (Fig. 1.2) is distributed along the rocky shores of both east and west coasts of the North Atlantic: throughout Europe, from the Arctic region to the southern limit, Portugal, and from Greenland to the north of United States of America (Crothers, 1985; Tam and Scrosati, 2011). This species is characterized by a round shell that is highly variable in colour (white, brown, grey) and in size or shape mainly due to the degree of exposure to the hydrodynamic action (Crothers, 1985). Usually these organisms can be found living gregarious next to the mussels and barnacles, on which they feed. Reproduction and breeding generally occurs preferentially during Spring in Ria de Aveiro (NW Portugal) (Galante-Oliveira et al., 2010), but it can vary from one location to another, nevertheless it is possible to observe fully mature adults, as well as egg capsules deposition, throughout the year (Gibbs, 1999; Galante-Oliveira et al., 2010). The number of egg capsules laid by each female may vary due to environmental factors and a capsule may contain up to 600 eggs, though only 25-30 emerge as juveniles (Graham, 1988). According to Galante-Oliveira et al. (2011), in Portugal the organisms achieve the adult stage (sexual maturity) with a shell height of around 17.5 mm, but they can grow twice this height.

This was the first species proposed as a bioindicator of TBT pollution (Gibbs et al., 1987) and is considered one of the most sensitive and, therefore one the most used species in biomonitoring TBT programs in Europe (Stroben et al., 1992a; Huet et al., 1995; Barroso et al., 2000). The imposex VDS scoring system used for *N. lapillus* in this thesis was developed by Gibbs et al. (1987), as seen in figure 1.2, comprehending 7 different stages, from 0 – normal female, to 6 – sterile female presenting aborted egg capsules inside the capsule gland. The imposex phenomenon in this species is initiated with the appearance of the vas deferens near the vulva (VDS = 1), followed by the appearance of a penis primordium behind the tentacle (VDS = 2). Both of these structures develop (VDS = 3) until

the vas deferens becomes complete, linking the base of the penis to the vulva at VDS = 4. At VDS = 5 the continuous growth of vas deferens will block the vulva making the female sterile. The final VDS of this scoring system is characterized by the already mentioned presence of aborted eggs (VDS=6).



Phylum: Mollusca
Class: Gastropoda
Subclass: Caenogastropoda
Order: Neogastropoda
Family: Muricidae
Genus: *Nucella*
Species: *Nucella lapillus*

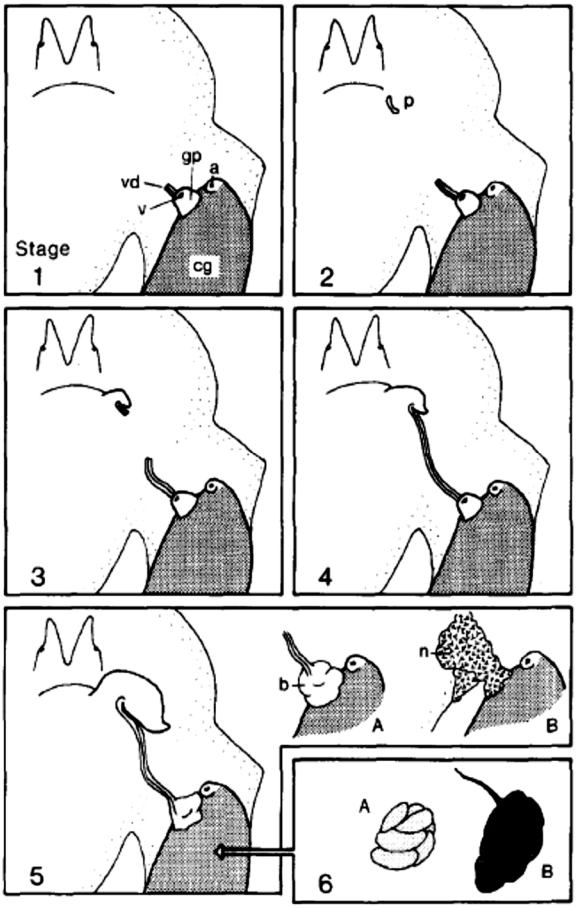


Figure 1.2 - *Nucella lapillus* illustration and its systematics. It is also represented the imposex development in *Nucella lapillus* corresponding to the VDS scoring system proposed by Gibbs et al., (1987) as explained in the text. Adapted from Gibbs et al. (1987). Drawing of the shells adapted from Galante-Oliveira et al. (2013). Abbreviations: a – anus; b – blister; gp – genital papilla; n – nodule; p – penis; v – vulva; vd – vas deferens.

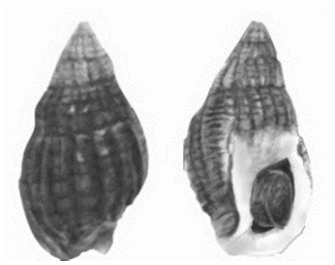
Nassarius reticulatus **Linnaeus, 1758**

The netted whelk *Nassarius reticulatus* has a wide range of distribution in Europe, from Norway to Azores and Canary Islands, being also found in Mediterranean and Black Sea (Graham, 1988). This species is very abundant along the Portuguese coast, being found in the coastal and offshore areas but also inside estuaries (Rato et al., 2006). The shell is a sharply pointed cone with a reticulate surface, presenting an oval aperture with internal

teeth in adults that can reach more than 30 mm height. This species is a scavenger, feeding essentially on dead organisms. In Ria de Aveiro (NW Portugal) reproduction may occur during Autumn/Winter with spawning occurring from February to July through capsule deposition (Barroso and Moreira, 1998). The netted whelk has an indirect development with a veliger larvae being formed inside a capsule that can have 50-350 eggs. These capsules can be normally found attached to shells, rocks or algae. After hatching, veliger larvae start a planktonic stage that lasts 1-2 months (Fretter and Graham, 1994).

N. reticulatus, as a bottom dwelling organism, is recommended as a good bioindicator species to evaluate TBT pollution in sediment (Barroso et al., 2000; Duft et al., 2007). The VDS scoring system (Fig. 1.3) used in this thesis for this species was proposed by Stroben et al. (1992b) and consists of 6 stages ranging from 0 (normal female) to 4+ (female where the vas deferens passes the vaginal opening). Unlike the previous species, the penis is the first morphological structure to be developed (VDS = 1) being followed for the penis duct (VDS = 2) and the appearance of the vas deferens (VDS = 3) growing from the basis of the penis to vaginal opening. The VDS = 4 is characterized by the continuous development of the vas deferens until the vaginal opening. If the vas deferens development passes this opening and continues up to the capsule gland, this corresponds to VDS = 4+. This is not considered another VDS since it does not imply the female sterility, with all pallial oviduct glands being functional as well as the copulation being conserved (Stroben et al., 1992b). However, Barroso et al. (2002a) suggested that this last VDS (4+) should be computed to the numerical value of 5 for a better discrimination between sites, and also because this stage is associated with the upsurge of sterile females. Unlike *N. lapillus*, there are no specific VDS classifying female sterility in this species, still this phenomenon can occur and is normally characterized by the vulva obstruction or the presence of aborted egg capsules inside the capsule gland (Barreiro et al., 2001b; Barroso et al., 2002a). The occurrence of

aphalic imposex development is also documented and it is classified as a *b-type* vas deferens development (Stroben et al., 1992b).



Phylum: Mollusca
Class: Gastropoda
Subclass: Caenogastropoda
Order: Neogastropoda
Family: Nassariidae
Genus: *Nassarius*
Species: *Nassarius reticulatus*

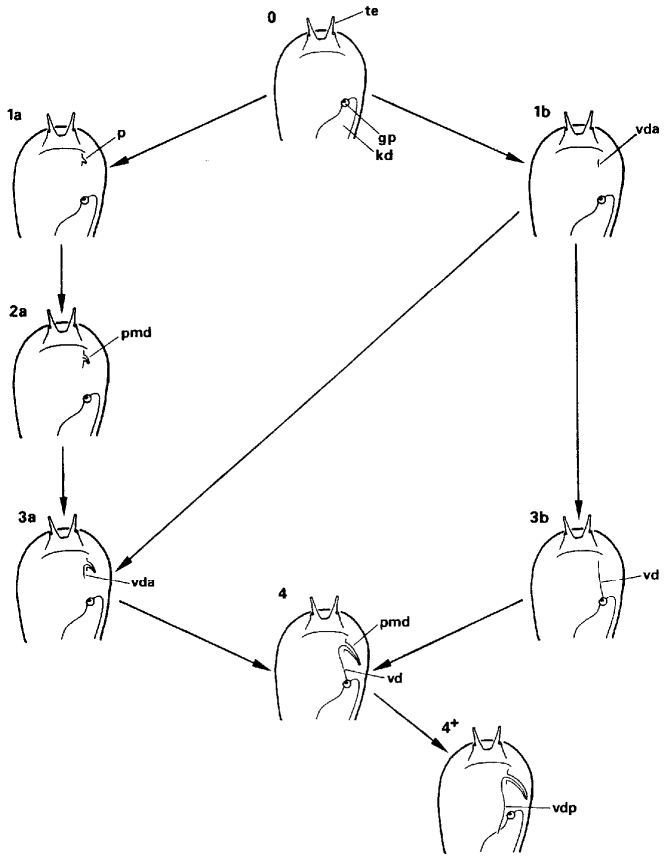
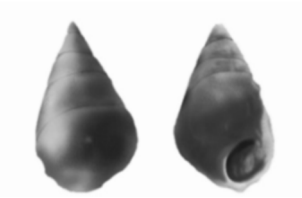


Figure 1.3 - *Nassarius reticulatus* illustration and its systematics. It is also represented the imposex development in this species and corresponding VDS scoring system proposed by (Stroben et al., 1992b) as explained in the text. Scheme adapted from OSPAR (2008). Drawing of the shells adapted from Galante-Oliveira et al. (2013). Abbreviations: 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.

Peringia ulvae **Pennant, 1777**

This gastropod species (Fig. 1.4), commonly known as mud-snail, occurs all over the Northeast Atlantic coast, from Norway to Senegal, including the Mediterranean (Graham, 1988). Characterized by its small size, this species can grow up to 10 mm height. Although it prefers estuarine conditions and wet banks of sand and mud, it also occurs on the open coast. The breeding period of this species occurs in two different peaks, in spring and summer (might slightly vary depending on the region) (Carlos Sola, 1996; Lillebo et al., 1999). This species lay the eggs in a gelatinous mass (egg capsules) over shells, preferably from the same species, sand grains or algae (Carlos Sola, 1996). The estimated lifespan for

this species is about 2 years (Lillebo et al., 1999). The feeding habitats of *P. ulvae* are diverse, since it preferably feeds on diatoms but can also feed on bacteria, silt or algae (Graham, 1988).



Phylum: Mollusca
Class: Gastropoda
Subclass: Caenogastropoda
Order: Littorinimorpha
Family: Hydrobiidae
Genus: *Peringia*
Species: *Peringia ulvae*

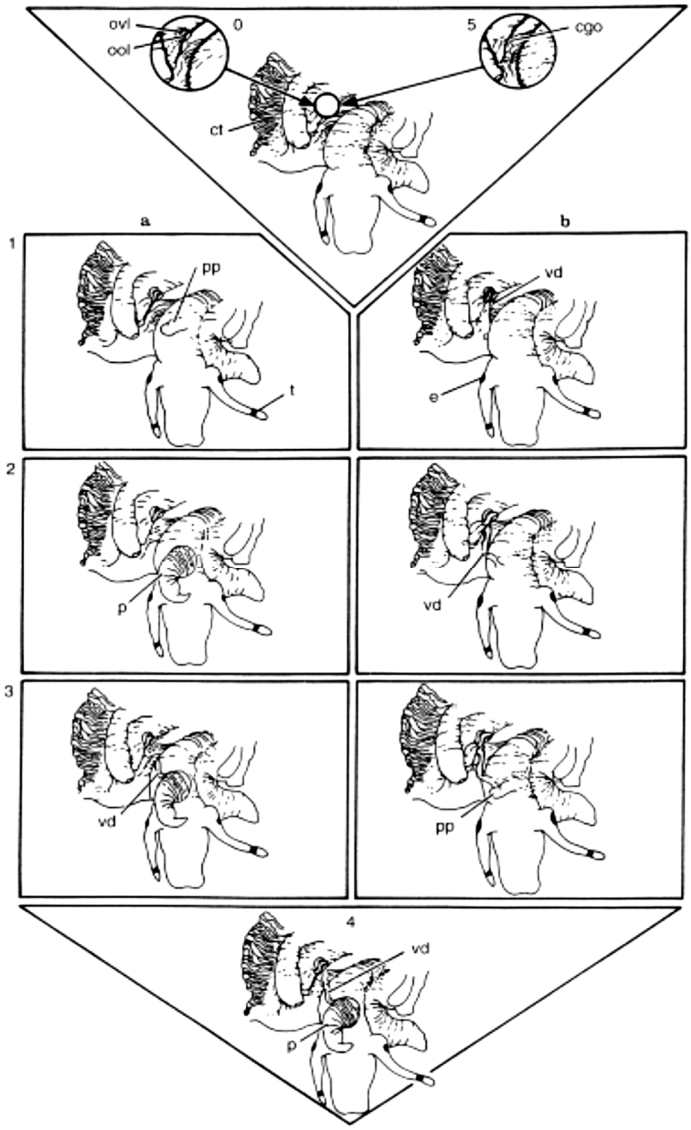


Figure 1.4 - *Peringia ulvae* illustration and its systematics. It is also represented the imposex development in this species and corresponding VDS scoring system proposed by Schulte-Oehlmann et al., (1997). Adapted from Schulte-Oehlmann et al. (1997). Drawing of the shells adapted from Galante-Oliveira et al. (2013). Abbreviations: cgo – closed genital openings; ct – ctenidium; e – eye; ool – orifice of the ovipar channel; ovl – orifice of the vaginal channel; p – penis; pp – penis primordium; t – tentacle; vd – vas deferens.

Even if considered one of the less sensitive species to TBT pollution in Europe (Barroso et al., 2000), it was suggested as a good TBT bioindicator in the most polluted areas where other species might be absent (Schulte-Oehlmann et al., 1997; Schulte-Oehlmann et al., 1998; Barroso et al., 2000). A VDS scoring system was proposed by

Schulte-Oehlmann et al. (1997) comprising 6 stages that goes from VDS = 0 (unaffected female) to VDS = 4 (a female with vas deferens and penis fully developed); and a VDS = 5 that corresponds to a sterile female without male characteristics. The imposex development in this species is similar to that of *N. reticulatus* previously described until VDS = 4 (see above). In this species it is also observed a *b-type* imposex development corresponding to an aphallic development with an initial development of the vas deferens at a VDS = 1b. The penis formation only occurs at VDS = 3b.

***Littorina littorea* Linnaeus, 1758**

Commonly named periwinkle, *Littorina littorea* (Fig. 1.5) is a shallow water species that lives in rocky and sandy shores, and estuarine areas, in the both sides of North Atlantic Ocean (at the east side from Norway to Portugal and at the west side along the USA, Canada and Greenland) (Graham, 1988). The shell might vary in colour, from dark grey to brown but usually banded, and vary in size and shape, from a more elongated shape (shell height length superior to the width) to globular morphology, with both shell height and width being similar (Kemp and Bertness, 1984). It can grow up to 40 mm height and adults can live up to 9 years. As all the previous mentioned species, the periwinkle reproduces sexually, after reaching maturity at the age of 12-18 months, and females lay planktonic egg capsules that will finally release veliger larvae (Bauer et al., 1995). *L. littorea* is a grazer that feeds preferably on algae but also on other vegetable detritus.

This species develops a different response to TBT pollution, yet related to imposex, termed intersex. This phenomenon was proposed as a biomarker for TBT pollution and the Intersex index (ISI) (Fig. 1.5) was firstly developed by Bauer et al. (1997) to be used in more contaminated areas due to the low sensitivity of this species to TBT pollution. Comprising 5 stages that goes from 0 (unaffected female) to 4, this phenomenon is a gradual transformation of the pallial tract of the females (Bauer et al., 1997). The ISI = 1 is characterized by a larger female genital opening and an opened bursa copulatrix due to an incomplete closure of the pallial oviduct. On ISI = 2 the complete pallial oviduct forms an open structure revealing female glands. This structure is then supplanted by a male

prostate gland in the ISI = 3. The last stage of this scoring system (ISI =4) is characterized by the presence of a small penis and a seminal groove (Bauer et al., 1997).

Phylum: Mollusca
Class: Gastropoda
Subclass: Caenogastropoda
Order: Littorinimorpha
Family: Littorinidae
Genus: *Littorina*
Species: *Littorina littorea*

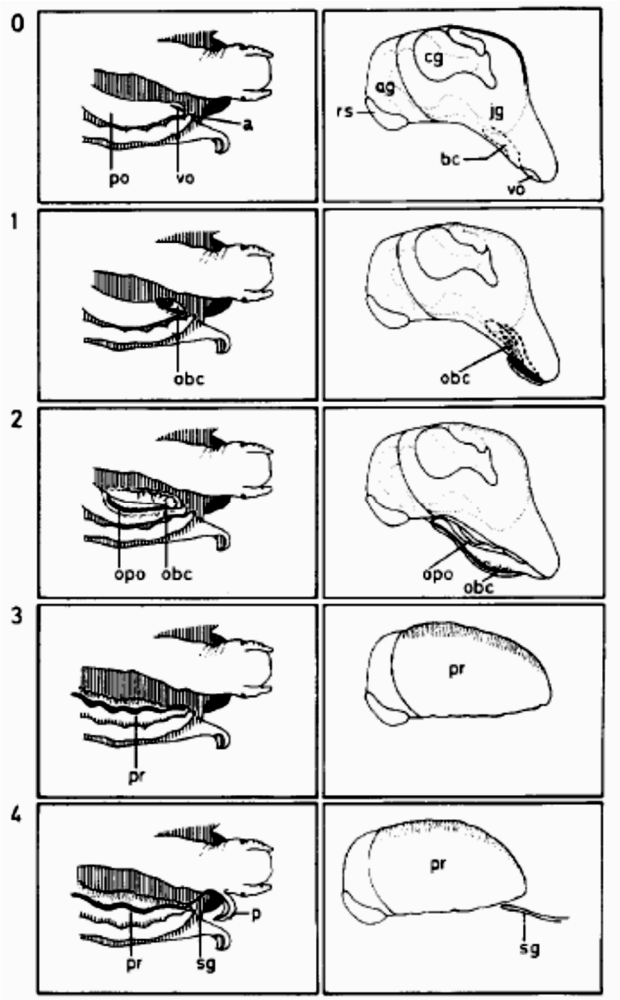


Figure 1.5 - *Littorina. littorea* illustration and its systematics. It is also represented the intersex development in this species and corresponding ISI scoring system proposed by Bauer et al. (1997) and further explained in the text. Adapted from Bauer et al. (1997). Drawing of the shells adapted from Galante-Oliveira et al. (2013). Abbreviations: a – anus; ag – albumen gland; bc –bursa copulatrix; cg – capsule gland; jg – jelly gland; obc – open bursa copulatrix; opo – open pallial oviduct; p – penis; po – pallial oviduct; pr – prostate; rs – receptaculum siminis; sg – seminal groove; vo – vaginal opening.

1.3.2. TBT pollution monitoring according to OSPAR

Understanding the great utility of this biomarker, organizations like OSPAR[1] and HELCOM[2] considered imposex a mandatory element under their monitoring programs (OSPAR, 2004; HELCOM, 2013). The OSPAR included organic tin compounds in the list of chemicals for priority action (OSPAR, 2007) and to effectively monitor this pollution, the assessment of TBT–specific biological effects (imposex/intersex) and TBT concentration in sediment or biota were considered mandatory elements under the OSPAR's Coordinated Environmental Monitoring Program (CEMP). These elements will help to describe ecosystem health and to understand if sites meet the proposed Ecological Quality Objective (EcoQo). To meet this objective OSPAR defined, for several matrixes, the TBT concentrations that should not cause any harmful biological effect - the Environmental Assessment Criteria (EAC). The EAC for TBT in water is 0.1 ng TBT/L , for sediment 0.01 µg TBT/Kg dw and mussel 12 µg TBT/Kg dw (OSPAR, 2011). Moreover, for TBT specific biological effects monitoring assessment, OSPAR developed the Assessment Criteria based on *Nucella lapillus* imposex levels, since it is one of the most sensitive species in Europe (OSPAR, 2004). This classification uses 6 classes divided by VDSI ranges and the EcoQO is reached for a VDSI lower than 2.0 (Assessment Class B). The interpretation of these assessment classes are presented in table 1.2.

[1] The successor of the Oslo and Paris commissions that is an intergovernmental organization responsible to administer The Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention).

[2] The Baltic Marine Environment Protection Commission or Helsinki Commission (HELCOM), is an intergovernmental organization that administrates the Convention on the Protection of the Marine Environment of the Baltic Sea Area (Helsinki Convention)

Table 1.2 - Interpretation of Assessment Classes as defined by OSPAR (adapted from OSPAR (2004)).

Assessment class	*N. lapillus* VDSI	Effects and impacts
A	VDSI = <0.3	The level of imposex in the more sensitive gastropod species is close to zero (0 - ~30% of females have imposex) indicating exposure to TBT concentrations close to zero, which is the objective in the OSPAR hazardous substances Strategy.
B	VDSI = 0.3 - <2.0	The level of imposex in the more sensitive gastropod species (~30 - ~100 % of the females have imposex) indicates exposure to TBT concentrations below the EAC derived for TBT. E.g. adverse effects in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT are predicted to be unlikely to occur.
C	VDSI = 2.0 - <4.0	The level of imposex in the more sensitive gastropod species indicates exposure to TBT concentrations higher than the EAC derived for TBT. E.g. there is a risk of adverse effects, such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT.
D	VDSI = 4.0 - 5.0	The reproductive capacity in the populations of the more sensitive gastropod species, such as *Nucella lapillus*, is affected as a result of the presence of sterile females, but some reproductively capable females remain. E.g. there is evidence of adverse effects, which can be directly associated with the exposure to TBT.
E	VDSI = > 5.0	Populations of the more sensitive gastropod species, such as *Nucella lapillus*, are unable to reproduce. The majority, if not all females within the population have been sterilised.
F	VDSI = -	The populations of the more sensitive gastropod species, such as *Nucella lapillus* and *Ocinebrina aciculata*, are absent/expired.

This classification scheme was also extended to other less sensitive species presenting different, yet equivalent, VDSI/ISI values ranges to *N. lapillus*. This relationship between species (shown in Table 1.3) was established based upon imposex correlations, obtained from comparison between the imposex levels of sympatric gastropod populations (OSPAR 2004). The assessment criteria for more than one species allows a more robust monitoring assessment with several advantages: i) in case of absence of *N. lapillus* other species can be used to detect TBT pollution; ii) effects on *N. lapillus* (one of the most sensitive species to TBT pollution) can be inferred based on the effects in other species; iii) a multi-species imposex monitoring approach allows a more comprehensive knowledge of TBT pollution due to the different spatial species distribution inside of a given study area;

iv) it allows an adequate use of the species based on their TBT sensitivity, for instance the assessment of imposex levels in *N. lapillus* should be employed in low polluted areas whilst for highly polluted areas the imposex assessment in species like *Littorina littorea* is recommended.

Table 1.3 - Biological effect assessment criteria for TBT presenting equivalent imposex levels between species as defined by OSPAR (adapted from OSPAR (2004)).

Assessment class	*N. lapillus* VDSI	*N. reticulatus* VDSI	*Buccinum undatum* PCI	*Neptunea antiqua* VDSI	*Littorina littorea* ISI
A	< 0.3	< 0.3	< 0.3	< 0.3	
B	0.3 - <2.0			0.3 - <2.0	< 0.3
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		0.3 - < 0.5
E	>5.0	> 3.5	> 3.5	.4.0	0.5 - 1.2
F	-				> 1.2

1.3.3. TBT pollution monitoring under Water Framework Directive and Marine Strategy Framework Directive.

In the year 2000, Water Framework Directive (WFD) was implemented and made way for a based ecosystem approach legislation that aimed to achieve at least a good ecological status of EU water bodies till 2015 by combining both biological, hydromorphological, chemical and physico-chemical elements. Two of the previously quality elements referred are directly linked with the assessment of TBT pollution and on the definition of the quality status of a water body based on the levels of this pollutant: the chemical and the biological elements. For the chemical elements, WFD defines Environmental Quality Standard (EQS) for all priority substances (like TBT) that should be respected to guarantee the good chemical status of a water body (EC, 2008). For the biological elements a five class ranking system (high, good, moderate, poor and bad) is used to evaluate the ecological quality status based on the analysis of specific community composition and abundance. Moreover, to guarantee comparability between EU member states, the biological monitoring results are presented as Ecological Quality Ratios (EQR). These ratios vary between one and zero so that values tending to zero indicate a major

deviation from the reference value (EQR near the value 1) and reflect a bad ecological status of the water body (EC, 2000). Tributyltin monitoring under the chemical quality elements is well defined with a fixed EQS for TBT of 0.2 ng TBT/L (Annual Average - AA-EQS) in water. The quantification of this value in water requires really sensitive chemical methods that are at the present time an analytical challenge (Moscoso-Pérez et al., 2015; Richter et al., 2015). On the other hand, and even if the assessment of the biological quality elements are well defined (with the evaluation of abundance and composition of phytoplankton, aquatic flora, benthic invertebrate fauna and in some cases fishes) it does not consider the deleterious biological effects of TBT pollution. The need to develop and use specific effect-based tools, like imposex/intersex, under WFD to link the chemical with the ecological quality status was already sensed (Martinez-Haro et al., 2015; Wernersson et al., 2015) and would be of extreme utility in the case of TBT since the chemical determination of this substance EQS in water is still expensive and challenging.

Afterwards, in 2008 the implementation of the Marine Strategy Framework Directive (MSFD) gave an integrated policy to EU marine environment, which was previously covered only by the Regional Sea Conventions: the OSPAR Convention for the North Eastern Atlantic, the Helsinki Convention for the Baltic Sea, the Barcelona Convention in the Mediterranean, and the Bucharest Convention in the Black Sea. The MSFD directive aims to achieve and preserve a good environmental status in EU marine environment by 2020, based on the fulfilment of 11 qualitative descriptors present in the Annex I of this directive. However, in contrast with WFD, there are no specific guidelines or targets on how the good environmental status should be achieved (view European Commission decision 2010/477/EU) and should be the member states to adopt necessary measures to achieve this goal. Nevertheless, a good environmental status achieved under MSFD should always be in agreement with other directives demands (e.g. WFD's EQS) and performed in coordination with member states sharing the same marine region, with regional sea conventions (like OSPAR or HELCOM) playing a central role for this purpose. The imposex/intersex, which is a mandatory element in the monitoring programs of the referred regional sea conventions, can also be an excellent tool to be applied in MSFD. However, there is not always a perfect agreement between the EU directives demands and

those from Regional Sea Conventions. For instance, the chemical objective in WFD for TBT in water is 0.2 ng TBT/L (annual average; the maximum allowable concentration is 1.5 ng TBT/L) while in OSPAR the EAC is 0.1 ng TBT/L.

1.3.4. Comparison between WFD, MSFD and OSPAR

As said, there are some differences between the European directives and the Regional Sea Conventions regarding TBT pollution. These main differences are summarized in table 1 where OSPAR is evidenced since Portugal integrates the area covered by this convention.

Table 1.4 - Main differences between WFD, MSFD and OSPAR, regarding TBT monitoring assessment. EU – European Union

		WFD	MSFD	OSPAR
	Objectives	Achieve or maintain at least the good status in EU water bodies by the year 2015	Achieve or maintain at least the good environmental status in EU marine environment by the year 2020.	Protect the marine environment of the North-East Atlantic.
	Types of waters	Inland surface waters, transitional waters, coastal waters and groundwaters.	Waters, seabed and subsoil from the seaward side of the territorial waters baseline to the outmost area where a Member State exercises jurisdictional rights, including the coastal waters defined by WFD.	Transitional waters and territorial seas of the Contracting Parties, the sea beyond and adjacent to the territorial sea under the jurisdiction of the coastal state to the extent recognized by international law, and the high seas, including the bed of all those waters.
Regarding TBT pollution	**TBT as priority substance**	Yes	Set by member states	Yes (organic tin compounds)
	Assessment criteria for water	Environmental Quality Standards (EQS) = 0.2 ng TBT/L (Annual Average - EQS)	Set by member states	Environmental Assessment Criteria (EAC) = 0.1 ng TBT/L
	Assessment criteria for sediment	Not defined	Set by member states	EAC = 0.01 ng TBT/g dw (dry weight)
	Assessment criteria for biota	Not defined	Set by member states	EAC = 12 ng TBT/L DW in mussels
	Biological effect monitoring tools	Not considered	Set by member states	Imposex assessment monitoring is a mandatory element under OSPAR's CEMP. Assessment Criteria defined for several species.
	Biological objectives	The values of the biological quality elements for the surface water body type must show none or only low levels of distortion in the taxa abundance and composition resulting from human activity. It is not defined a biological effect objective for this pollutant.	Should be assessed under descriptor 8: "Concentrations of contaminants are at levels not giving rise to pollution effects." It is not defined a biological effect objective for this pollutant.	An Ecological Quality Objective is well defined regarding this pollutant. Imposex levels indicate exposure to TBT concentrations in water below the OSPAR's EAC. This is obtained for a *N. lapillus* population with VDSI levels between 0.3 < 2.0 (~30 to ~100% of affected females).

Analysing this table is possible to compare the different legislative frameworks and perceive that: i) exists an overlap on the legislation application in coastal waters between

WFD, MSFD and OSPAR and in transitional waters between WFD and OSPAR; ii) there is a divergence in the TBT chemical objectives for water between WFD and OSPAR; iii) TBT chemical objectives for sediment and biota are not referred in WFD and MSFD; iv) the use of biological effect tools in monitoring (eg., biomarkers such as imposex and intersex) is defined under the OSPAR JAMP but is not explicitly required by the WFD or MSFD; v) in OSPAR, the ecological objectives are reached for VDSI levels that will correspond to TBT concentrations in water lower to EAC, while in WFD or MSFD (even if there are not specific objectives for TBT pollution), the objectives are reached without a direct relation between biological elements and specific contaminant concentrations in the environment. Therefore it is possible to perceive that there are some differences between these pieces of legislation while at the same time there is an overlap in some of their application areas. Most importantly, there is a clear lack of effect based tools in EU directives regarding TBT pollution.

1.4. Aims and rationale of the thesis

The recent EU Directives created new challenges and requirements at the TBT pollution monitoring level, as this chemical makes part of WFD list of priority substances. Still, as seen in table 1.4, it is undeniable that there are some important gaps in these Directives regarding this priority substance since, for example, they do not include any quality element to directly assess the ecological impact of TBT pollution. Hence, and in accordance with recent recommendations (Allan et al., 2006; Martinez-Haro et al., 2015; Wernersson et al., 2015), it is desirable to develop new biological effect tools to properly monitor TBT pollution, respecting these directives requirements. Therefore, the work presented in this thesis aims to:

i) develop new imposex/intersex-based monitoring tools to evaluate the ecological quality status of EU water bodies regarding TBT pollution;

ii) test their effectiveness to assess spatial and temporal trends of ecological quality status regarding TBT pollution in the Portuguese coast;

iii) choose the most suitable bioindicators for the above tools and check if imposex/intersex do represent a specific biological response to TBT pollution.

The current thesis is divided into five main chapters: the present introductory chapter plus three main research chapters (2-4; see below) and a general conclusion.

Chapter 2 addresses the use of gastropods to assess the ecological quality status of EU water bodies regarding TBT pollution. This chapter is divided into three parts. The first one proposes a multi-species tool to be used *in situ* for the evaluation of the ecological status, the second part proposes a sediment bioassay tool to be used in the laboratory in order to assess the bioavailable-TBT level in sediments, and the third part integrates both tools for TBT pollution monitoring under the scope of EU directives.

Chapter 3 addresses the use imposex as a biomarker for TBT pollution monitoring along the Portuguese coast and comprises two parts. The first part describes the current TBT pollution levels along the Portuguese coast as evidenced by the imposex in two gastropod species, *Nucella lapillus* and *Nassarius reticulatus,* and evaluates the TBT pollution trends over the last 15 years. Based on these results, the second part evaluates the current ecological quality status of the Portuguese coast and its temporal evolution, under the perspective of OSPAR and WFD. It is also discussed the integration of the imposex monitoring tool into the MSFD.

Chapter 4 appraises if imposex is the perfect biomarker for TBT pollution or if there are any exceptions to the rule. Considering that the previous chapters present specific monitoring tools based on imposex/intersex to evaluate the ecological quality status of EU water bodies, it is of foremost importance to further validate the bioindicators and biomarkers in use. Therefore, we must verify if there are some cases where the chosen bioindicator species fails to correctly deliver the proper ecological quality status regarding TBT pollution and, on the other hand, ascertain if other contaminants, apart from TBT, have the capacity to induce imposex. To check these points and properly accomplish the third objective of the thesis, this chapter presents three distinct parts. The first one aims to test the capacity of the gastropod *Peringia ulvae* to assess TBT pollution. This gastropod species is suggested to be a good bioindicator for TBT pollution monitoring, for instance by

HELCOM on their TBT monitoring programs, but was recently considered a counter current TBT pollution bioindicator. Therefore it is important to confirm if this is a valid species to be included in the tools proposed in the current thesis. The second part aims to confirm the hypothesis that other triorganotin - TPT - is able to induce imposex in *N. lapillus*, the key bioindicator species for the tool proposed in chapter 2.1. The third part aims to test the capacity of a wide variety of environmental contaminants to activate nuclear receptors by means of a reporter gene assay, as a preliminary essay to evaluate their potential to induce or interfere with the imposex response.

At the beginning of chapters 2, 3 and 4 it is presented an abstract with the reasoning and specific objectives of these sections, as well as the main results obtained.

So far, the research work developed during this thesis originated three research papers, while other ones will be submitted soon:

Chapter 2.1: Laranjeiro, F., P. Sánchez-Marín, S. Galante-Oliveira and C. Barroso (2015). "Tributyltin pollution biomonitoring under the Water Framework Directive: Proposal of a multi-species tool to assess the ecological quality status of EU water bodies." Ecological Indicators 57: 525-535.

Chapter 2.2: Laranjeiro, F., S. Pérez, P. Navarro, J. A. Carrero and R. Beiras (2015). "The usefulness of a sediment bioassay with the gastropod *Nassarius reticulatus* in tributyltin monitoring programs." Chemosphere 139: 550-557.

Chapter 4.2: Laranjeiro, F., P. Sánchez-Marín, A. Barros, S. Galante-Oliveira, C. Moscoso-Pérez, V. Fernández-González and C. Barroso (2016). "Triphenyltin induces imposex in *Nucella lapillus* through an aphallic route." Aquatic Toxicology 175: 127-131

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CHAPTER 2 - THE USE OF GASTROPODS TO ASSESS THE ECOLOGICAL QUALITY STATUS OF EU WATER BODIES, REGARDING TBT POLLUTION

2. THE USE OF GASTROPODS TO ASSESS THE ECOLOGICAL QUALITY STATUS OF WATER BODIES, REGARDING TBT POLLUTION

"Far above the world,
Planet Earth is blue
And there's nothing I can do."

in David Bowie's *Space oddity*, 1969

Water Framework Directive (WFD) marked undoubtedly a new era in pollution control by aiming to restore, or maintain, at least a good ecological status of European Union (EU) aquatic ecosystems. According to WFD, the ecological quality status of a given water body can be classified into five categories, which are represented by a colour code and where the highest ecological quality status is given by the colour blue. But if water gives the planet Earth the blue colour, as seen from "far above the world", from the WFD perspective blue is not the true colour of the majority of European water bodies, and in fact there's still a lot that should be done to improve the ecological status of aquatic systems.

Perceiving the necessities recently identified about the use and development of effect-based tools in pollution monitoring under WFD, the current chapter pretends to give a step in that direction. Tributyltin, listed as a priority hazardous substance in WFD, is known as the causative agent of imposex/intersex in gastropods, which is here proposed as a useful effect-based tool under the referred directive. Therefore, this chapter aims to develop and adapt suitable imposex/intersex-based monitoring tools to evaluate the ecological quality status of water bodies regarding TBT pollution, within the European directives WFD and MSFD. In order to achieve the proposed objective this chapter is divided in the following three subchapters.

CHAPTER 2.1 - TRIBUTYLTIN POLLUTION BIOMONITORING UNDER THE WATER FRAMEWORK DIRECTIVE: PROPOSAL OF A MULTI-SPECIES TOOL TO ASSESS THE ECOLOGICAL QUALITY STATUS OF EU WATER BODIES

Laranjeiro, F., P. Sánchez-Marín, S. Galante-Oliveira and C. Barroso (2015). "Tributyltin pollution biomonitoring under the Water Framework Directive: Proposal of a multi-species tool to assess the ecological quality status of EU water bodies." Ecological Indicators **57**: 525-535.

2.1. Tributyltin pollution biomonitoring under the Water Framework Directive: proposal of a multi-species tool to assess the ecological quality status of EU water bodies Introduction

2.1.1. Abstract

The Water Framework Directive (WFD) is a key legislative action developed by the European Union in order to protect aquatic ecosystems. One of the concerning pollutants, listed in this directive as a priority hazardous substance, is tributyltin (TBT), a biocide largely used in antifouling paints and identified as a causative agent of imposex/intersex in gastropods. In order to integrate TBT pollution monitoring within this legislative framework, a practical exercise is here proposed to assess the evolution of surface water ecological status in Ria de Aveiro (NW Portugal). Three bioindicators - the caenogastropods *Nucella lapillus*, *Nassarius reticulatus* and *Littorina littorea* - were used under the general WFD benthic invertebrate quality element, and the vas deferens sequence index (VDSI) and the intersex index (ISI) were selected as biomarkers for the purpose of assessing the condition of this quality element regarding the impact of TBT pollution. Levels of VDSI in *N. lapillus* and *N. reticulatus*, and ISI in *L. littorea*, were surveyed in 2013 and compared with previous data available for the same species and study area in 1998 and 2005, providing a time lapse for a period of 15 years. VDSI and ISI values were converted into Ecological Quality Ratios (EQR) and EQR boundaries were set for each species in order to define the five ecological status classes (High, Good, Moderate, Poor and Bad). We propose *N. lapillus* as key bioindicator, however the combined use of further species is very useful to cover a wider study area. Based on the proposed method, it is concluded that the ecological status of the surface waters surveyed in Ria de Aveiro, concerning the impact of TBT pollution on the above benthic invertebrate taxa, improved considerably since 1998 and achieved a Good Ecological Status in 2013, thus meeting the WFD environmental objectives for this priority hazardous substance even before 2015.

2.1.2. Introduction

The introduction of new legislative frameworks in the European Union (EU) water policy marked a new era in environmental risk assessment and raised new challenges in order to develop quick and low-cost tools for a better evaluation of water bodies' status. The Directive 2000/60/EC, known as Water Framework Directive (WFD) (EC, 2000), made way for a based ecosystem approach legislation combining all biological, hydromorphological, chemical and physico-chemical elements. The WFD advocates the use of community structure traits specified in its Annex V as biological quality elements, and demands the classification of the studied biological variables into a five class ranking system (High, Good, Moderate, Poor and Bad) that is used to evaluate the ecological status, upon regular assessment, of a water body. Moreover, to guarantee comparability between EU member states, the biological monitoring results must be presented as Ecological Quality Ratios (EQR), which vary between one (the best possible ecological status of the water body) and zero (maximum deviation from the reference conditions and reflecting the worst status) (EC, 2000). The definition and harmonization of EQR boundaries between the five classes of ecological status has been one of the bigger tasks for the EU scientific community, with special attention to the definition of the boundary between Good and Moderate status, which will define if a member state has to take actions to improve a certain water body quality (Birk et al., 2013). Another trending topic of discussion is the introduction of ecotoxicological assessment methods (Marín-Guirao et al., 2005; Beiras and Durán, 2013; Gonçalves et al., 2013; Hamers et al., 2013), including the use of biomarkers (Hagger et al., 2008; Sanchez and Porcher, 2009; Martinez-Haro et al., 2015), that can provide more sensitive, early-warning responses, and reduce costs compared to the faunistic methods proposed in the WFD. The use of these alternative methods was already referred in the WFD's Common Implementation Strategy (CIS) guidance documents (EC, 2009; EC, 2010; EC, 2011). Moreover, effect-based tools were recently identified in a technical report by the European Commission, as links between chemical and ecological assessments in monitoring programs under the WFD (EC, 2014).

Tributyltin (TBT) compounds are priority hazardous substances (WFD Annex X) (EC, 2013) that must be monitored in order to classify the chemical status of a water body. These compounds were used worldwide as biocides in antifouling systems (AFS) since the mid 1960's. Due to their high toxicity, the use of TBT-AFS was banned in EU by the Regulation Nº 782/2003 (EC, 2003) and in 2008 a global ban was implemented by the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention) (IMO, 2001). TBT pollution caused serious impacts on aquatic ecosystems throughout EU coastal areas (Bryan et al., 1986; Alzieu, 2000; Barreiro et al., 2001; Barroso et al., 2002a), being therefore important to evaluate if there has been a recovery after entry into force of the above legislative acts, and if the EU waters achieve the WFD target of Good ecological and chemical status by 2015 regarding this aquatic pollutant.

The WFD Environmental Quality Standard (EQS) defined for TBT in water is: (i) 0.2 ng TBT/L, corresponding to the annual average concentration (AA-EQS), and (ii) 1.5 ng TBT/L, corresponding to the maximum acceptable concentration (MAC-EQS) that can be found in a water body (EC, 2008). However, the WFD does not include any guidance concerning the specific biological effects caused by this pollutant. A well-known biomarker used in TBT pollution biomonitoring is the imposex/intersex phenomenon. Imposex was defined by Smith (1971) as the superimposition of male sexual characters onto the reproductive tract of gastropod females, the same author that reported, 10 years later, the cause-effect relationship between imposex and the exposure to TBT (Smith, 1981). This causative relationship was then firmly confirmed by several other studies in the following years (e.g., Bryan et al., 1986; Gibbs et al., 1987; Stroben et al., 1992a). A very similar syndrome – intersex – was also described for the gastropod *Littorina littorea*, whose intensity is also dependent on the degree of TBT environmental contamination (Bauer et al., 1995). In extreme cases, imposex and intersex can lead to the complete sterilization of the affected specimens (Gibbs and Bryan, 1996). In our view, imposex and intersex are among the best examples of specific and dose-dependent biological effects caused by an environmental pollutant, and probably constitute the most successful biomarkers used to date in pollution biomonitoring. Due to these unravelled properties, they are mandatory elements under the OSPAR Coordinated Environmental Monitoring Program (CEMP)

(OSPAR, 2004), being the European Commission, representing the European Union, a signatory to the OSPAR Convention. Therefore, a similar approach could be adopted in the WFD, after proper adjustments respecting the specific goals of this legislative framework. The WFD-United Kingdom Technical Advisory Group (WFD-UKTAG) already proposed the use of imposex in the species *Nucella lapillus* as a tool for the WFD ecological status assessment of coastal waters in the UK (WFD-UKTAG, 2014). Oehlmann (2002) also recommended TBT assessment criteria using intersex in *L. littorea* regarding the WFD. The current work aims to give a further step regarding the use of more species (multi-species approach) for the assessment of the ecological status in coastal and transitional waters and proposes new boundaries for the ecological status classes. We tested this approach with a well-studied case scenario – the Ria de Aveiro (NW Portugal) – and discuss the classification of the ecological status of water bodies centered on the definition of EQR based on imposex/intersex values. The monitoring of imposex/intersex levels was performed in three gastropod species – *Nucella lapillus, Nassarius reticulatus* and *Littorina littorea* – common along the EU coast and among those recommended by OSPAR, and the evolution of the ecological status for these water bodies assessed under the WFD from 1998 to 2013, i.e., comprising the moment from which the WFD was established (2000) and 1.5 years before the first deadline (2015) for achieving a Good status in EU. This case study constitutes an exercise to evaluate how this approach can be helpful for monitoring the ecological status of EU water bodies regarding TBT pollution in order to accomplish the WFD objectives.

2.1.3. Methods

Study area

Ria de Aveiro (Fig. 2.1) is a shallow estuarine system located in NW Portugal, comprising three main and narrow channels that are connected to the ocean through an artificial and constricted opening – the lagoon mouth. The lagoon covers an area of about 83 km^2 in high tide and 66 km^2 in low tide, therefore creating important intertidal zones like mud flats and salt marshes (Dias et al., 2000). The Port of Aveiro and the main shipyards

are located in the main navigation channel, between the mouth of the lagoon and the city of Aveiro (Fig. 2.1). The port consists of several specialized terminals extending along 5 km of docking area (www.portodeaveiro.pt). The most important fishing ports are situated in the main navigation channel and in the north of Mira channel (Fig. 2.1). Also, several marinas, small fishing ports and small shipyards are dispersed throughout the channels, increasing the potential sources of TBT (for an extensive description of this estuarine system and the identification of major TBT sources see Barroso et al., 2000). Ria de Aveiro has been subjected to regular TBT pollution monitoring surveys during the past 15 years (Barroso et al., 2000, 2005; Sousa et al., 2007; Galante-Oliveira et al., 2009; 2010; Laranjeiro et al., 2010) and therefore constitutes an excellent study area to assess the temporal evolution of the ecological status by using the imposex/intersex tool. Ria de Aveiro water bodies, defined by the Portuguese administration, are also represented in figure 2.1 (for more information see Fidélis and Carvalho (2012).

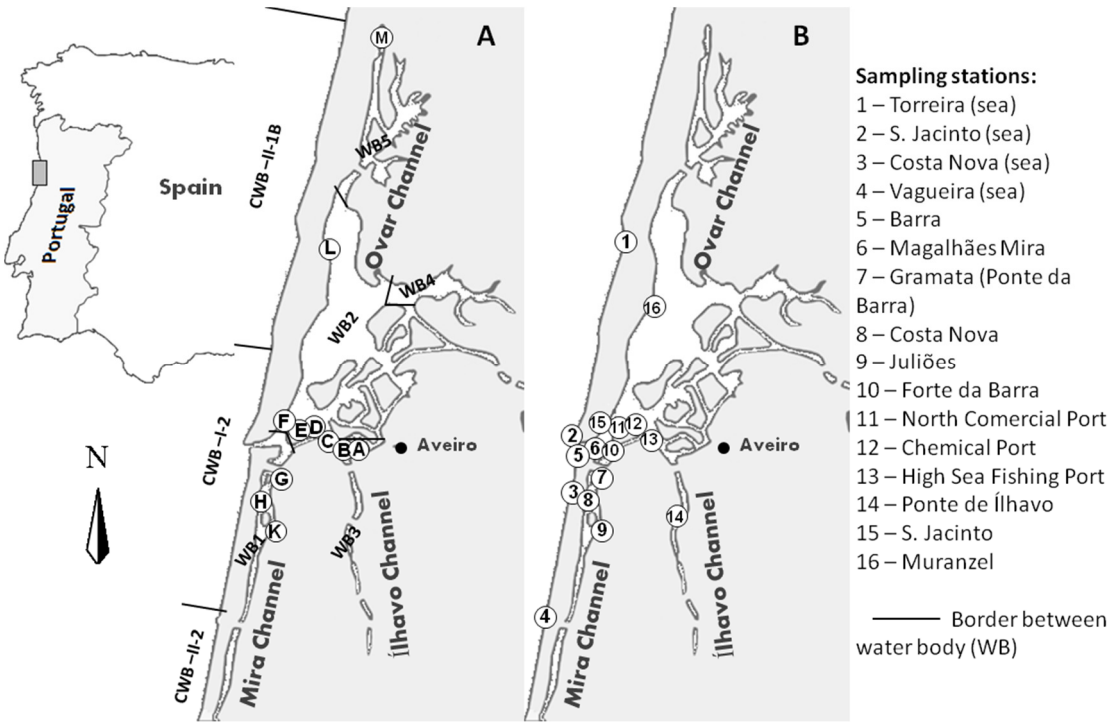

Figure 2.1 - Ria de Aveiro and adjacent seacoast. (A) WFD water bodies of the lagoon, as named by the Portuguese administration (Fidélis and Carvalho, 2012) with letters indicating the location of the main TBT pollution sources: A-South Commercial Port; B-Aveiro dockyards; C- High Sea Fishing Port; D- Chemical Port; E- North Commercial Port; F- S. Jacinto Marina; G- Gafanha Marina; H- Costa Nova Marina; K- Cais da Bruxa Marina; L-Torreira Marina; M- Ovar Marina; (B) Sampling stations location.

Indicator species and imposex analysis

Imposex or intersex levels were assessed in adult specimens of the dog-whelk *Nucella lapillus*, the netted-whelk *Nassarius reticulatus* and the periwinkle *Littorina littorea*, collected in Ria de Aveiro during low tides in July and August 2013. Specimens were collected by hand in intertidal areas, while baited hoop nets were used for *N. reticulatus* sublittoral sampling. The same laboratory procedures were followed for these three species. Shell heights were measured with Vernier callipers to the nearest 0.1 mm and then, animals were cracked open with a bench vice, sexed and dissected under a stereo microscope. Parasitized specimens were discarded from the analysis. Dog-whelks were analysed without narcotisation and the percentage of imposex-affected females (%I), the mean female penis length (FPL), the mean male penis length (MPL), the relative penis size index (RPSI = FPL^3x100/MPL^3) and the vas deferens sequence index (VDSI) were determined at each location. The VDS was classified according to the scoring system developed for this species by Gibbs et al. (1987). In contrast, netted-whelks were narcotised by immersion in $MgCl_2$ 7% solution in distilled water for 45 min prior to imposex analysis and the following indices were used to classify the imposex at each location: %I, FPL, MPL, the relative penis length (RPL = FPLx100/MPL) and VDSI. The VDS in *N. reticulatus* was classified according to the scoring system developed by Stroben et al. (1992b) and VDS stage 4+ was computed as 4 for VDSI final calculation (as recommended by these authors and by OSPAR). The periwinkle develops a related syndrome – intersex – that is characterized by the progressive masculinization of the female pallial genital tract (Bauer et al., 1995). *L. littorea* specimens were narcotised by immersion in 3.5% $MgCl_2$ in distilled water, and intersex levels were assessed by means of the Intersex Index (ISI) according to Bauer et al. (1995).

Ecological Quality Ratios (EQR)

The EQR values were obtained following the equation (Eq. 1):

$EQR_{\text{imposex/intersex}} = \frac{(M-O)}{M}$, where M is the maximum VDSI or ISI score that a population may attain and O is the observed VDSI or ISI value for a population at one given

site. For instance, in the case of *N. lapillus*, as the VDS varies between level 0 and 6, the maximum observable VDSI is 6, thus M assumes the value 6.

N. lapillus is one of the most sensitive gastropods to TBT pollution in EU waters. It is the key bioindicator under the OSPAR CEMP and was also adopted as the key bioindicator in the present work. In table 2.1 we present the interpretation given by OSPAR for *N. lapillus* VDSI values regarding the assessment of TBT pollution in the North-East Atlantic, which defines six classes A-F (OSPAR, 2004). We have adapted the general OSPAR criteria to define the required five WFD ecological status categories (table 2.1), adjusting the boundaries and objectives. The VDSI boundaries defined by OSPAR were computed as EQRs and a correspondence between the OSPAR A-F and the WFD High-Bad classes was established. Our class-boundaries proposal for *N. lapillus* differ from those used by WFD-UKTAG (2014) as we advise a lower VDSI limit between Good/Moderate and Moderate/Poor ecological status (table 2.1). The current study also proposes new corresponding EQRs for *N. reticulatus* and *L. littorea* and a practical exercise to test the efficacy of a multi-species approach to assess the ecological status of water bodies in Ria de Aveiro (table 2.2). The correspondence of EQR boundary values between the three species was based on the OSPAR's VDSI/ISI intercalibration key provided in the Provisional Joint Assessment and Monitoring Program (JAMP) Assessment Criteria for TBT (OSPAR, 2004) and on interspecific VDSI/ISI equations available in the literature (table 2.2). These relationships were established for sympatric populations of different species before the definitive ban of TBT that was implemented in EU (OSPAR, 2004), so further validation is desirable for a post-ban scenario. EQRs were calculated for *N. reticulatus* and *L. littorea* using Eq. 1, where O and M were substituted by the observed and maximum score of VDSI in the nassariid (M = 4) or ISI in the littorinid (M=4), respectively. The EQRs for *N. reticulatus* and *L. littorea* can always be adapted if the boundaries for *N. lapillus* change after intercalibration between member states is finally set.

Table 2.1 - EQR values calculated for each OSPAR's imposex assessment class and their correspondence to our proposal of WFD ecological status, based on the VDSI levels of Nucella lapillus. Classes definitions were based on the information included in OSPAR (2004) and in the WFD (2000). The "smile" symbols are here proposed as an alternative to WFD ecological status classification colours, for black-and-white printing purposes. The major focus of WFD is population abundance and community response, so WFD class boundaries here proposed ponder the effect of TBT on reproduction activity of females (population level), and the Good status (VDSI < 3) should be achieved till 2015. OSPAR class boundaries reflect mainly the biological effects at the specimen level, as an indication of TBT exposure, providing an Ecological Quality Objective of VDSI < 2.

OSPAR's Imposex assessment class	OSPAR class definition for *Nucella lapillus*	VDSI and correspondent *EQR* range	WFD ecological status classification class	WFD colour code	General class definition	Proposed VDSI and *EQR* ranges
A	Imposex levels close to zero (0 to ~30% of affected females) indicating an exposure to TBT concentrations close to zero, which is the ultimate objective in the OSPAR hazardous substances strategy.	[0 - 0.3[*[1.00 - 0.95[*	**High**	**Blue**	No, or minor, anthropogenic alterations in the quality element comparing to that normally associated with undisturbed water body. TBT concentration below the limits of detection of the most advanced analytical techniques in general use. This is the type-specific condition. MS action is not required.	[0 - 0.3[*[1.00 - 0.95[*
B	Imposex levels (~30 to ~100% of affected females) indicate exposure to TBT concentrations below the OSPAR Environmental Assessment Criteria (EAC) defined for TBT (0.1 ng TBT/L). Adverse effects in the more sensitive taxa caused by long term exposure to TBT is unlikely to occur.	[0.3 - 2.0[*[0.95 - 0.6(6)[*	**Good**	**Green**	There is no risk of population extinction. TBT has a negligible impact on population abundance due to the absence of sterile females. Therefore, the values for the biological quality element present low levels of distortion at the population level as a result of anthropogenic activities, deviating only slightly from undisturbed conditions. Action is not required but MS should be alert and prompt for action if VDSI>2.	[0.3 - 3.0*[*[0.95 - 0.50*[*
C	Imposex levels indicate exposure to TBT concentrations above the EAC. Long term exposure can cause adverse effects such as a reduced growth and recruitment in the more sensitive taxa of the ecosystem.	[2.0 - 4.0[*[0.6(6) -0.3(3)[*				
D	The reproductive capacity of populations of the more sensitive gastropod species, such as *N. lapillus*, is affected due to the presence of sterile females, but some reproductively capable females may remain.	[4.0 - 5.0] *[0.3(3) - 0.1(6)]*	**Moderate**	**Yellow**	There is low risk of population extinction. The quality element presents moderate signs of distortion from those normally associated with undisturbed conditions in terms of population abundance. Sterilized females may appear very rarely in the lower limit of the class. Sterility rises sharply in the range of 4<VDSI<4.5, probably affecting less than half of the females in the population. MS action is required.	[3.0 - 4.5*[*[0.50 - 0.25*[*
E	Populations of the more sensitive gastropod species, such as *N. lapillus*, are unable to reproduce as the majority or all females are sterilized.	> 5.0 *< 0.1(6)*	**Poor**	**Orange**	There is a high risk of population extinction and a major deviation from undisturbed conditions in terms of population abundance. Probably more than half of the females are sterilized. MS action is required.	[4.5 - 6.0] *[0.25 - 0.00]*
F	The populations of the more sensitive gastropod species (e.g.: *N. lapillus* and *Ocinebrina aciculata*) are absent/expired.	**	**Bad**	**Red**	Extinct population. The quality element shows a severe deviation from undisturbed conditions as massive female sterilisation caused population extinction. MS action is required.	**

* We propose a different limit between Good/Moderate (VDSI = 3.0; EQR = 0.5) and Moderate/Poor status (VDSI = 4.5; EQR = 0.25), comparing to WFD-UKTAG (2014). Based on the relationship between VDSI and the incidence of sterile females provided by Oehlmann et al. (1998), a VDSI = 4.5 corresponds to about 50% of sterile females in a population.
** This ecological status class classification is based on the absence of the studied organism and therefore no VDSI or EQR values are applied.

Table 2.2 - Provisional EQR values and ecological status classes are shown for the three species. Ecological status classes for *N. reticulatus* and *L. littorea* were defined based on the OSPARs VDSI/ISI intercalibration key (OSPAR, 2004) and the relationships provided in the literature between the VDSI in *N. lapillus* and *N. reticulatus* (Stroben et al., 1992a) and between the VDSI in *N. lapillus* and the ISI in *L. littorea* (Oehlmann, 2002), established for sympatrically living populations. Pop. Abs. – population absence.

OSPAR				Proposal for the Water Framework Directive								
Imposex assessment class	VDSI		ISI	*Nucella lapillus*			*Nassarius reticulatus*			*Littorina littorea*		
	Nucella lapillus	*Nassarius reticulatus*	*Littorina littorea*	Ecological status class	Proposed EQR classes	Corresponding VDSI	Ecological status class	Proposed EQR classes	Corresponding VDSI	Ecological status class	Proposed EQR classes	Corresponding ISI
A	< 0.3	< 0.3	< 0.3	High	[1.00 - 0.95[	[0.00 - 0.30[	At Least Good*	[1.00 - 0.93[	[0.00 - 0.28**[	At Least Good*	[1.00 - 0.95[	[0.00 - 0.20[
B	[0.3 - 2.0[			Good	[0.95 - 0.50[	[0.30 - 3.00[						
C	[2.0 - 4.0[	[0.3 - 2.0[					Good	[0.93 - 0.80[	[0.28 - 0.80[			
D	[4.0 - 5.0]	[2.0 - 3.5]	[0.3 - 0.5[	Moderate	[0.50 - 0.25[	[3.00 - 4.50[	Moderate	[0.80 - 0.40[	[0.80 - 2.40[	Moderate	[0.95 - 0.91[	[0.20 - 0.36[
E	]5.0 - 6.0]	]3.5 - 4.0]	[0.5 - 1.2]	Poor	[0.25 - 0.00]	[4.50 - 6.00]	At best Poor*	[0.40 - 0.00]	[2.40 - 4.00]	Poor	[0.91 - 0.70[	[0.36 - 1.20[
F	Pop. Abs.		]1.2 - 4.0]	Bad	Pop. Abs.					Bad	[0.70 - 0.00]	[1.20 - 4.00[

* *N. reticulatus* cannot always discriminate between High/Good and between Poor/Bad, so it may only indicate a status of At Least Good or At Best Poor. *L. littorea* cannot discriminate between High/Good, so it may only indicate a status of At Least Good.

** This VDSI boundary in WFD differs slightly from OSPAR due to EQR value rounding.

2.1.4. Results

Spatial and temporal evolution of imposex and intersex levels

Imposex and intersex levels registered in 2013 in Ria de Aveiro are shown in table 2.3. Low levels of imposex were found in *N. lapillus* and *N. reticulatus* while *L. littorea* females showed no signs of intersex. The %I varied between 35 and 100% in the dog-whelk and between 3 and 36% in the netted-whelk. The values of VDSI and RPSI varied between 0.35 - 1.38 and from 0.00 - 4.00% in the dog-whelk, respectively, while for the nassariid the VDSI and RPL ranged between 0.03 - 0.36 and 0.05 - 2.71%, respectively. Temporal evolution of imposex/intersex values in Ria de Aveiro for the three species are also presented in table 2.3, all showing lower levels in 2013. The VDSI in dog-whelks decreased between 1998 (VDSI = 4.00) and 2005 (2.53 ≤ VDSI ≤ 3.77) but the decline was more pronounced from 2005 (1.43≤VDSI≤4.40) to 2013 (0.35≤VDSI≤1.38), when comparing common stations for each pair of years. The netted whelks presented a slight increase of imposex from 1998 (0.00 ≤ VDSI ≤ 3.92) to 2005 (0.09 ≤ VDSI ≤ 4.00), and a decline from 2005 (1.20 ≤ VDSI ≤ 3.70) to 2013 (0.03 ≤ VDSI ≤ 0.36). The intersex in periwinkles showed a complete recovery from 1998 (0.27 ≤ ISI ≤ 0.51) to 2013 (ISI = 0.00).

Table 2.3 - Levels of imposex/intersex found in the study area in the current study (2013), in 2005 (Sousa et al., 2007; Galante-Oliveira et al., 2009) and 1998 (Barroso et al., 2000). Number of female and males analysed in the 2013 survey (N♀ and N♂, respectively); mean female and male shell height (SH♀ and SH♂); percentage of females affected by imposex or intersex (%I); relative penis size index (RPSI) in *Nucella lapillus*; relative penis length (RPL) in *Nassarius reticulatus*; vas deferens sequence index (VDSI); intersex index (ISI). n.a. – not analysed. Mean values are displayed with respective standard deviation as "mean±s.d.". Sterile females were only observed for *N. lapillus* at Stns. 12 (in 1998) and 6 (in 2005).

Station	N ♀	SH ♀	N ♂	SH ♂	% I	RPSI/RPL 2013	VDSI/ISI 2013	VDSI/ISI 2005	VDSI/ISI 1998
Nucella lapillus									
1 - Torreira (sea)	36	22.1±1.9	23	21.2±2.3	58	0.00	0.58±0.50	2.61±0.96	n.a.
2 - S. Jacinto (sea)	37	23.3±1.6	21	22.7±1.6	35	0.00	0.35±0.48	1.43±1.28	n.a.
3 - Costa Nova (sea)	39	23.4±1.5	21	22.3±1.3	49	0.22	0.49±0.51	2.82±0.61	4.00±0.00
4 - Vagueira (sea)	35	22.7±1.6	20	22.5±1.9	43	0.00	0.43±0.50	2.53±0.59	4.00±0.00
5 - Barra	39	30.4±3.0	20	29.6±2.0	54	0.00	0.54±0.51	3.77±1.30	4.00±0.00
6 - Magalhães Mira	16	27.5±3.2	22	26.4±1.8	100	4.00	1.38±0.50	4.40±0.55	n.a.
10 - Forte da Barra	28	27.3±1.8	17	27.9±3.1	68	0.00	0.68±0.48	3.25±1.03	4.00±0.00
12 - Chemical Port	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4.43±0.79
Nassarius reticulatus									
1 - Torreira (sea)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.09±0.55	0.00±0.00
2 - S. Jacinto (sea)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.11±0.77	0.00±0.00
3 - Costa Nova (sea)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.25±0.99	0.17±0.64
4 - Vagueira (sea)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.15±1.59	0.00±0.00
5 - Barra	30	25.6±2.5	19	25.3±2.0	3	0.05	0.03±0.18	2.15±1.28	1.21±0.63
6 - Magalhães Mira	32	25.3±2.7	22	23.5±3.2	19	1.48	0.31±0.78	3.50±0.51	1.88±0.85
7 - Gramata	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.68±1.21
8 - Costa Nova	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.25±0.83
10 - Forte da Barra	30	24.3±2.2	17	21.9±1.3	7	0.16	0.07±0.25	3.70±0.47	3.50±0.70
11 - North Comercial Port	30	23.2±1.5	25	21.8±1.0	13	0.31	0.13±0.35	3.53±0.73	3.58±0.69
12 - Chemical Port	22	22.4±2.8	22	22.8±2.8	14	1.63	0.23±0.69	n.a.	n.a.
13 - High Sea Fishing Port	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4.00±0.00	3.92±0.40
15 - S. Jacinto	34	25.9±1.6	23	24.5±1.8	36	2.71	0.36±0.49	2.43±1.03	1.12±0.77
16 - Muranzel	30	23.4±1.5	25	22.3±1.3	10	0.10	0.10±0.31	1.20±1.32	1.62±1.15
Littorina littorea									
7 - Gramata	1	25.6	4	19.8±2.1	0	-	0.00	n.a.	0.32±0.50
9 - Juliões	9	19.4±1.4	6	21.6±3.5	0	-	0.00	n.a.	0.31±0.40
13 - High Sea Fishing Port	5	26.8±2.4	4	24.1±2.5	0	-	0.00	n.a.	0.43±0.40
14 - Ponte Ílhavo	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.51±0.40
16 - Muranzel	7	23.7±2.8	16	24.1±2.4	0	-	0.00	n.a.	0.27±0.50

For a better assessment of the temporal trends of imposex in dog-whelks and netted-whelks, we selected two representative stations in Ria de Aveiro for which populations were sampled more frequently (Fig. 2.2): one located at the mouth of the lagoon, where all the water converges (Stn. 5; WB1), and the other located in the main

navigation channel (Stn. 10; WB2). Despite some fluctuations, the graphs confirmed the general trends described above: a clear decline of imposex in both species between 2005 and 2013, leading to an almost complete recovery of imposex in *N. reticulatus* (VDSI close to zero in 2013) and clear recovery for *N. lapillus* (VDSI 0.54 - 0.68), (see Fig. 2.2). However, a dissimilar pattern in the VDSI temporal trend is perceived, in both species in different sampling stations, before 2005. Highest levels in *N. lapillus* were registered soon in 1998 and persisted (only with a slight reduction) till 2005 at Stn. 5 (WB1), whilst at Stn. 10 (WB2) the VDSI decreased notably from 1998 to 2005. On the other hand, from 1998 to 2003 *N. reticulatus* imposex levels increased markedly at Stn. 5 and only slightly at Stn. 10.

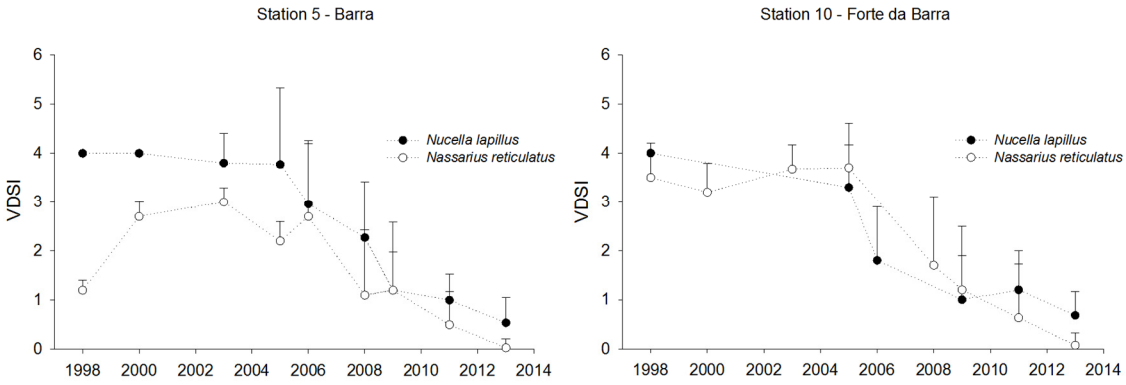

Figure 2.2 - VDSI levels in *Nucella lapillus* and *Nassarius reticulatus* over the years for station 5 (Barra) and station 10 (Forte da Barra). Past data on imposex levels were taken from the following publications: 1998 (Barroso et al., 2000); 2000 (Barroso and Moreira, 2002); 2003 (Sousa et al., 2005; Galante-Oliveira et al., 2006); 2005 (Sousa et al., 2007; Galante-Oliveira et al., 2009); 2006 (Galante-Oliveira et al., 2009; Rato et al., 2009); 2008 (Sousa et al., 2009; Galante-Oliveira et al., 2011); 2009 (Laranjeiro et al., 2010); 2011 (unpublished data).

Ecological Quality Status in Ria de Aveiro

The WFD ecological status of Ria de Aveiro regarding the impact of TBT is presented in figure 2.3. The location of the sampling stations for *N. lapillus* covered all the three water bodies outside the Ria de Aveiro for most of the years but just two out of the five water bodies inside the lagoon, because the species distribution is confined to polyhaline areas near the mouth. As a consequence, the area surveyed using this species is relatively small compared to the whole area occupied by the transitional and coastal waters of this basin. The use of more species improves the assessment of the ecological status, as the surveyed area is expanded. Biomonitoring with *N. lapillus* revealed that all water bodies assessed

outside and inside the lagoon presented in 1998 a Moderate ecological status. The situation improved considerably in 2005 but only Stns. 3 - 4 located in the seacoast achieved a Good ecological status. Stns. 1 - 2, surveyed in 2005 for the first time along the seacoast, also indicated a Good status. However, inside the lagoon, dog-whelks still presented an EQR within the range of Moderate ecological status in 2005. Finally, in 2013, a Good ecological status was observed at all surveyed sites as a result of a decline of TBT pollution in the area.

Table 2.2 shows the VDSI and EQR correspondence between species based on the OSPAR imposex assessment classes (compare table 2.1) and on the relationships between VDSI and ISI available in the literature. As can be seen in figure 2.3, the area surveyed increases considerably inside the lagoon by adding two more bioindicators, mainly due to the larger tolerance of *N. reticulatus* and *L. littorea* to lower salinity, providing a wider scope in the assessment of WB1, WB2 and WB3. The use of these species reveals worse ecological status in some sites that could not have been assessed by using only the dog-whelk. In fact, *N. reticulatus* showed EQRs within the range of At Best Poor ecological status at four sites in 1998 and at five in 2005, and Moderate status in the remainder locations except Stns. 1 - 4 in 1998 and Stns. 1 - 2 in 2005. Based on the ISI levels observed in the periwinkle, the Poor ecological status was also evidenced at two sites in 1998, while the rest of the surveyed sites presented Moderate status. The situation improved considerably in 2013 and At Least the Good status (could be High as well) was reached in all surveyed sites when using the netted-whelk and the periwinkle. At its right side, figure 2.3 shows the final proposal for the ecological status classification across sites. This was determined based exclusively on *N. lapillus*, regardless the results obtained for the other species. When *N. lapillus* was absent, the ecological status was defined by *N. reticulatus* or *L. littorea* using the following criteria: if only one of these species was available at a given site, that species determined the final status; if both species were available, the final status corresponded to the worse status identified by the nassariid or the littorinid.

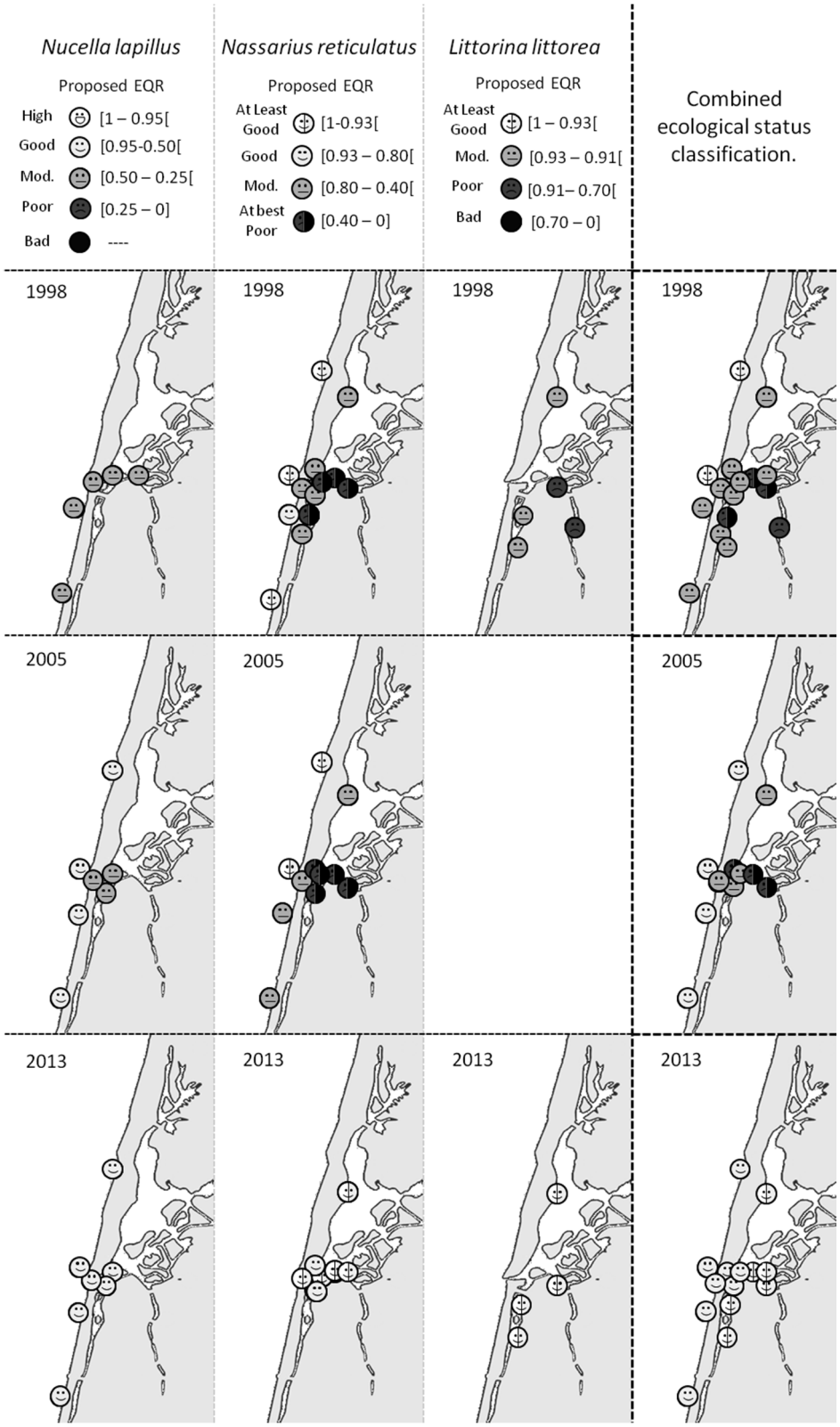

Figure 2.3 - Map showing the temporal evolution of EQR from 1998 to 2013 across sites and the corresponding ecological status in Ria de Aveiro, for each species separately and in combination (multi-species approach) to render the final classification.

2.1.5. Discussion

Imposex assessment is a simple and reliable tool for monitoring TBT pollution as it gives an indication of the average long-term levels of TBT environmental concentration and, simultaneously, shows the effective impact of this hazardous chemical on gastropod populations (Matthiessen and Gibbs, 1998; Barroso et al., 2000; Horiguchi, 2009). Its value is recognized by OSPAR as a mandatory element of the CEMP (OSPAR 2003). OSPAR guidelines for monitoring TBT-specific biological effects (imposex/intersex) were developed for several gastropods species – *Nucella lapillus*, *Nassarius reticulatus*, *Buccinum undatum*, *Neptunea antiqua* and *Littorina littorea* – of which *N. lapillus* is considered the key bioindicator, and the remainder are complementary, i.e. useful at sites where *N. lapillus* is absent or when more sensitive species are required. In this sense, for instance, as the periwinkle is less sensitive to TBT, it is more appropriate for highly polluted water bodies whilst the dog-whelk in better suited for less polluted areas (Bauer et al., 1997). Following the recent discussion on the use of effect-based tools (EC 2014), the current work applied the OSPAR methodology on imposex/intersex as an effect-based tool to classify the ecological status of Ria de Aveiro under the scope of the WFD, regarding the impact of this priority hazardous substance. As a tool with high ecological significance, our classification took into consideration the definition of the ecological status in transitional and coastal waters for the benthic invertebrate fauna, listed as a biological quality element in the WFD Annex V.

<u>Defining class boundaries for imposex and intersex</u>

According to OSPAR, the key imposex/intersex parameter to be monitored is the VDSI or the ISI because they correlate well with the level of TBT pollution and have biological meaning as they reveal the reproductive capability of a population. The reference situation, corresponding to an undisturbed condition, is the complete absence of imposex/intersex in the sampled population (i.e., VDSI/ISI = 0) since this phenomenon is considered to be specifically caused by this man-made synthetic chemical (TBT).

Triphenyltin can also induce imposex in *N. reticulatus* but has less environmental relevance as justified by Barroso et al. (2002b). We define *N. lapillus* as the key bioindicator for WFD monitoring (as in OSPAR) due to its high sensitivity to TBT and ubiquity in the EU Atlantic coast. Therefore, the critical step is to find the appropriate EQR boundaries for imposex levels in this species that best define the five surface water ecological status categories of WFD: High, Good, Moderate, Poor and Bad. The WFD-UKTAG (2014) proposed the following VDSI class boundaries for *N. lapillus* to be implemented in the UK: High/Good (EQR = 0.95), Good/Moderate (EQR = 0.34) and Moderate/Poor (EQR = 0.17), which correspond respectively to VDSI = 0.30, VDSI = 3.96 and VDSI = 4.98 (WFD-UKTAG, 2014). Our classification proposal uses different boundaries for Good/Moderate and Moderate/Poor status as we believe they better fulfil WFD premises. Our interpretation of these values, compared to OSPAR and WFD framework, is presented in the following paragraphs (see also table 2.1).

The High status is achieved if no (or minor) anthropogenic alterations occur, meaning that imposex levels are close to zero and so the correspondence of this category to OSPAR class A (VDSI < 0.30) is adequate. This status matches water concentrations of TBT close to zero (or at least below the detection limits of the most advanced analytical techniques in general use) and constitutes the definitive goal of both OSPAR and the WFD.

The next WFD category is the Good status. The lower boundary is VDSI = 0.3, as seen above, but what should be the higher boundary? Considering that the main focus of the WFD is the diversity and abundance of benthic invertebrate fauna, the occurrence of female sterility is the deciding factor for a site not achieving a Good ecological status, as this would represent an important deviation from undisturbed conditions due to human activity. The large data set available for the EU coast reveal that sterilised females may start to appear, though rarely, for VDSI > 3.0, particularly when the variance is high (Oehlmann et al., 1998; Ruiz et al., 1998; Galante-Oliveira et al., 2006), so we adopted the value VDSI = 3.0 as the upper limit of the class. The probability of the occurrence of sterilised females remains very low in the interval 3.0 < VDSI < 4.0, but above this value the sterility rises steadily (see below). Therefore, the Good status is defined in the current work as 0.3 ≤ VDSI < 3.0 which guarantees that sterile females do not occur and population breeds normally.

The adoption of these criteria will force the ecological targets of OSPAR and WFD to differ: while the OSPAR Ecological Quality Objective (EcoQO) for imposex in dog-whelk is to achieve classification A or B (VDSI < 2.0), the WFD target for 2015 would be getting all EU water bodies at least into Good status (EQR > 0.5; VDSI < 3.0).

The Moderate status implies, given the general definition of the WFD, that the values of the biological quality elements show moderate signs of distortion resulting from human activity. An incidence of sterilised females in dog-whelk populations over 50% represents more than just a moderate deviation from undisturbed conditions caused by a chemical pollutant. As 50% sterility relates approximately to a VDSI = 4.5 (see below), we propose that the Moderate class should be defined by the boundaries 3.0 ≤ VDSI < 4.5, instead of 3.96 < VDSI ≤ 4.98 applied by the WFD-UKTAG (2014).

If the VDSI surpasses 4.5 (which may indicate a female sterility incidence over 50%) then we propose that the water body should be classified as Poor ecological status because this corresponds to the condition where reproductive activity becomes severely curtailed and population faces a higher risk of extinction. Therefore the Poor status should be defined by the boundaries 4.5≤VDSI≤6.0.

The Bad ecological status refers to a situation where *N. lapillus,* and other most sensitive species like *Ocinebrina aciculata* (Oehlmann et al., 1996), became extinct due to high TBT pollution levels. This WFD category corresponds to OSPAR class F. The Bad status classification should only be applied when there is a clear evidence that natural populations of dog-whelks have existed before in the area under assessment and have declined following a Poor status, as other causes for extinction may exist (Gibbs, 1999). As *N. lapillus* may need several years to recolonize a given site, it is difficult to determine the real ecological status after extinction, as pollution may have declined meanwhile.

The impossibility to obtain adequate sample sizes (Poor status) or the total absence of *N. lapillus* (Bad status or inappropriate habitat) may force the use of alternative species (table 2.2). This approach was advised by Oehlmann (2002) regarding the use of *L. littorea* for WFD monitoring, but in the current work we added a third species besides the periwinkles: the netted-whelk *N. reticulatus*. We applied the OSPAR's VDSI/ISI

intercalibration key (OSPAR, 2011) and VDSI/ISI relationships available in the literature (table 2.2) to calibrate these three gastropod species (a more detailed explanation is provided below), in order to accommodate their different sensitivity to TBT. In fact, *N. lapillus* is the most sensitive species and a useful bioindicator for low TBT environmental concentrations, while *N. reticulatus* is ranked as the second most sensitive species and more appropriate at moderate TBT levels, whereas *L. littorea* represents the least sensitive among the three species and more suitable for high TBT pollution scenarios (Huet et al., 1995; Oehlmann et al., 1998; Barroso et al., 2000; Barroso and Moreira, 2002).

Comparing OSPAR and WFD criteria for ecological status assessment

The OSPAR JAMP Guidelines for TBT-specific biological effects (OSPAR, 2009) were partially transposed into the recommendations adopted here regarding the assessment and interpretation of imposex in *N. lapillus*. However, as previously stated, the major discrepancy between the OSPAR and WFD classification here proposed relies on the Good status being accepted in the WFD for VDSI < 3.0, while OSPAR defines an EcoQO of VDSI < 2.0. Despite the fact that a definitive boundary is yet to be established, and regardless the ambiguity of this issue, in our view the "spirit" of the WFD may differ from OSPAR in the sense that the former tends to focus on the practical ecological impacts of pollution in terms of population abundance and community diversity, rather than on the individual. In fact, a VDSI < 3.0 indicates that *N. lapillus* breeding is normal, which complies with the WFD requirements and does not demand member states to take an action. By no way this means that member states should be comforted when VDSI < 3.0, on the contrary, the correct behaviour is to be vigilant wherever imposex is detected in wild populations, and particularly alert whenever VDSI > 2.0. So, the establishment of the boundary between Good and Moderate status at 3.0 makes the WFD objective at the short term less ambitious than the OSPAR EcoQO regarding TBT pollution. The objectives for the chemical status of surface waters also differ in the sense that the WFD AA-EQS (0.2 ng TBT/L) is slightly higher than the OSPAR Environmental Assessment Criteria (EAC) (0.1 ng TBT/L).

Setting the boundary between Moderate and Poor status

The most relevant ecological outcome of imposex/intersex monitoring is the assessment of the impact of TBT on the reproductive activity of gastropod populations. Extinction of *N. lapillus* populations presumably happened at many sites of the EU coast as a consequence of TBT pollution (see, for example, Bryan and Gibbs, 1991). Reduced breeding is particularly alarming in this species because it lacks a dispersive pelagic larval phase and, besides, adults and hatchlings move very slowly, making more difficult the recruitment of new individuals coming from adjacent less affected populations. Consequently, a high incidence of female sterility (%S) may drive populations towards extinction due to lack of recruitment. Moreover, if fertile females are very scarce and dispersed, it may eventually affect the typical aggregative breeding behaviour and jeopardise reproduction (Gibbs, 1999).

Oehlmann et al. (1998) studied the relationship between VDSI and %S in dog-whelks collected from 178 sampling stations along the coasts of Ireland and France during 1988 - 1996. This relationship revealed an almost linear increase in the incidence of sterile females, from 6% to 93% within the interval 4≤VDSI≤5, where 50% of sterile females correspond approximately to VDSI = 4.5. We analysed equivalent data reported by Ruiz et al. (1998) in Galicia (NW Spain) during 1996 and we have found a remarkably similar relationship between %S and VDSI, although %S did not surpass 54% in this geographical area, where 50% sterilisation also coincided with a VDSI of approximately 4.5. As it can be seen, the single interval of 4≤VDSI≤5 comprises a full range of %S values, i.e., it comprises the most relevant and critical information regarding the ecological impact of TBT on wild populations without performing any discrimination. So we think that the VDSI boundary for the Moderate/Poor classes should rely on the distinction between a situation of low and high risk of extinction. Some evidences point that populations can recover after attaining high levels of %S. For example, 70 - 80% sterilisation was registered in a population of dog-whelks at Renney Rocks (SW England) and this value dropped gradually to nearly 10% in 1993, following the restrictions on TBT paint usage implemented in 1987 (Gibbs, 1999). There are further examples of population recovery after achieving %S as high as 93% or more (Harding et al., 1997), but we do not know whether these recoveries relied on the

recruitment of new individuals from neighbor populations or exclusively to the maintenance of self-sustaining populations, which may vary from case to case. Hence, it is not granted that such high values did not dictate extinctions of other populations in the past. Having this in mind, we take the precautionary and provisional value of VDSI = 4.5, corresponding to about %S = 50, as the limit to distinguish a Moderate status where the population faces a lower risk of extinction (VDSI < 4.5) and a Poor status where the population faces a higher risk of extinction (VDSI > 4.5). Obviously, this does not preclude the possibility of population extinction at a VDSI below 4.5 because populations can be subjected to natural and anthropogenic pressures that may interact with TBT to determine the course of the events.

Biological and chemical elements

The degree of imposex exhibited by adult whelks represents a dose-dependent but irreversible response to the total TBT body burden integrated throughout their lives (Bryan et al., 1986; Stroben et al., 1992a). Dog-whelk females mature at 2 - 3 years old and the adults specimens collected for biomonitoring may be much older since their longevity reaches 7 - 10 years (Gibbs, 1999), so a few years lag may occur between the imposex levels exhibited by adult females and the current TBT concentration in waters. Therefore, under a declining pollution scenario (the dominant trend nowadays) the current level of TBT water contamination may be lower than that predicted by imposex. Moreover, it is difficult to establish a relationship between imposex intensity and the TBT seawater concentration for very low levels of pollution as imposex may initiate for concentrations below the limit of chemical detection. According to Gibbs (1999) a VDSI < 4 relates to concentrations below ≈1 ng TBT/L. It should be noted that no deleterious effects in biota are described in the literature for TBT concentrations in water below 1 ng TBT/L, except the occurrence of imposex in gastropods (see, e.g. Nell and Chvojka (1992); Alzieu (2000); Kusk and Petersen (1997)). The Good status defined by 0.30 ≤ VDSI < 3.0 may indicate levels that may stand below or above the WFD AA-EQS of 0.2 ng TBT/L. As WFD also compel European member states to perform chemical monitoring on priority substances, direct measurements of TBT

in ambient water could decide whether to accept or decline the Good status applying the one-out all-out principle, i.e., the ecological status of the water body equates to the lower status of either the biological quality elements or the chemical elements.

Utility of the multi-species imposex/intersex tool for the WFD

The boundaries between ecological status classes in *N. reticulatus* and in *L. littorea* should correlate with the VDSI boundaries defined above for *N. lapillus*. To define these values we used the OSPAR's VDSI/ISI intercalibration key (OSPAR, 2011) and the relationships between VDSI in *N. lapillus* and VDSI in *N. reticulatus* (Stroben et al., 1992a), and also between VDSI in *N. lapillus* and ISI in *L. littorea* (Oehlmann, 2002), established for sympatric populations (see table 2.2). Therefore, the boundary between Good/Moderate in *N. reticulatus* was set as VDSI = 0.8 and for *L. littorea* was established as ISI = 0.2. Regarding female sterilisation, in the nassariid it may occur sporadically at high TBT pollution levels where VDSI approaches the maximum value of 4.0 (Barroso et al., 2002a), whilst for *L. littorea* at least some females will be definitely sterilised for ISI above 0.8 and 50% sterilisation will probably occur for ISI around 1.6 - 1.7 (Oehlmann et al., 1998). Hence, populations of netted-whelks and periwinkles are less vulnerable than dog-whelks, as sterilisation occurs for higher levels of TBT and also because their life cycle comprise a pelagic dispersive larva that may provide new recruits from less affected populations. When applying the above criteria to classify the evolution of the ecological status of water bodies in Ria de Aveiro, the assessment based on *N. lapillus* shows that all sites fall into the category Moderate status in 1998 as populations presented 4.00 ≤ VDSI ≤ 4.43. Sterile females were found only at one location (Stn. 12; WB2) showing VDSI = 4.43 and %S = 29. The situation improved considerably by 2005 as some surveyed sites fall into the category of Good status, while Stns. 5, 6 and 10 still presented a Moderate status; high imposex levels were still observed at Stn. 6 (WB1), at the inner part of the Ria, where VDSI = 4.40 and %S = 40. In 2013 the situation changed drastically: there was a clear recovery of imposex in all studied area, with VDSI declining to values between 0.40 - 1.40, indicating that Good status was finally reached in all surveyed area.

N. lapillus was very useful to assess the temporal and spatial evolution of the ecological status regarding the impact of TBT pollution, but the distribution of this gastropod in this basin is restricted to less polluted sites nearby the mouth of the lagoon, and so the ecological status in 1998 and 2005 for the whole study area could have been underestimated if *N. lapillus* had been the only species used. A more complete picture, specially for transitional waters, comes when *N. reticulatus* and *L. littorea* are included in the monitoring program, as these species are available at locations further inside the lagoon and nearer hotspots of TBT pollution, like ports, shipyards and marinas. The netted-whelk revealed situations of At best Poor status in 1998 (Stn. 7 in WB1 and Stn. 10, 11 and 13 in WB2) and 2005 (Stn. 6 in WB1 and Stn. 10, 11, 13 and 15 in WB2) at locations closer to TBT hotspots, and Moderate status at five sites in 1998 (Stn. 5, 6 and 8 in WB1 and Stn. 15 and 16 in WB2) and at four sites in 2005 (Stn. 5 in WB1, Stn. 16 in WB2, Stn. 3 in CWB-I-2 and Stn. 4 in CWB-II-2). The improvement between 2005 and 2013 is also clearly depicted when using *N. reticulatus*, with a decline of VDSI from 0.09 - 4.00 to values close to zero (0.03 - 0.36). This species is not sensitive enough to distinguish between High and Good status, but indicate that At Least the Good status was achieved in 2013 for the majority of the surveyed area, with Stn. 6 and 15 indicating Good ecological status. Similarly, periwinkles showed a Poor status in 1998 at the two locations closer to hotspots (Stn. 13 in WB2 and Stn. 14 in WB3), a Moderate status at the remainder sites (Stn. 7 and 9 in WB1 and in Stn. 16 in WB2) (figure 2.3). Intersex in *L. littorea*, reassessed in 2013 for the first time since 1998, revealed the complete recovery of this species as only normal females were found (ISI = 0 and %I = 0), which agrees with the recovery pattern also shown by the other two bioindicators. The periwinkle has not enough sensitivity to distinguish between High and Good status, but indicates that in 2013 all surveyed sites reached the classification of At Least Good. The use of these three indicator species proved to be very useful for tracking the evolution of the ecological status regarding the impact of TBT pollution in Ria de Aveiro.

We applied the OSPAR's intercalibration key of table 2.2 and relationships available in the literature to correlate the VDSI/ISI response between these three gastropod species, in order to deal with different specific sensitivities to TBT. However, it is our understanding

that the exact regressions between VDSI and ISI may be subject to variations with time. The intercalibration performed was based on relationships between imposex/intersex registered in sympatric gastropod populations in pre-ban periods, when TBT pollution levels were high (OSPAR, 2004) and so further research should address this topic for post-ban scenarios as well, with lower fresh TBT inputs. Possible changes may be due to differences in TBT bioavailability in several environmental compartments. For instance, while the dog-whelk seems to accumulate TBT mostly from water, with an estimated interference of less than 50% from its feeding habits (Bryan et al., 1989), other TBT exposure routes seem to be more relevant on the netted-whelk, with food uptake accounting for more than 50% of TBT body burden (Stroben et al., 1992a) and with contamination also possibly arising from the direct contact with TBT in interstitial water, subjected to adsorption/desorption processes from the sediments. As a result, different TBT accumulation may eventually occur between pre- and post-ban periods producing biased intercalibrations of VDSI/ISI. Moreover, these intercalibrations result from regression trends among a scatter plot of points, meaning that they are subjected to geographical variability. Although the correspondence between VDSIs for *N. lapillus* and *N. reticulatus* in table 2.2 has been confirmed for the Portuguese coast (Barroso and Moreira, 2002), the relationships observed for Stn. 5 and Stn. 10 (figure 2.2) is somehow different from that predicted from OSPAR (2004) and denotes spatial divergence between two sampling stations that are only about 1 km distant from each other. This is also reflected in the ecological status registered at Stn. 10 in 2005, which is Moderate according to *N. lapillus* but At Best Poor according to *N. reticulatus*.

Despite this weakness, the use of different bioindicators undoubtedly provides a more complete and accurate assessment of the temporal and spatial evolution of the ecological status of member state's water bodies, because it allows monitoring larger geographical areas, wider variety of water bodies (coastal and transitional, soft-bottoms to rocky shores), and broader TBT pollution gradients. In contrast, the single use of dog-whelks is confined to polihaline waters, rocky shore substrates and low-polluted environments. Therefore, we consider that the best approach to be adopted for monitoring TBT-specific biological effects under the WFD consists: (i) on the use of *N. lapillus* as key

bioindicator to assess the ecological status of a water body; (ii) when *N. lapillus* is absent one should look for alternative bioindicators; in this case, using the precautionary principle, the ecological status should correspond to the worse status identified among the alternative species, if more than one is available at that site. We used this approach to obtain the global ecological status across all surveyed sites in Ria de Aveiro (figure 2.3).

According to the multi-species approach outlined above, we conclude that the coastal (CWB-II-2, CWB-I-2, CWB-II-1-B) and transitional (WB1, WB2, WB3) water bodies surveyed in Ria de Aveiro recovered considerably. In fact, all surveyed sites reached the Good ecological status in 2013, thus achieving the WFD target before 2015, an improvement that is clearly linked to the legislation effectiveness. TBT concentrations in water, sediments and biota, along with TBT-specific biological effects, such as imposex and oyster shell thickening, have declined in Ria de Aveiro following the ban on TBT-AFS imposed in 2003 by the EU Regulation Nº 782/2003 (Sousa et al., 2007; Galante-Oliveira et al., 2009). Afterwards, the IMO AFS Convention that entered into force in 2008 may have also contributed to this decline, as we are now reporting the lowest imposex levels ever recorded for all bioindicators species in the last 15 years in Ria de Aveiro. This monitoring tool will eventually become redundant in the future, but surveillance must be carried on to confirm that this recovery trend in the ecological status is effective in the whole EU coast following the TBT-ban.

2.1.6. Bibliography

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CHAPTER 2.2 - THE USEFULNESS OF A SEDIMENT BIOASSAY WITH THE GASTROPOD *NASSARIUS RETICULATUS* IN TRIBUTYLTIN POLLUTION MONITORING PROGRAMS

Laranjeiro, F., S. Pérez, P. Navarro, J. A. Carrero and R. Beiras (2015). "The usefulness of a sediment bioassay with the gastropod Nassarius reticulatus in tributyltin monitoring programs." Chemosphere **139**: 550-557.

2.2. The usefulness of a sediment bioassay with the gastropod *Nassarius reticulatus* in tributyltin pollution monitoring programs

2.2.1. Abstract

Despite the use of tributyltin (TBT) had been banned worldwide in 2008 there is still evidence of its deleterious presence in environment. We evaluate the usefulness of a 28 days sediment bioassay with *Nassarius reticulatus* females to monitor TBT pollution using imposex as endpoint. In addition, butyltins were determined in sediments and tissues, and imposex was assessed in native *N. reticulatus* from the same sites where the sediments were sampled, whenever present. In bioassay, a significant increase in imposex parameters was obtained in three sediments (Vi2, Vi3, and Vi4). No correlation was found between this and TBT concentrations in sediment although a good correlation was obtained for TBT in tissues, putting in evidence TBT bioavailability in sediment. A significant decrease in imposex from 2008 to 2013 in native snails was only observed at sites that did not cause any effect in the bioassay. In contrast, imposex levels in 2013 were kept as high as 2008 in one of the sites where a significant imposex increase in the bioassay was observed. The bioassay proves thus to be a practical and ecological relevant tool, as: i. it can be conducted in sites with no native populations of snails, ii. it provides early identification of polluted sites anticipating future imposex levels or early identification of recovering, and iii. it yields information on the bioavailable fraction of the TBT in the sediment. Therefore, this tool can be of extreme usefulness under the scope of recent European legislative frameworks, as well as OSPAR.

2.2.2. Introduction

In order to ensure water quality and prevent hazardous effects observed in aquatic environment, new legislative frameworks have been introduced in Europe, namely Water Framework Directive (WFD) and Marine Strategy Framework Directive (MSDF) (EC, 2000;

EC, 2008). These directives demand European Member states to perform effective monitoring plans at ecological and chemical level in order to achieve good ecological status in all water bodies by 2015. One of the compounds listed as a priority substance on WFD's Annex X is tributyltin (TBT) (Directive 2013/39/EU), considered as the most toxic compound ever released and introduced into the environment (Goldberg, 1986) from its use as biocide in antifouling paints. One of the deleterious effects of TBT is the imposex phenomenon defined as the superimposition of male characters, such as penis and vas deferens, in females of many gastropod species. It was first described by Blaber (1970) in *Nucella lapillus* and since then this phenomenon was reported in more than 260 gastropods species (Titley-O'Neal et al., 2011) and is globally dispersed (Abidli et al., 2011; Titley-O'Neal et al., 2011; Choi et al., 2013; Cuevas et al., 2014; Lopes-dos-Santos et al., 2014; Paz-Villarraga et al., 2015). In extreme cases imposex can cause female sterility leading to population's decline, as reported in sites severely contaminated by TBT (Bryan et al., 1986; Gibbs and Bryan, 1996). As a response to these observations, several restrictions on the use of this compound in antifouling paints were applied. In Europe, restrictions on TBT were initially imposed by the directive 89/677/CEE that banned the application of TBT based antifouling paints in small vessels (< 25 m) but proved to be inefficient (Barreiro et al., 2001; Barroso and Moreira, 2002; Santos et al., 2002). This, ultimately, led to a global ban on the use of TBT by the IMO International Convention on the Control of Harmful Anti-fouling Systems on Ships that only entered into force in 2008 (IMO, 2001). Previously, European Union introduced Regulation (EC) No 782/2003 that banned the application of organotin antifouling paints on EU boats after 1 January 2003 and forbid its use after 2008 on any boat sailing in European waters.

Seen as the best biomarker for TBT pollution, imposex has been intensively used since the 80's to monitor TBT pollution in marine environment (Bryan et al., 1986; Barreiro et al., 2001; Sousa et al., 2009). It was also considered by OSPAR a mandatory element under the Coordinated Environmental Monitoring Program (CEMP) (OSPAR, 2004) and recently, has been regarded as a useful effect-based tool in monitoring programs under European Frameworks linking chemical and biological elements of quality (EC, 2014). Even with the relevant information obtained from imposex monitoring, this is a phenomenon

that may not reflect current TBT levels in the environment as it is an irreversible phenomenon (Gibbs et al., 1987; Oehlmann et al., 1998) and so it can mirror a history of TBT accumulation by the organism throughout life. Therefore, alternative approaches could be used to better assess realistic levels of TBT contamination. Quintela et al. (2000) and Smith el al. (2006) concluded that *in situ* exposures of *Nucella lapillus* with low levels of imposex were highly useful on detecting differences in TBT levels between sites. Rodríguez et al. (2010) found that caging transplant of *Nassarius reticulatus*, a less sensitive but more ubiquitous species, was also useful in highly contaminated areas. In addition, this species was also responsive to organotin contamination after laboratory exposure to sediment samples collected in the field (Duft et al., 2007). *N. reticulatus* is considered as the most reliable gastropod species in Europe for monitoring TBT contamination in sediments (Barroso et al., 2000), and therefore, can be regarded as a good candidate to be used in sediment bioassays, that ultimately may be adopted in monitoring programs as also pointed out by Oehlmann and Duft el al. (Oehlmann et al., 2000; Duft et al., 2007). Within this perspective, this paper intends to be a further contribution on contamination monitoring efforts under the increasing demands from international institutions, such as OSPAR, ICES or European Union. Moreover, the scientific community identified the use of bioassays and biomarkers in monitoring programs under WFD, as useful tools to complement the information obtained by the assessed quality elements and to investigate the causative agents of a reduced ecological quality status in water bodies (Marín-Guirao et al., 2005; Hagger et al., 2008; Martinez-Haro et al., 2015). Therefore, using Ría de Vigo as a case study, we evaluated the effectiveness of 28-day sediment bioassay with *N. reticulatus*, a species with a wide distribution along the North Atlantic in Europe, and compared it with the imposex trends observed in individuals inhabiting the same sites where sediments were collected.

2.2.3. Methods

Study area

This study was performed in Vigo harbour (Ría de Vigo, Galicia, NW of Spain) as a part of a comprehensive and multidisciplinary study on the environmental status of this and other Spanish harbours (Durán and Nieto, 2012; Montero et al., 2013; Vidal-Liñán et al., 2014). The study area comprises 5 sampling sites submitted to an intense naval traffic, up to $33.3*10^6$ G.T. in 2010 (www.apvigo.com), and therefore possible hotspots for TBT pollution as well as other site (Vi0 To), used as control, away from these sources of pollution. As indicated in figure 2.4, site Vi2 is located in a small marina surrounded by an intense shipyard activity with several companies operating in this enclosed area, site Vi3 inside the fishing port, sites Vi4 and Vi0 Ch situated inside yacht marinas with a capacity for 430 boats and 130 boats respectively and finally site Vi5 is located near the commercial harbour and next to another shipyard. Also, sites Vi2, Vi3 and Vi4 are found in enclosed areas which do not allow a proper water exchange with external water bodies being more likely exposed to contamination and accumulation of pollutants.

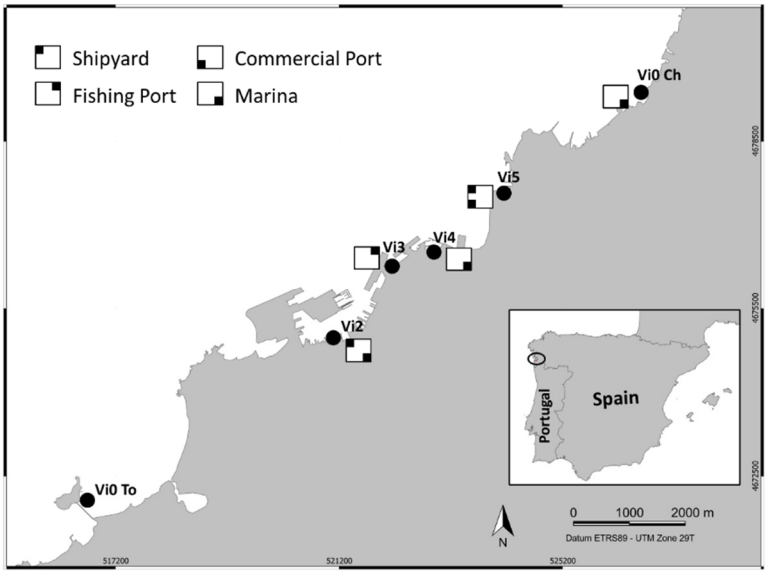

Figure 2.4 - Location of Vigo harbour with sampling sites and main sources of TBT pollution in the area.

Sediment and gastropod sampling

Surface sediments (< 5 cm sediment depth) were collected to polyethylene zipper bags, in June 2010, at each site shown in figure 2.4, with the help of a Van Veen grab and stored in the dark at 4 °C for five days until the beginning of the bioassay. Organic matter (OM) was quantified after incineration at 450 °C for 24 h while the fine fraction of sediment (< 63µm) was determined after wet sieving the sediment through a 63 µm mesh. Sediment subsamples were preserved at -20 °C in glass vials for subsequent butyltin species quantification.

Adult individuals of *N. reticulatus* were collected from a control site previously known to have the lowest imposex levels in the study area (Vi0 To). After narcotisation with a solution of $MgCl_2$ in distilled water (7%), the organisms were sexed under a stereo microscope and the presence of a penis in females, and its length, was registered by gently pushing the foot allowing the observation of the body to some extent. Only females with a penis smaller than 1 mm were selected for the bioassay and left to recover naturally from narcotisation during one week in 40 L tanks with constant seawater flow. The discarded organisms were returned to the environment.

Sediment bioassay

The sediment bioassay was adapted from the work by Duft et al. (2005; 2007) that cited previous works from Tillmann, (2004) and from Schulte-Oehlmann et al. (2001). Before the beginning of the test small stones, shells and other organisms present in the sediment were removed. The sediment collected at each site was then placed in three 2L glass beakers in order to form a 2 - 3 cm layer. One litre of filtered seawater was added to each beaker in order to achieve a ratio sediment/water of 1:5 and the mixture was left to settle overnight. Afterwards, 10 animals were added to each beaker starting the 28 - day bioassay at 18±1 °C, under constant water aeration, and with weekly water renewal without any food supply. The sediment collected at the same site as the animals served as control (Vi0 To). At the end of the experiment, the organisms were narcotized, as before, and measured with callipers to the nearest 0.5 mm. The shells were cracked open with a

bench vice and the animals were sexed and dissected under a stereo microscope with image analysis (Niss Elements, V4.00.06). The mean female penis length (FPL) and the vas deferens sequence (VDS) were measured in all organisms according to the scoring system developed by Stroben et al. (1992) with minor alterations proposed by Barroso et al. (2002). The organism tissues of each treatment were then pooled and preserved in glass vial at -20 °C for butyltin species quantification. To disclose differences between treatments, statistical analyses were performed with IBM SPSS Statistics 19 Software (the same software was used to perform the statistical analysis described in the following sections). Differences between treatments were evaluated through one-way ANOVA, followed by Dunnett's post hoc test.

Butyltin species determination

The analysis of the organotin species, monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT), in freeze-dried sediment samples and in gastropod samples was carried out by gas chromatography (Clarus 500, Perkin Elmer, Ontario, Canada,) coupled to inductively coupled plasma-mass spectrometry (NexION 300, Perkin Elmer) using isotope dilution (^{119}MBT, ^{119}DBT and ^{119}TBT) as described in the literature (Rodriguez-Gonzalez et al., 2005; Navarro et al., 2011). Isotope dilution corrects for non-quantitative recoveries, matrix effects or instrumental signal drift, improving accuracy and precision.

The extraction of the samples was carried out using a dry block heater Tembloc (J.P.Selecta S.A., Barcelona, Spain). Briefly, 250 mg of sediment were extracted in 5 mL of HAc:MeOH (3:1) at 80 ^{o}C for 5 min. For biota samples, 500 mg were extracted in 5 mL of tetramethylammonium hydroxide (TMAH, 25% in water) in the same conditions. The extracts were centrifuged; an aliquot was propylated ($NaBPr_4$) at pH 5 and recovered in hexane. All concentrations are given on a dry weight basis, as Sn. The accuracy and precision of the method were evaluated using the certified reference materials, PACS-2 (marine sediment, NRCC) and BCR-477 (mussel tissue, IRMM), analysed in triplicate. Blank analyses were also performed accordingly. A Spearman Rank correlation was performed

between TBT concentrations in sediment and tissues and the VDSI increase observed in the bioassay.

Imposex analysis

Imposex values were assessed 3 years after the bioassay, at the same sites where sediments were collected and compared with previous imposex data registered before the experiment in order to detect possible trends observed in the field and relate them with bioassay results. About 60 to 80 adult organisms of *Nassarius reticulatus* were collected whenever possible, at each sampling site between June and July in 2008 and 2013, with a baited hoop net and brought to the laboratory where they were kept in aquaria and observed within 2 days. The organisms were narcotized, as described before, and observed under a stereo microscope for imposex analysis. Parasitized specimens were discarded. The percentage of females affected by imposex (%I), mean males penis length (MPL), mean female penis length (FPL), the relative penis length (RPL = FPLx100/MPL), the vas deferens sequence index (VDSI) and the average oviduct stage (AOS) were determined at each site. The VDSI was classified with the scoring system previously described and AOS was classified according to Barreiro et al. (2001). Statistical comparisons were performed to distinguish differences in imposex parameters between 2008 and 2013 using the Mann-Whitney U test. The Student's t-test was used to evaluate differences in shell height between the same years to guarantee a proper comparison.

2.2.4. Results

Sediment bioassay

The variation of Female Penis Length (FPL) and Vas Deferens Sequence Index (VDSI), after 28 days of exposure, in the sediment bioassay is shown in figure 2.5. Mortality among sampling sites varied from 3.3% in Vi0 To to 30% in Vi4. Pronounced differences in mean FPL ($p < 0.001$) were induced in sediments from sites Vi2 and Vi3 in relation to control, whereas no significant differences were observed in the 3 other treatments. The greatest difference was found in site Vi2, with a mean increase of 4 mm in the FPL, in relation to control. For the mean VDSI statistically significant differences were registered at 3 sites –

Vi2 ($p < 0.001$), Vi3 ($p < 0.005$) and Vi4 ($p < 0.005$). Again Vi2 showed the highest increase in relation to control.

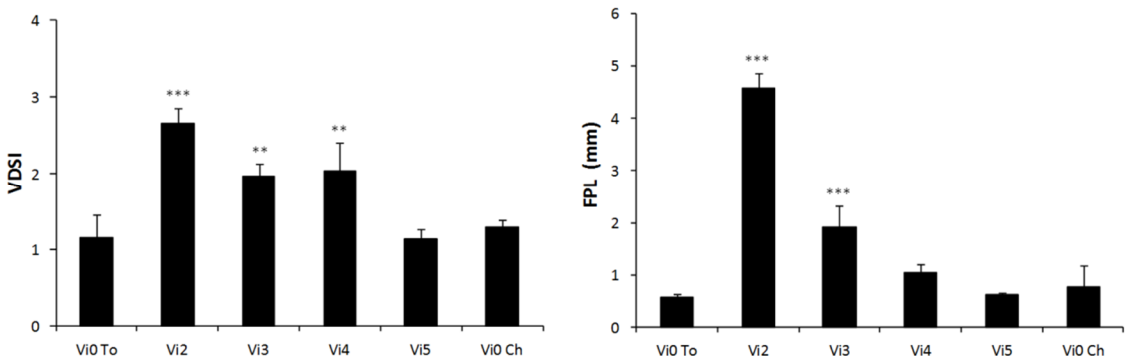

Figure 2.5 - Vas Deferens Sequence (VDSI) and mean female penis length FPL registered in test organisms after 28 days of exposure to sediments collected from Vigo harbour. Statistically significant differences to control (Vi0 To) are represented by ** ($p < 0.005$) and *** ($p < 0.001$).

Butyltin species levels

For the evaluation of the accuracy and precision of the butyltin species determination PACS-2 and BCR-477 certified reference materials were analysed in triplicate. Experimental results (table 2.4) were in good agreement with certified values, whereas the precision was in the range 0.5 - 2.9% (N = 3).

Table 2.4 - Butyltin species concentration (ng/g as Sn) in PACS-2 and BCR-477 certified reference materials.

	PACS-2		**BCR-477**	
	Certified	**Experimental**	**Certified**	**Experimental**
MBT	600*	582 ± 16	1013 ± 189	963 ± 28
DBT	1047 ± 64	1100 ± 8	785 ± 61	798 ± 22
TBT	890 ± 105	886 ± 13	901 ± 78	913 ± 20

* Indicative value

Concentrations of butyltins (ng Sn/g dry weight (dw)) in sediments and in the tissues of organism used in the bioassays are shown in table 2.5. In sediments, Vi4 exhibited the highest butyltins concentration in the study area while Vi0 To, used as control in the bioassay, presented the lowest values. TBT ranged from concentrations of 36 to 306 ng Sn/g dw representing 51.0% of the total butyltins (ΣBTs = MBT + DBT + TBT), while DBT

levels varied from 13 to 273 ng Sn/g dw representing 34.2% from the ΣBTs and MBT levels ranged 6.8 to 150 ng Sn/g dw being 14.8% ΣBTs. Also the butyltin degradation index (BDI = ([MBT] + [DBT])/[TBT])) proposed by Diez et al., (2002), reveal that sites Vi0 To, Vi1 and Vi2 had recent TBT inputs indicated by a BDI < 1. Despite the high levels of butyltins found in the sediment, no significant correlations were found ($p > 0.05$) between the imposex parameters registered in the bioassay (VDSI and FPL) and the levels of TBT; VDSI vs TBT ($r = 0.31$; $p > 0.05$) and FPL vs TBT ($r = 0.37$; $p > 0.05$). To complement butyltin analysis OM content and the percentage of fine particles (< 63 µm) was quantified at each site. OM varied between 5.2% in Vi0 To and 12.8% in Vi5 and consistently, Vi0 To showed a low percentage of fine sediment particles (1.3%) while Vi3 registered the highest percentage of < 63 µm sediment fraction (45.7%).

In tissue samples analysed after the exposure, butyltin levels varied significantly from the sediment; organisms exposed to sediments from Vi2 revealed the highest level of butyltins while the lowest was found in the organisms exposed to sediments from Vi0 To. The levels found in Vi2 and Vi3 were substantially higher than in sediments with enrichment factors of 15.6 for Vi2 and 3.0 for Vi3. TBT levels found in the tissues ranged from 16.5 to 1840.6 ng Sn/g dw which represented 55.5% of the ΣBTs; DBT levels varied from 11.9 to 724.1 ng Sn/g dw and MBT´s from 4.7 to 73.1. These levels accounted 12.4% and 32.1%, respectively of MBT and DBT, for the ΣBTs. In contrast with the levels of TBT observed in sediment, significant correlations were found between TBT in tissues and imposex values after the bioassay: VDSI *vs.* TBT ($r = 0.89$; $p < 0.05$) and FPL vs TBT ($r = 1.00$; $p < 0.01$).

Table 2.5 - Sediment parameters and butyltin concentrations (ng Sn/g dw) quantified in sediments collected in Vigo harbor area and in tissues samples from *Nassarius reticulatus* after the experiment. OM – Organic Matter, MBT – Monobutyltin, DBT – Dibutyltin, TBT – Tributyltin, BDI – Butyltin Degradation Index.

	Sediment samples						**Tissue samples**		
	< 63 µm	**OM (%)**	**MBT**	**DBT**	**TBT**	**BDI**	**MBT**	**DBT**	**TBT**
Vi0 To	1.3	5.2	6.8	13.0	35.7	0.6	10.3	15.4	+16.5
Vi2	24.0	5.8	18.0	85.0	118.0	0.9	73.1	724.1	1840.6
Vi3	45.7	11.6	13.0	39.0	80.0	0.7	16.6	103.9	239.1
Vi4	8.2	5.9	150.0	273.0	306.0	1.4	13.9	68.6	140.1
Vi5	21.2	12.8	55.0	139.0	156.0	1.2	4.7	11.9	17.7
Vi0 Ch	22.3	7.0	36.0	58.0	65.0	1.4	9.8	14.8	18.6

Imposex monitoring

The imposex levels found in *Nassarius reticulatus* from 2008 and 2013 are shown in table 2.6. In 2008, the percentage of females affected by imposex was always 100% except in Vi0. The FPL and RPL varied from 1.14 to 9.21 mm and 10.52 to 97.19 %, respectively. The mean VDSI levels ranged between 2.40 and 4.73 while AOS values varied from 0 to 1.32. In Vi2 13% females were found to be sterile being the only site where sterility was observed. In addition, in site Vi5 it was observed signs of penis reduction in adult males: two presented a penis lower than 1 mm while one was aphallic (with no penis). This was a rare observation in this species being to our best knowledge the second case registered (Sousa 2009). No organisms were found in Vi3.

In 2013, a statistically significant decrease on imposex levels was observed in three of the four sites monitored ($p < 0.001$), when compared to 2008. Imposex comparability between these 2 years is ensured by the lack of statistically significant differences in shell heights at each site between both years evidenced by Student's t-test performed. FPL, RPL, VDSI and AOS decreased significantly in sites Vi0 To, Vi5 and Vi0 Ch. On the other hand, site Vi4 seems to maintain high imposex levels. FPL ranged from 0.13 to 6.31, RPL from 1.08 to 65.06 and VDSI from 0.43 to 4.47. In the 2013 survey, no organisms were found at Vi 2 despite an intense sampling effort and again in Vi3.

Table 2.6 - Imposex levels in native *N. reticulatus* collected in Vigo harbor in 2008 and in 2013. N: number of organisms analysed, MPL: mean male penis length, FPL: mean female penis length, RPLI: relative penis length index, VDSI: vas deferens sequence index classified according to Barroso et al (2002), VDSI (4) : vas deferens sequence index classified according to Stroben et al. (1992), AOS: average oviduct stage, %I: percentage of females affected by imposex.

		Males			Females							
	Sites	N	Shell Height (mm)	MPL (mm)	N	Shell Height (mm)	FPL (mm)	RPL (%)	VDSI	VDSI (4)	AOS	%I
2008	Vi0 To	29	22.68 ± 3.48	10.88 ± 2.94	30	24.69 ± 2.02	1.14 ± 0.99	10.52	2.40 ± 1.22	2.40 ± 1.22	0.00 ± 0.00	90
	Vi2	30	20.04 ± 3.47	9.48 ± 1.77	30	20.78 ± 2.43	9.21 ± 1.99	97.19	4.73 ± 0.45	4.00 ± 0.00	0.38 ± 0.63	100
	Vi4	26	16.24 ± 2.67	8.10 ± 1.61	14	18.21 ± 3.59	6.53 ± 2.03	80.64	4.50 ± 0.65	3.93 ± 0.27	0.62 ± 0.65	100
	Vi5	30	23.13 ± 2.95	10.97 ± 3.70	30	24.84 ± 2.37	7.45 ± 1.63	67.91	4.03 ± 0.67	3.80 ± 0.41	1.32 ± 0.72	100
	Vi0 Ch	30	20.46 ± 2.38	10.82 ± 1.27	30	20.93 ± 3.48	7.15 ± 1.19	66.11	4.37 ± 0.61	3.93 ± 0.25	0.79 ± 0.82	100
2013	Vi0 To	20	22.86 ± 3.26	12.05 ± 1.01	30	25.36 ± 2.99	0.13 ± 0.49	1.08	0.43 ± 0.82	0.43 ± 0.82	0.00 ± 0.00	30
	Vi2	--	----------	----------	--	----------	----------	-----	----------	----------	----------	--
	Vi4	16	18.72 ± 3.21	9.70 ± 1.67	19	19.41 ± 2.44	6.31 ± 1.26	65.06	4.47 ± 0.61	3.95 ± 0.23	0.79 ± 0.79	100
	Vi5	15	22.40 ± 1.49	10.95 ± 1.04	26	24.10 ± 2.64	3.02 ± 1.95	27.53	2.96 ± 0.96	2.96 ± 0.96	0.00 ± 0.00	92
	Vi0 Ch	24	20.9 ± 2.83	11.6 ± 1.44	30	22.2 ± 2.52	4.08 ± 2.09	35.24	3.17 ± 0.59	3.17 ± 0.59	0.00 ± 0.00	100

Relation between bioassay and imposex monitoring

Figure 2.6 illustrates the VDSI increase observed in the bioassay and relates it with the VDSI levels observed in 2008 and 2013. When all the data is observed, it is possible to depict a relation between the bioassay result and the imposex monitoring values. For instance site Vi4, whose sediments caused a significant VDSI increase in the bioassay does not show a statistical difference between VDSI levels registered in 2008 and 2013 as levels were found to be extremely high at both years. On the other hand, sites that did not cause a significant effect in the bioassay registered a significant VDSI decrease between 2008 and 2013. In 2013, no animals were found at sites Vi2 which does not allows a more robust conclusion.

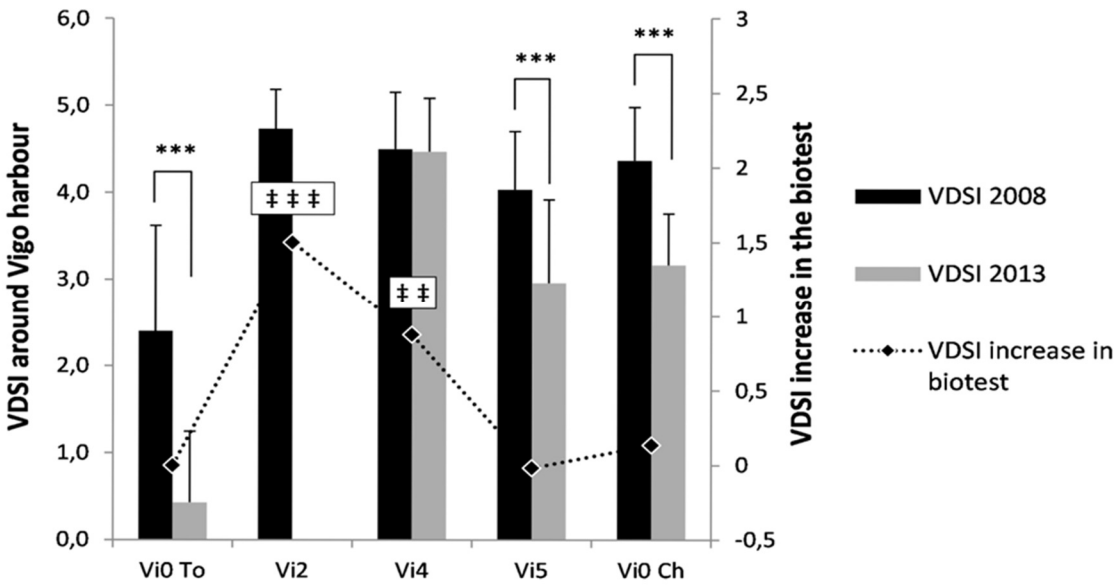

Figure 2.6 - VDSI levels observed between 2008 and 2013 in monitoring and VDSI increase registered in bioassay. Statistically significant differences in VDSI levels observed between 2008 and 2013 in the same site (***, $p < 0.001$). Statistically significant VDSI increase to control (Vi0 To) observed in bioassay (‡‡, $p < 0.01$; ‡‡‡, $p < 0.001$).

2.2.5. Discussion

The results obtained in the bioassay showed a significant FPL and VDSI increase in gastropod females of *Nassarius reticulatus*, exposed to collected sediments, but it does not significantly correlate with TBT concentrations found in sediment. Nevertheless, significant and positive correlations were obtained between imposex parameters and TBT accumulated in the gastropod tissues during the bioassay; VDSI *vs.* TBT in tissues ($r = 0.89$; $p < 0.05$) and FPL vs. TBT in tissues ($r = 1.00$; $p < 0.05$). As there is a better correlation between TBT in the tissues rather than TBT in sediments we may assume that only a part of this compound is bioavailable to be uptake by the organisms. On his experiments with *N. reticulatus*, Pope (1998) showed that TBT would be more available to the gastropod in sandy sediments (low % < 63 µm) with low organic matter (OM) while sediments rich in fine particles (high % < 63 µm) and OM would have less available TBT as its sorption capacity would be enhanced. However, in our bioassay, the sites that showed significant imposex increase presented some variability regarding the fine grained sediments and the presence of OM. For instance, Vi4 presented low values for both fine content and OM but the

accumulated TBT body burden was lower than expected from the TBT concentration in sediment which goes against Pope's prediction. Moreover, sites Vi2 and Vi3 showed some variability for these parameters that do not completely justify TBT availability according to literature (Radke et al., 2008; Langston et al., 2009). Tough, it should be noted that recent TBT inputs (BDI < 1) were registered in these two last sites what might possibly justify observed TBT bioavailability. This fresh TBT most likely accumulates in sediment upper layer, being subjected to effects of bioturbation by dwelling organisms and aeration in sediments, causes that are known to affect positively TBT bioavailability (Pope, 1998; Langston et al., 2009). On the other hand, a fact that can affect the lack of correlation between the TBT concentrations in sediment and bioassay results might be the presence of TBT paint particles in sediment common in sites as the ones assessed, like marinas and shipyards (Schulte-Oehlmann et al., 2001; Turner, 2010). The full TBT content of these particles is chemically measured however it has been suggested that when in paint particles TBT can be less bioavailable than when adsorbed to sediment particles (Langston et al., 2009). Therefore, even if sediments physico-chemical properties does not justify the obtained results in the bioassay we should keep in mind that this is an environmental complex matrix and conclusions should not be completely straightforward. On the other hand the tissues analysis disclosed the ecological relevance of the bioassay indicating the actual threat for organism which does not preclude that other sediments might be important TBT sources for the future, namely Vi4 and Vi5.

A set of studies conducted by Oehlmann and co-workers reported some other effects in gastropods that should also be taken into account when interpreting our results. These studies revealed that environmental contaminants with estrogenic or antiandrogenic action could suppress the imposex development and reduce the penis length in male individuals of this species (Oehlmann et al., 2000; Tillmann et al., 2001). In site Vi4 there is a significant increase in VDSI level, justified by the TBT presence both in sediments and tissues, still, a significant increase in the penis length was not observed as it was the case in other sites with significant VDSI increase. It is possible, as suggested by the previous authors, that other compounds had interfered with penis length development justifying the observed result in Vi4. Recently a study detected high seawater concentrations of

nonylphenol, a well-known xenoestrogenic, in Vigo harbor close to sites Vi2 to Vi4 (Salgueiro-González et al., 2015), possibly the result of direct discharges of untreated domestic and industrial effluents into this area. This compound was found, when mix with other xenoestrogens in an effluent sample, to decrease imposex severity and interfere with imposex development, specially RPS, in *Nucella lapillus* (Santos et al., 2008). It was also recently suggested that TBT in environment could be sometimes underestimated by imposex values due to the presence of xenoestrogenics in environment which may interfere with imposex development (Borges et al., 2013). Therefore, based on our results and the above-cited literature, we suggest that the VDSI determination in a bioassay is preferable as it is specific response for TBT pollution. As for FPL should also be assessed but results must be analysed having into account the previously discussed confounding factors. Still, the fact that the penis growth is a faster response, in a short period of time, might be useful at sites where low TBT concentrations cause insignificant VDSI growth and as it may also indicate the presence of other compounds in sediments, makes FPL a useful parameter for overall sediment pollution assessment.

In the adult snail, imposex development depends on timing, duration and level of exposure of gastropods to TBT while imposex severity represents a dose-dependent and irreversible response to the total TBT uptake throughout its life (Gibbs and Bryan, 1996). Therefore, organisms collected in imposex surveys will reflect past conditions and not the present TBT contamination. Sediment bioassays or gastropod transplanting represents a more realistic attempt to describe contemporary TBT pollution in the environment (Quintela et al., 2000; Bech et al., 2002; Duft et al., 2007). Our work identifies Vi2, Vi3 and Vi4 as sites where it will be more prone to find TBT pollution effects in gastropods. In fact, a reflection of the bioassay result can be seen on imposex levels found in 2013, since significant VDSI differences between sites can be found as in the bioassay. This is a shift as previous imposex levels registered in 2008 showed no significant difference among stations with highly VDSI values found at all sites, except Vi0 To. As seen in figure 2.6, the significant VDSI increase, observed at Vi4, in the bioassay seems to be related with the maintenance of high imposex levels between 2008 and 2013. This might indicate that since tested sediment has bioavailable TBT, imposex at this site would not decrease in the near future

(at least after 3 years). Despite the fact that we only could prove this for one site, as we were unable to find organisms in Vi2, these results may be a hint for the importance of the bioassay in monitoring programs or on the definition of pollution remediation areas. Moreover, a VDSI levels decrease in 2013 is observed in all sites that did not cause a significant imposex increase in the bioassay. This, however, does not confirm the fact that TBT is not present in the sediment and any conclusion must be taken carefully.

Water Framework Directive (WFD) (EC, 2000), and then Marine Strategy Framework Directive (MSFD) (EC, 2008), marked a new era in environmental risk assessment in Europe and new challenges were raised in order to develop quick and low-cost tools for monitoring purposes. Duft and co-workers (Duft et al., 2005; Duft et al., 2007 including the references therein) had already described the utility of the bioassay under WFD and defined Ecological Status Class (see Annex V of WFD (EC 2000) based on the bioassay results with *N. reticulatus*. In the proposed classification, Duft el al. (2007) assumes a maximum VDSI increase of 1.0 for this species in 28 days, after high TBT concentrations exposure and consequently the definition of the ecological classes were based on this premise. Since in our work a maximum increase in the VDSI of 1.5 is attained at Vi2, we decided not to follow the same classification, as it could mislead our conclusions. Also, Rodriguez et al. (2010) suggested that the transplant of *N. reticulatus* has some potential to be used in WFD for investigative monitoring (see WFD's Annex V (EC, 2000)) however, it lacks sensibility in low contaminated areas and therefore might not be useful for surveillance and operational monitoring. In their work, non-affected imposex females were transplanted, which can influence the comparison with our work where organisms were previously selected with low imposex levels. This aspect can enhance TBT sensibility in the gastropod promoting a faster imposex development during the bioassay biotest and, thus, partly overcome the limitation stressed by Rodríguez et al. (2010). In addition, the 28 day laboratory bioassay with *N. reticulatus* shows evident benefits in simplicity and cost-effectiveness compared to caging (see also biotest advantages in Duft et al. (2007)), and we can recommend it as an effect-directed biological tool for monitoring under the WFD. The bioassay, here presented, will not establish a full cause-effect response for the bulk contamination of the sediment samples tested, necessary to report the environmental status under the referred directives,

however we were able to demonstrate its effectiveness in a specific response to a contaminant that is listed on the Annex X's list of priority substances in WFD (EC, 2013) and on the Oslo and Paris (OSPAR) Commission 'List of chemicals for priority action' (OSPAR, 2013). Moreover, it might assume high relevance under the descriptor 8 of MSFD, helping to point out areas that are causing TBT pollution effects on organisms. The distribution of this species makes this bioassay suitable to be used in Europe, however this tool can also be adapted for other species sharing the same biological characteristics and TBT sensitivity in other parts of the world where concerning TBT levels are still found in environment, namely in South America (Borges et al., 2013; Paz-Villarraga et al., 2015), Africa (Okoro et al., 2013) or in Asia (Kim et al., 2014; Dong et al., 2015).

Despite all the current restrictions on TBT use, the butyltins concentrations and imposex were still found at extremely high levels in our study, similar to the previously described in highly industrialized harbours from Galicia (Barreiro et al., 2001; Ruiz et al., 2005) before the European ban in 2003 and as the ones observed in other Iberian Peninsula harbours (Couceiro et al., 2009; Rodríguez et al., 2009; Sousa et al., 2009) in surveys performed after the European ban but previous to the worldwide ban by IMO convention. A complete study performed in 1998/1999 in 5 Galician Rías by Barreiro and co-workers (2001), detected high VDSI levels inside Ría de Vigo but no sterile females were found. Also, a study performed in Vigo harbor (close to Vi2) in 2007 detected extremely high TBT values in mussels (4086 ng g^{-1} dry weight) (Vidal-Liñán et al., 2010). In 2008, and despite all the restrictions on TBT, imposex levels found were still very high and sterile females were observed in this site (13% in Vi2). Coincidently, sediments from this site caused the biggest imposex parameters increase in the bioassay and no organisms were found after several attempts in the 2013 survey. Even when there are recordings of gastropod population disappearance due to TBT pollution (Bryan et al., 1986), the known migration habits of *N. reticulatus* (Lambeck, 1984) and no previous knowledge of this population dynamics make us be precocious to infer that TBT pollution was the cause for this disappearance. Nevertheless, the entry into force of the IMO convention few months after our first survey might have been responsible for the decrease of imposex observed in 2013 at some sites,

a significant trend already observed in other studies (Lahbib et al., 2011; Cuevas et al., 2014).

2.2.6. Conclusion

In this study the species *Nassarius reticulatus* was used as part of a holistic approach to assess TBT pollution around Vigo harbour. Our results allow us to understand not only past and present TBT contamination but also to disclose which sites may still pose a risk to gastropods populations and which ones may be on the way to recover from TBT pollution. The 28 days bioassay confirmed the existence of bioavailable TBT in 2010 at some sites inducing a significant imposex increase in gastropods. The imposex levels monitored in 2013, confirmed the sites of concern previously identified in the bioassay. Therefore, the ecological significance together with a fast outcome makes this bioassay a cost-effective tool to evaluate TBT pollution suitable to be used in monitoring programs such as WFD, MSFD or OSPAR's CEMP. It can also be a useful tool to identify TBT pollution at sites where is not possible to sample an imposex indicator species or at other studies involving sediments such as dredging activities in harbours and historical hotspot for TBT pollution (Duft et al., 2007). This bioassay, should however be tested under different conditions to prove its efficacy. Further work should also be addressed in order to develop indexes associated to the bioassay as well as the role of other substances in the outcome. The imposex levels and recent TBT inputs evidenced in this study are still alarmists, however with the entrance into force of IMO convention levels are thought to decrease.

2.2.7. Bibliography

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CHAPTER 2.3 - COMBINATION OF IMPOSEX MONITORING AND SEDIMENT BIOASSAY TO ASSESS THE ECOLOGICAL QUALITY STATUS OF TWO ESTUARINE AREAS (MINHO AND LIMA, NW PORTUGAL) REGARDING TBT POLLUTION

2.3. Combination of imposex monitoring and sediment bioassay to assess the ecological quality status of two estuarine areas (Minho and Lima, NW Portugal) regarding TBT pollution

2.3.1. Abstract

Recent recommendations for the European Union (EU) directives concerning aquatic environment propose the use of effect based tools in pollution monitoring for a better integration of chemical and biological data. In order to meet those recommendations, the previous subchapters developed and proposed two different tools based on the imposex response to monitor TBT pollution under EU directives, since TBT is considered a priority substance under such legislative acts of general application in EU member states. The present study applies the previously described tools and evaluates its combined effectiveness on the assessment of TBT environmental pollution and the ecological quality status, regarding this substance, in two transitional water bodies: Minho and Lima estuarine systems (NW Portugal), and adjacent coastal waters. For this, the levels of imposex were assessed in the bioindicator species, *Nucella lapillus* and *Nassarius reticulatus*, in the two study areas, and the ecological quality status obtained according the classification developed in chapter 2.1. Also, the bioassay with *N. reticulatus* was performed with sediments collected in both estuarine systems, following the methods described in chapter 2.2. Additionally, a classification for the bioassay results was developed in order to identify sediments that may be potentially toxic to organisms, concerning its TBT concentration. Imposex expression allowed the identification of the different ecological quality status of the two estuarine areas assessed, with Minho presenting "Good" ecological status while inside the Lima estuary a "Moderate" to "At Best Poor" ecological quality was evidenced. Sediment bioassay results were in accordance to the classification of the ecological quality status obtained from imposex assessment, with Minho sediments presenting no toxicity while in Lima potentially toxic sediments at several stations (BL1, BL2, BL4 and BL7) were recorded. The combined use of these tools increases the monitored area since the absence of imposex bioindicator species can be, sometimes,

a shortcoming in TBT pollution biomonitoring. These results are discussed and highlight the relevance of the application of the proposed tools within EU Directives.

2.3.2. Introduction

Tributyltin (TBT) is a well-known substance due to its use as a biocide in antifouling paints, which contributed for an overall spread of this pollutant throughout the aquatic ecosystems. One of the most well-known biological adverse effect of TBT pollution is the female masculinization (imposex and intersex) of gastropods, a phenomenon already described for more than 260 species (Titley-O'Neal et al., 2011). At more advanced imposex or intersex stages, reproductive failure may occur as females become sterile, leading to population decline or extinction (Bryan et al., 1986; Oehlmann et al., 1996). In Europe the directive (89/677/CEE) banned the application of TBT based antifouling paints in small vessels (< 25 m) but this was proved to be inefficient in many coastal areas (Barreiro et al., 2001; Barroso and Moreira 2002; Santos et al., 2002). Subsequently, in 2002, the European Union (EU) introduced the Directive 2002/62/EC that banned the application of organotin antifouling paints in all kind of vessels after 1 January 2003 and forbad the circulation of any boat with these paints in EU waters after 2008.

Over the last decades, the EU has striven to maintain or recover the aquatic ecological status with directives such as the Water Framework Directive (WFD; Directive 2000/60/EC) (EC, 2000) or the Marine Strategy Framework Directive (MSFD; Directive 2008/56/EC) (EC, 2008). Herewith, new opportunities and challenges to develop quick and low-cost monitoring tools emerged to meet these directive demands. Regarding TBT, a priority substance listed in WFD's Annex X, several efforts have already been taken to develop new tools to monitor *in situ* TBT biological effects (Oehlmann, 2002; WFD-UKTAG, 2014; Laranjeiro et al., 2015b) and environmental concentrations (Alasonati et al., 2015; Moscoso-Pérez et al., 2015), and also bioassays to detect TBT in the environment (Rodríguez et al., 2010; Laranjeiro et al., 2015a).

The aim of this work is to test the effectiveness of the new tools developed in this thesis (previous sub-chapters) for TBT pollution monitoring under the WFD, namely (i)

imposex field surveys for ecological quality status assessment (Laranjeiro et al., 2015b) and (ii) laboratory bioassays for TBT sediment contamination assessment (Laranjeiro et al., 2015a). To accomplish this objective, the ecological quality status regarding TBT pollution in two estuarine areas (Minho and Lima), located in the northwest of Portugal, was evaluated through these tools' combined use.

2.3.3. Methods

The imposex levels in the gastropod species, *Nucella lapillus* (dog-whelk) and *Nassarius reticulatus* (netted-whelk), were assessed from January to July 2012 at stations located at the river, estuary or in the adjacent marine coast of Minho and Lima basins (see figure 2.7); for the sake of simplicity these areas will be referred henceforth only as "Minho" and "Lima". Individuals of both species were collected by hand in intertidal areas while for *N. reticulatus* baited hoop nets were also used for underwater sampling. In the laboratory, organisms were kept in aquaria and observed within 2 days. Before imposex analysis, shell heights were measured with Vernier callipers to the nearest 0.1 mm, being afterwards cracked open with a bench vice. Then organisms were sexed and dissected under a stereo microscope.

Dog-whelks were analysed without narcotisation and the percentage of imposex-affected females (%I), the mean female penis length (FPL), the mean male penis length (MPL), the relative penis size index (RPSI = FPL^3x100/MPL^3) and the vas deferens sequence index (VDSI) were determined at each location. The VDS was classified according to the scoring system developed by Gibbs and co-workers (1987) for this species. On the other hand, netted-whelks were narcotised by immersion in $MgCl_2$ 7% solution in distilled water for about 45 min prior to imposex analysis and the following indices were used to classify imposex levels at each location: %I, FPL, MPL, the relative penis length (RPL = FPLx100/MPL) and VDSI. *N. reticulatus* VDS was classified according to the scoring system developed by Stroben et al., (1992) and VDS stage 4+ was computed as 4 for VDSI final calculation.

Following the criteria proposed by Laranjeiro et al. (2015b), that intends to integrate imposex assessment within WFD monitoring, we also classified the ecological status

regarding TBT pollution for each station. Hence, to proceed accordingly to the WFD, imposex values were computed to Ecological Quality Ratios (EQR) through the equation: $EQR_{\text{imposex}} = \frac{(M-O)}{M}$, where M is the maximum VDSI score that a population may attain and O is the observed VDSI value for a population at one given site. This procedure renders a value between 0 and 1 as demanded by the WFD to guarantee comparability between EU member states in all biological monitoring results. For an extended description of this classification scheme please consult Laranjeiro et al. (2015b).

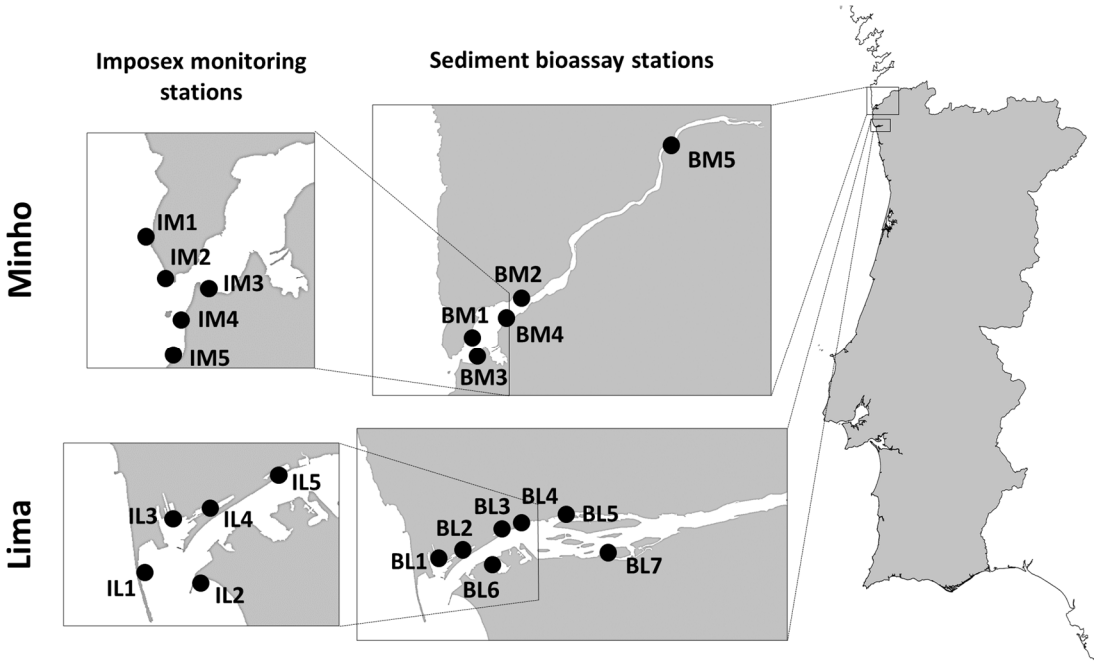

Figure 2.7 - Map showing the sampling stations in Minho and Lima for field imposex monitoring (IM and IL, respectively) and for the collection of sediments to be used in laboratory bioassays (BM and BL, respectively).

Sediment bioassay

Surface sediments (< 5 cm sediment depth) were collected in polyethylene bags in May (in Minho) and July (in Lima) in 2012, at each station (BM and BL in figure 2.7). The sampling was made with a Van Veen grab or a shovel, depending on the depth, and stored in the dark at 4 °C for a maximum of five days until the beginning of the bioassay. Selected sediments were frozen at -20 °C for TBT chemical quantification.

The sediment bioassay with *N. reticulatus* was adapted from the work of Duft et al. (Duft et al., 2005; Duft et al., 2007) and described in Laranjeiro et al. (2015a). Adult individuals of *N. reticulatus* employed in the assay were collected at a station previously known to have low imposex levels (Vi0 To, as termed in section 2.2.) located in Ria de Vigo, Spain (Laranjeiro et al., 2015a). Before the bioassay, an assessment of the imposex levels of this population indicate that 38% of the females in this population were affected by imposex and presented VDSI = 0.62. After narcotization in $MgCl_2$ 7% in distilled water, organisms were sexed under a stereo microscope and the presence of the penis in females, and its length, was registered by gently pushing the foot, allowing the observation of the body to some extent. Only females with penis length < 1 mm and presenting signs of imposex (VDSI = 1, 2 or 3, where the vas deferens, if exists, does not extend far from the base of the penis) were selected for the bioassay and left to recover from narcotization during one week in 40 L tanks with constant seawater flow. The discarded organisms were returned to the environment.

At the beginning of the bioassay, sediment collected at each site was placed in 2L glass beakers (3 replicates) in order to form a 2 - 3 cm layer. One liter of 1 µm filtered seawater was added to each beaker in order to achieve a volume ratio sediment/water of 1:5, and the mixture was left to settle overnight. Afterwards, 10 animals were added to each beaker starting the 28 - day bioassay at 18±1 °C, under constant seawater aeration, and with weekly water renewal without any food supply. The "control" sediment was collected at the same site as the organisms employed in the bioassay (Vi0 To), while other sediment, also collected at Ria de Vigo (Spain) and known to be highly polluted (Vi2, see section 2.2; Laranjeiro et al., 2015a), was used as positive control.

At the end of the experiment, the organisms were again narcotized and measured with callipers to the nearest 0.5 mm. The shells were cracked open with a bench vice and the animals were sexed and dissected under a stereo microscope for imposex analysis. The imposex indices FPL and VDS (according to the scoring system of Stroben et al. (1992) as described above for this species) were assessed in all exposed organisms.

A simultaneous experiment with TBT-spiked sediments at increasing concentrations was performed. This aims to reach a maximum FPL and VDSI increase in 28 days, allowing therefore a better understanding of the impact of the sediment exposure to the organisms. Hence, specimens were exposed (also 3 replicates) to the following TBT concentrations in the sediment: 10, 20, 40, 80, 160 and 320 ng TBT/g wet weight (ww), using DMSO as a solvent. For the spiking procedure, control sediment (Vi0 To) was collected, weighted, and placed in 2 L beakers. Sediments were spiked with a correspondent volume of TBT solution (670 µL was the maximum volume of solution used at the highest concentration) and homogenized by stirring. No water was added in this process; however, due to the presence of interstitial water, the sediment was not completely dried. Sediment was left overnight to allow TBT adsorption to sediment particles. Afterwards, seawater was added to each beaker and left to settle as previously described. This assay was performed respecting the same parameters, duration, and endpoints, as the previous bioassay.

To disclose differences between treatments in both bioassays, statistical analyses were performed with IBM SPSS Statistics 22 Software. Statistically significant differences to control were evaluated through one-way ANOVA, followed by Dunnett's post hoc test, after confirming data normality and homoscedasticity.

Since the aim of this work is to describe the ecological quality status in Minho and Lima, a classification for the sediment bioassay results was developed. For this, the spiking bioassay is essential to understand the maximum VDSI development that exposed organisms can attain for the duration of the bioassay. A direct comparison between organisms exposed to natural sediments and the control might not be so informative since a maximum VDSI increase is not predictable for 28 days. On the other hand, VDSI development can depend on several factors like, for instance, seasonality (Sternberg et al., 2008)) and the provenience of the animals used in the bioassay. Consequently, a maximum VDSI increase might differ from one experiment to another. The combined performance of sediment bioassay and spiking bioassay is, therefore, required in this classification proposal, which is based on the percentage of female's VDSI increase induced by natural sediments. This is obtained following the equation 1:

$$\%VDSI_{\text{increase natural sediments}} = \frac{N-C}{S-C} \times 100,$$

where N is the VDSI obtained at a given station at the end of the bioassay, S is the maximum VDSI obtained at the spiking bioassay (a maximum effect should be observed) and C is the VDSI registered in the control group. This ensures comparability between different bioassays because it expresses the ratio of effect caused by natural sediments.

Several works on sediment toxicity tests consider three toxicity categories: non-toxic, potentially toxic and toxic (Reynoldson et al., 2000; Chapman and Anderson, 2005; Feiler et al., 2013). In this work, three classes are also considered even if the WFD classification defines five ecological status classes. This seems the most appropriate decision since the definition of five categories would be extremely difficult based only in a single endpoint (VDSI increase). Chapman and Anderson (2005) consider that a sediment can be potentially toxic if the bioassay endpoint differs more than 20%, and is statistically significant, in relation to control and toxic if this effect surpasses 50%. Here, the same values are adopted to define the toxicity categories, but the percentage of effect is referred not to the control but to the maximum VDSI increase observed in the spiking bioassay. A sediment causing a VDSI increase larger than 20% of the maximum increase observed in the spiking bioassay, and if statistically significant in comparison to control, should be considered to present an endocrine disruption potential, while a sediment causing more than 50% of VDSI increase should have doubtlessly an endocrine disruption effect. Setting a comparison with WFD, a sediment presenting an endocrine disruption potential (causes a VDSI increase greater than 20%) would correspond to the “Moderate” ecological quality status, as at this class it is observed a significant deviation from undisturbed conditions and action by member states is required in order to improve the ecological quality.

Butyltin analysis

Monobutyltin (MBT), dibutyltin (DBT) and TBT were quantified in *N. lapillus* tissues of females collected around Minho and Lima. The chemical analysis of this species tissues allow a better evaluation of current TBT contamination in water since *N. reticulatus* is a

sediment dweller, which is more prone to reflect sediment contamination. The analysis of netted-whelk tissues can therefore distort the evaluation of the current TBT pollution since sediment can act as a sink of TBT, accumulating the substance during decades. Dog-whelk samples consisted of a pool of the whole soft tissues of 10 - 15 females after imposex analysis. Butyltins' concentrations were also quantified in the collected sediments. Both matrices analyses were performed in the certified laboratory UT2A – Ultra Trace Analyses Aquitaine (Pau, France), using the methods described by Lopes-dos-Santos et al. (2014). Detection limits (DL) in both matrices, expressed in ng Sn/g dry weight (dw), were 2 for MBT and 0.5 for DBT and TBT.

2.3.4. Results

Imposex monitoring and Ecological Quality Status

Results from imposex monitoring performed in Minho and Lima are shown in table 2.7. Low levels of imposex were found in *N. lapillus* and *N. reticulatus* in Minho but a different scenario was observed in Lima where higher levels of imposex were registered. In Minho, the %I varied between 54 and 94% in the dog-whelk and between 44 and 72% in the netted-whelk. The values of VDSI and RPSI varied between 0.55 - 1.63 and from 0.00 - 0.03% in the dog-whelk, respectively, while for the nassariid the VDSI and RPL ranged between 0.56 - 0.72 and 0.13 - 0.89%, respectively. In Lima, the %I varied between 82 and 100% in the dog-whelk and between 69 and 100% in the netted-whelk. The values of VDSI and RPSI varied between 1.00 - 3.23 and from 0.01 - 9.62% in the dog-whelk, correspondingly, while for the nassariid the VDSI and RPL ranged between 1.28 - 3.79 and 12.47 - 70.53%.

Table 2.7 - Levels of imposex found in this study. Number of females and males analysed (N♀ and N♂, respectively); mean female and male shell height (SH♀ and SH♂, respectively); percentage of imposex-affected females (%I); relative penis size index (RPSI) in *Nucella lapillus*; relative penis length (RPL) in *Nassarius reticulatus*; vas deferens sequence index (VDSI).

	Station	N ♀	SH ♀	N ♂	SH ♂	% I	RPSI/RPL	VDSI
Minho	***Nucella lapillus***							
	IM1	30	21.58 ± 3.91	29	21.52 ± 2.51	88	0.00	1.08 ± 0.57
	IM2	30	23.57 ± 1.55	30	24.10 ± 1.41	55	0.00	0.55 ± 0.50
	IM3	14	30.13 ± 2.45	26	29.29 ± 2.86	94	0.03	1.63 ± 0.81
	IM4	30	26.13 ± 2.86	30	25.90 ± 1.84	66	0.00	0.69 ± 0.47
	IM5	30	27.74 ± 2.08	30	24.86 ± 2.17	54	0.00	0.59 ± 0.55
	Nassarius reticulatus							
	IM2	32	24.43 ± 2.52	34	23.86 ± 1.91	72	0.13	0.72 ± 0.46
	IM5	34	24.67 ± 1.78	23	22.84 ± 2.16	44	0.89	0.56 ± 0.79
Lima	***Nucella lapillus***							
	IL1	30	23.35 ± 1.76	23	23.33 ± 2.14	100	9.62	3.23 ± 0.77
	IL2	34	23.60 ± 1.63	21	23.41 ± 1.35	82	0.01	1.00 ± 0.60
	Nassarius reticulatus							
	IL3	34	26.20 ± 1.95	19	22.98 ± 1.12	100	70.53	3.79 ± 0.41
	IL4	27	27.59 ± 2.16	19	22.94 ± 0.92	100	66.31	3.33 ± 0.48
	IL5	32	26.65 ± 2.74	19	22.34 ± 1.11	69	12.47	1.28 ± 1.14

The ecological status of the studied areas, regarding TBT pollution, is presented in figure 2.8. Following the classification proposed by Laranjeiro et al. (2015b), and also displayed in figure 2.8, both bioindicators evidence a good ecological status regarding TBT pollution at all Minho stations as a consequence of the low imposex levels observed in this area. However, a different scenario is depicted in Lima. The biomonitoring with *N. lapillus* reveals that the station located outside the estuarine area (IL2) evidence a good ecological status while the station located inside this area (IL1) indicates a moderate ecological status regarding TBT pollution. Since we could not find *N. lapillus* further inside the estuarine area, the use of more TBT bioindicators is essential. Therefore biomonitoring with *N. reticulatus* allowed us to identify the at best poor ecological status in stations IL3 and IL4 and a moderate status at station IL5.

Nucella lapillus			*Nassarius reticulatus*		
Ecological status class	EQR classes	Corresponding VDSI	Ecological status class	EQR classes	Corresponding VDSI
High	[1.00 - 0.95[	[0.00 - 0.30[	At Least Good	[1.00 - 0.93[	[0.00 - 0.28[
Good	[0.95 - 0.50[	[0.30 - 3.00[	Good	[0.93 - 0.80[	[0.28 - 0.80[
Moderate	[0.50 - 0.25[	[3.00 - 4.50[	Moderate	[0.80 - 0.40[	[0.80 - 2.40[
Poor	[0.25 - 0.00]	[4.50 - 6.00]	At best Poor	[0.40 - 0.00]	[2.40 - 4.00]
Bad	Population Absent				

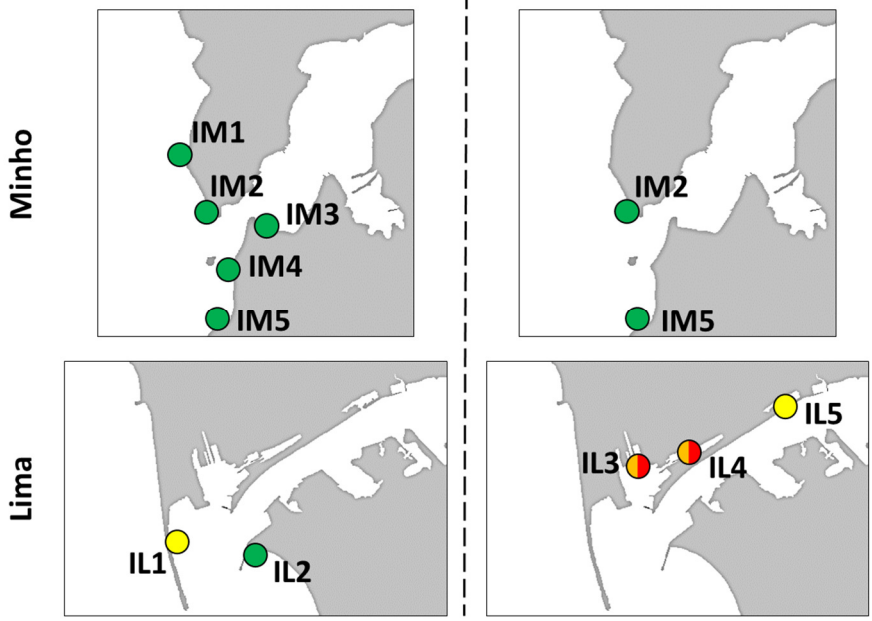

Figure 2.8 - Classification of the ecological quality status of Minho and Lima regarding TBT pollution based on the imposex levels of two gastropod species*: Nucella lapillus* and *Nassarius reticulatus*. This assessment is performed according to the WFD Ecological Quality classification proposed by Laranjeiro et al. (2015b).

The results of the sediment bioassay are shown in figure 2.9. Statistically significant differences in VDSI, in relation to control, were observed in organisms exposed to sediments from site Vi2 (sediment used as positive control) ($p < 0.001$) and BL1 ($p < 0.01$), whereas no significant differences were observed for the other sediments. Even so, most of the sediments collected in Lima induced an increase in VDSI, with BL2 being close to statistical significance ($p = 0.104$). Organisms exposed to sediments from station Vi2 had a VDSI increase slightly higher than 1.0. The same sediments caused statistically significant

rises in FPL: Vi2 ($p < 0.001$) and BL1 ($p < 0.05$). Again, Vi2 showed the highest increase (about 1.30 mm) in relation to control.

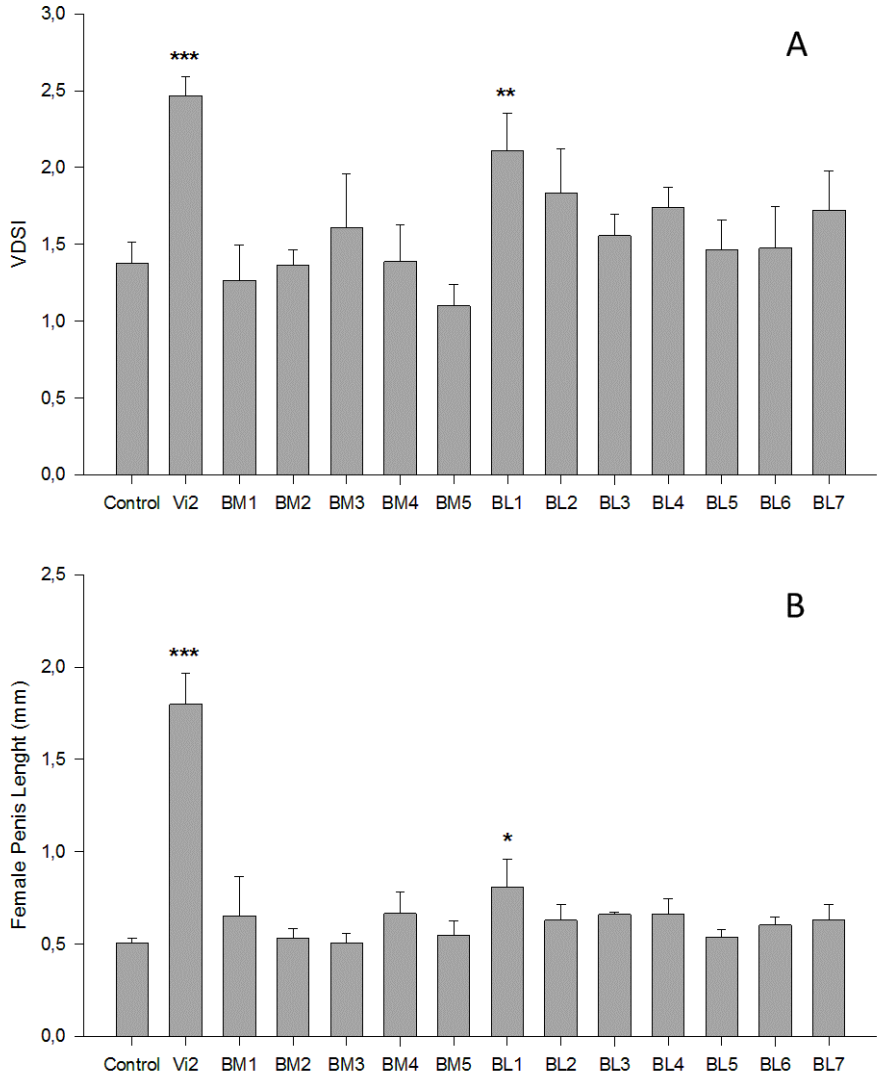

Figure 2.9 - Vas Deferens Sequence index (VDSI) and mean female penis length (FPL) registered in test organisms after 28 days of exposure to sediments collected in Minho and Lima (BM1-BM5 and BL1-BL7, respectively). Statistically significant differences to Control are represented by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

In order to better understand the magnitude of the effects, a simultaneous test with TBT spiked sediments was performed and the results are presented in figure 2.10. The idea was to obtain the maximum VDSI and FPL increase after 28 days of exposure. This maximum increase, in both VDSI and FPL, reaches a plateau at the 3 last concentrations tested (80, 160 and 320 ng TBT/g ww), which are significantly different ($p < 0.001$) from the control. For VDSI, statistically significant differences are already observed at TBT concentrations as

low as 20 TBT/g ww ($p < 0.05$). For FPL, statistically significant differences started to be observed at a TBT concentration of 40 ng ($p < 0.05$) and then a sharp FPL increase was observed in the following concentrations (about 3 mm).

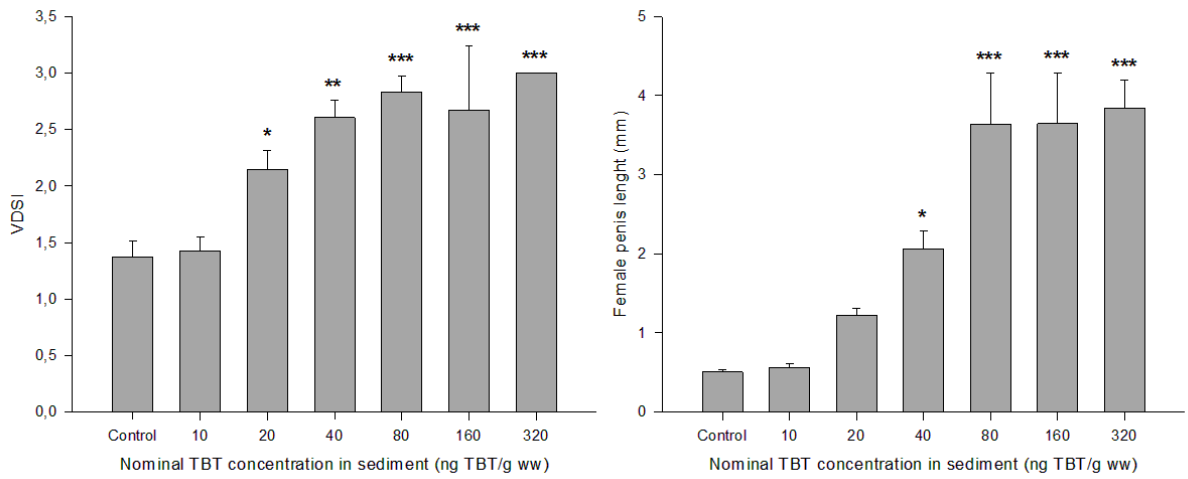

Figure 2.10 - Vas Deferens Sequence Index (VDSI) and mean female penis length (FPL) registered in test organisms after 28 days of exposure to spiked sediments with increasing TBT concentrations. Statistically significant differences to Control are represented by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Figure 2.11 indicates the percentage of VDSI increase induced by the natural sediments, computed following equation 1 described in the Methods section. It is possible to observe that sediments from station Vi2 and BL1 caused about 50% of the maximum VDSI increase. Sediments from other stations – BL2, BL4 and BL7 – induced a VDSI increase of more than 20%. However, these are not considered to have endocrine disruption potential because they do not differ significantly from the control (see figure 2.9 A).

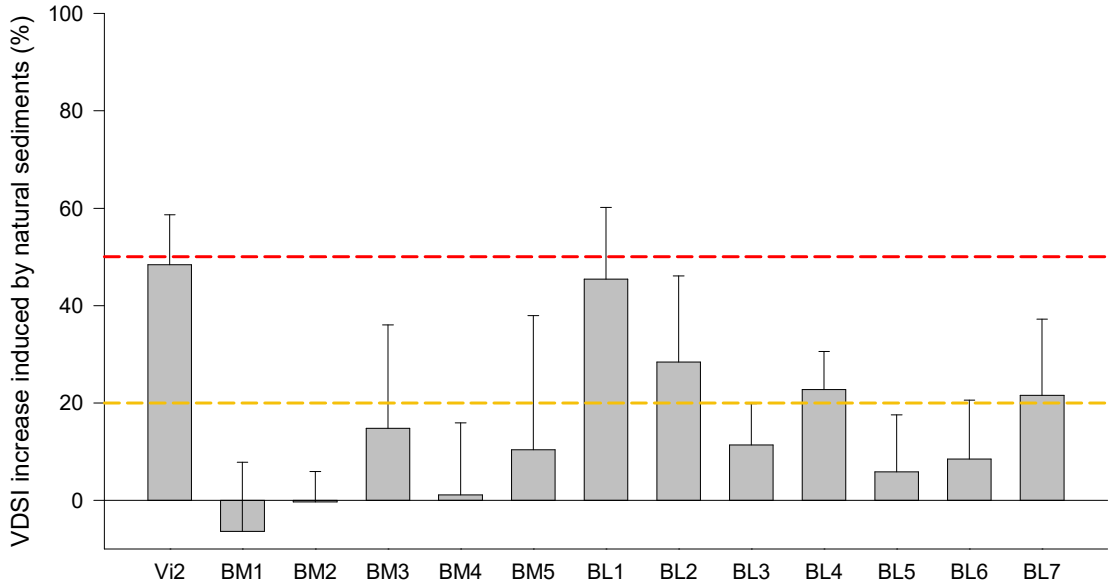

Figure 2.11 - The percentage of VDSI increase induced by the natural sediments, obtained following equation 1. Dashed lines represent the limits between non-toxic to potentially toxic (yellow line at 20%) and between potentially toxic to toxic (red line at 50%).

Butyltin analysis

Butyltin concentrations in the soft tissues of *N. lapillus* females analysed during the imposex monitoring study, as well as in the sediments collected for the sediment bioassay are shown in table 2.8. Butyltin levels in tissues were found at relatively low concentrations in both Minho and Lima. TBT ranged between 8.3 - 37 ng Sn/g dw while DBT and MBT ranged between 5 - 24 ng Sn/g dw and < LOQ – 4.7 ng Sn/g dw, respectively. Dog-whelks collected in IL1 presented the highest TBT level as expected from imposex analysis as this was the site where females were more masculinised. Butyltin degradation index [BDI = ([MBT] + [DBT])/[TBT])] proposed by Diez et al. (2002), revealed that 3 stations from Minho (IM2, IM3, IM4) and the 2 stations from Lima (IL1 and IL2) experienced recent TBT inputs indicated by BDI < 1. The butyltin levels found in sediments reveal that Lima is far more contaminated by these organotins than Minho. In fact, the TBT concentrations found in Minho sediments ranged between < LOQ - 4.7 ng Sn/g dw, while DBT and MBT varied between < LOQ – 3.0 and < LOQ - 4.7 ng Sn/g dw, respectively. In Lima sediments, butyltin levels varied between 0.7 - 352 ng Sn/g dw for TBT, 0.5 - 141 ng Sn/g dw for DBT and < LOQ

- 73 ng Sn/g dw for MBT. Stations BL1 and BL2 exhibited the highest TBT concentrations, 329 and 352 ng Sn/g dw, respectively. BDI also indicate the occurrence of recent TBT inputs in almost half of the sites surveyed.

Table 2.8 - Butyltin concentrations (in ng Sn/g dw) quantified in *Nucella lapillus* tissues samples, collected during imposex monitoring assessment, and in sediments used in the bioassay. MBT – Monobutyltin, DBT – Dibutyltin, TBT – Tributyltin, BDI – Butyltin Degradation Index, LOQ – Limit of Quantification.

	Station	MBT	DBT	TBT	BDI
***N. lapillus* tissues concentrations**	**IM1**	3.7 ± 0.1	19 ± 3	15.5 ± 0.9	1.46
	IM2	< LOQ	6.3 ± 0.9	8.3 ± 0.5	0.88
	IM3	< LOQ	5 ± 1	10.6 ± 0.9	0.57
	IM4	2.1 ± 0.1	6.8 ± 0.3	18 ± 1	0.49
	IM5	3.5 ± 0.2	24 ± 1	21 ± 1	1.31
	IL1	4.7 ± 0.2	22 ± 3	37 ± 5	0.72
	IL2	< LOQ	6.8 ± 0.9	9.2 ± 0.8	0.85
Sediment concentrations	**BM1**	< LOQ	< LOQ	< LOQ	-
	BM2	< LOQ	< LOQ	< LOQ	-
	BM3	4.7 ± 0.5	3.0 ± 0.4	4.7 ± 0.5	1.64
	BM4	2.2 ± 0.4	1.55 ± 0.05	4.2 ± 0.8	0.89
	BM5	< LOQ	< LOQ	< LOQ	-
	BL1	56 ± 6	129 ± 2	329 ± 30	0.56
	BL2	73 ± 8	141 ± 25	352 ± 85	0.61
	BL3	12 ± 0.6	6.7 ± 0.7	14 ± 2	1.34
	BL4	13 ± 0.9	10.1 ± 0.5	24 ± 2	0.96
	BL5	12.2 ± 0.3	6.6 ± 0.1	15 ± 2	1.25
	BL6	< LOQ	0.5 ± 0.1	0.7 ± 0.2	2.14
	BL7	2.7 ± 0.5	2.2 ± 0.7	3.2 ± 0.7	1.53
	LOQ	2	0.5	0.5	

2.3.5. Discussion

The aim of this work was to assess the ecological quality status of Minho and Lima regarding TBT pollution, by combining the effect based tools proposed in previous sub-chapters (sections 2.1. and 2.2.). So, the results obtained from the imposex field survey using *N. lapillus* and *N. reticulatus* as bioindicator species (as proposed by Laranjeiro et al. (2015b) were compared with those acquired from the laboratory sediment bioassay using *N. reticulatus* (as proposed by Laranjeiro et al. (2015a). The first tool provides an indication

of the current levels of imposex in the populations while the second tool gives an indication of the presence of bioavailable TBT in sediments.

Imposex values evidenced *in situ* by the indicator species show a low level of TBT pollution in Minho. VDSI values observed are generally low and none of the stations register 100% of imposex-affected females. Even so, it is possible to observe some TBT pollution spatial trend since the highest value, as shown by *N. lapillus*, was found inside the estuary at station IM3 (VDSI = 1.63). This might be related with regular boat activity, such as the ferry boat linking the two banks of River Minho, or with the presence of a large number of small fishing boats. These should still represent the main sources of TBT contamination in this area as previously identified by Carvalho et al. (2009). Moreover, the influence of the estuary as a TBT pollution source can also be perceived by the higher imposex values evidenced by *N. reticulatus* near the river mouth and by the BDI analysis in *N. lapillus* tissues, with station IM3 located inside the estuary and stations IM2 and IM4 near the mouth evidencing recent TBT inputs. Still, the TBT pollution can be considered low and prone to cause negligible effects in gastropod populations.

A different scenario is found in Lima where imposex levels are quite higher, which was predictable given the previous imposex studies performed in this area (Barroso et al., 2002; Rato et al., 2009; Sousa et al., 2009). These studies reported the shipyard "Estaleiros de Viana do Castelo" (nearby station IL3) as the main TBT source within this estuary. The present study confirms that the highest VDSI value for *N. reticulatus* is, in fact, found in the immediate vicinity of this site (IL3), being observed a gradual VDSI decrease associated with the distance to this point, both downstream (IL3 > IL1 > IL2) and upstream (IL3 > IL4 > IL5). Station IL4, located inside a fishing port, also presents considerably high imposex levels, with fishing boats traffic being most likely the reason for such an occurrence. On the other hand, the marina (IL5), also considered a source of TBT contamination in previous studies and with similar VDSI levels of that of the shipyard in 2000 (VDSI = 3.9 in both sites; Barroso et al., 2002), seems that is now recovering rapidly, with a VDSI = 3.0 in 2008 (Sousa et al., 2009) and VDSI = 1.28 currently. As for *N. lapillus*, station IL1, located inside the estuary and directly influenced by the previously identified TBT sources, presents higher VDSI values than IL2, which is located outside the estuary and may receive less polluted currents

from offshore. The chemical analysis of *N. lapillus* female tissues also confirms this pattern, with higher concentrations of TBT being found inside the estuary (IL1); still, recent TBT inputs (BDI < 1) are registered in both stations.

Following the proposal of Laranjeiro et al. (2015b), where OSPAR's imposex assessment classes were adapted to meet the WFD requirements (figure 2.8), the ecological quality status regarding TBT pollution in the two areas are distinct. In Minho, both bioindicators evidence the "Good" ecological status in all assessed stations, which is in agreement with previous studies that describe this as a low contaminated area (Rodrigues et al. 2014; Capela et al. 2016). Regarding Lima, even if the station located outside the estuary (IL2) presents "Good" ecological status, the situation inside this estuary is considerably worse. Stations IL1 and IL5 present "Moderate" ecological status given by *N. lapillus* and *N. reticulatus*, respectively, but stations IL3 and IL4 are far worse as they present "at best Poor" ecological status, according to the imposex levels observed in *N. reticulatus*. As a sediment dweller, the netted-whelk is exposed to the sediments and consequently will more likely reflect TBT contamination found in this compartment (Barroso et al., 2000). Although being currently forbidden TBT application in boats, TBT compounds easily accumulate in the sediment and, through remobilization or desorption processes, might become available to organisms, including the ones in the water column (Sarradin et al., 1995; Langston et al., 2015; Ruiz et al., 2015). Since this is an area subjected to intense boat traffic and shipyard activities, TBT accumulation in sediments may have occurred for years and may presently constitute the main source of pollution by TBT compounds. The fact that sediments at BL1 and BL2 are still highly contaminated (in the range of 300 - 400 ng Sn/g dw), despite TBT-based antifouling paints have been banned in 2008, supports the concept that sediments act as a sink for this pollutant. Laranjeiro et al., (2015b) proposed *N. lapillus* as a key bioindicator within WFD monitoring programs for TBT pollution due to the species high sensitivity to this pollutant, and to the fact that it better reflects water TBT contamination over other species, since it inhabits this compartment. Nevertheless, the present results confirm the idea that the use of more species, such as *N. reticulatus*, is extremely useful in monitoring as it allows to cover a wider area and types of habitat.

Even if imposex is considered one of the best biomarkers and effect based tool in environmental pollution monitoring (Forbes et al., 2006; Wernersson et al., 2015), it is also an irreversible phenomenon that produces permanent effects in organisms. Therefore, imposex levels found *in situ* expresses an effect that corresponds to the TBT accumulated throughout the organism life and most likely not the current status of contamination. The sediment bioassay that was performed can better retrieve a result that expresses the current levels of bioavailable TBT in the environment, evidencing its great ecological relevance. The results indicate that TBT concentrations found in Minho sediments (table 2.8) do not cause a significant increase in the VDSI or FPL values during the exposure period considered (28 days) and all were classified as "non-toxic". This result supports the classification of a good ecological status evidenced by the imposex monitoring in this area. By the contrary, sediments BL1 and BL2 from Lima were considered potentially toxic and one almost reached the classification of "toxic" (BL1), which also corroborates the imposex data observed *in situ.* The classification scheme for the bioassay was developed in order to categorize the sediment toxicity since the aim of this work is to describe the ecological quality status in both estuarine areas. The use of this bioassay under the WFD was previously suggested by Duft et al. (2007). These authors proposed a classification of the results in five ecological status classes as defined by WFD, considering a maximum VDSI increase of 1.0 in one month. The moderate or lower ecological status (that demands actions by EU member states to improve ecological quality at that site) is defined by these authors for a VDSI increase superior to 0.2. This is in good agreement to the boundary of 20% set in this work between a non-toxic and potentially toxic sediment, following their assumption of a maximum VDSI increase of 1.0. However, the results presented in figure 2.9-A show a VDSI increase superior to 1.0 in sediments from Vi2, and it is even greater in the spiking bioassay that defines the maximum VDSI increase in 28 days. Therefore, if this is larger than 1.0 the classification proposed by Duft et al. (2007) cannot be applied in this work. Furthermore, the definition of other three boundaries for the five ecological quality status, as defined by WFD, does not seem to be an easy and assertive task. By comparing the bioassay classification proposed with ecological quality status given by imposex monitoring assessment it is possible to spot some affinity, even if, as referred above, the

former detects current contamination while the latter expresses the result of a bioaccumulative process. The sediments from stations BL1 and BL2 that are considered potentially toxic were collected at the same stations that were considered as having "at best poor" quality status by the imposex registered in *N. reticulatus*. Sediments from BL3 were considered non-toxic while at the same station a moderate quality status was evidenced, however, as previously said, a rapidly recover in imposex levels has been registered in later years. This might indicate that the TBT bioavailable in the sediments is currently at low levels and was not enough to cause a significant increase during the bioassay, which does not prevent that the population still evidences a moderate quality status as specimens were exposed for a longer time to this pollutant (a bioassay of more than 28 days should be considered in future studies).

One of the most important limitations of assessing imposex in natural populations of gastropods is the lack of samples in some areas, even if a multi-species approach is applied. This is of paramount importance because many times there are no bioindicators available in a given study area, in spite of an intense prospection effort devoted for searching these species, as it was the case in the current study. In fact, in Minho we could only found bioindicators around the mouth of the estuary, which may reflect a better pollution condition at that region. In turn, in Lima we were able to cover a little bit more upstream the estuary but, even so, we were not be able to survey the whole estuarine area. In this sense, the sediment bioassay may be very useful because it provides the assessment of a wider area and allows a more complete evaluation of the ecological status of transitional water bodies.

2.3.6. Conclusions

Minho and Lima showed a distinct level of TBT pollution and, consequently, a distinct ecological quality status regarding this specific priority substance. The imposex levels assessment performed indicated that Minho presents “Good” ecological status in all assessed stations, while Lima is more severely polluted with all sites located inside the

estuarine area presenting “Moderate” or worse ecological status. The sediment bioassay applied confirms the worse ecological quality status of this area.

Both tools applied gave comparable results, meaning that the single use of one of them would contribute to assess the ecological quality status of a given area. However, further studies are still needed to confirm the suitability of the sediment bioassay under the WFD monitoring. Anyway, the combined use of both procedures has several advantages, such as an extended area coverage, the assessment of the ecological quality status in different environmental matrices/compartments or the comparison between the current bioavailability of TBT in sediments and *in situ* imposex levels, which can be an instrument to predict future imposex trends.

The classification developed in this work for the sediment bioassay aims to give a categorization of the VDSI increase observed. Even if does not respect the classification in five ecological quality status classes, as demanded by the WFD, it can help to make a decision on whether or not the ecological quality status of a given site should be improved. Therefore, it can be a useful tool to be used by member states under WFD monitoring programs.

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CHAPTER 3 - IMPOSEX AS A BIOMARKER FOR MONITORING TBT POLLUTION ALONG THE PORTUGUESE COAST

3. IMPOSEX AS A BIOMARKER FOR MONITORING TBT POLLUTION ALONG THE PORTUGUESE COAST

"In this great future,
You can't forget your past… "

in Bob Marley's *No woman, no cry*, 1974

The continuous evolution in the toxicology and ecotoxicology research field must not underestimate the significance of the environmental pollution monitoring. This jeopardizes the construction of historical data sets on environmental pollution, which is of extreme importance to assist future generations in decision making and to avoid History repeating itself.

Recognizing the major importance of historical data, as also acknowledged in WFD, this chapter aims to analyse TBT and imposex data since the year 2000, which will allow to understand the current TBT pollution trends and its spatial distribution along the Portuguese coast. It is also intended to depict the current ecological quality status of Portuguese coastal and transitional water bodies based on the tool developed in Chapter 2.1 and look in perspective the evolution of this status over the last decades.

CHAPTER 3.1 - FIFTEEN YEARS OF TBT POLLUTION MONITORING ALONG THE PORTUGUESE COAST

3.1. Fifteen years of TBT pollution monitoring along the Portuguese coast

3.1.1. Abstract

IMO's Anti-Fouling Systems convention banned the use of organotin-based antifouling systems in 2008 as the ultimate effort to stop tributyltin (TBT) inputs into the marine environment. One of the hazardous effects of TBT is imposex (the superimposition of male sexual characters onto gastropod females), a phenomenon that may cause female sterility and the decline of gastropod populations in polluted locations. Despite previous European Union legislation had already been shown effective in reducing the imposex levels along the Portuguese coast, this study intends to confirm these decreasing trends after 2008 and describe the global evolution in the last 15 years. Imposex levels were assessed in two bioindicators – the dog-whelk *Nucella lapillus* and the netted-whelk *Nassarius reticulatus* (Gastropoda, Prosobranchia) – in 2011 and 2014, and the results were compared with previous years. Both species showed progressive decreasing trends in imposex levels over the last 15 years; within this time-lapse median values of the vas deferens sequence index (VDSI) fell from 3.96 to 0.78 in *N. lapillus* and from 3.39 to 0.29 in *N. reticulatus*. Imposex in *N. lapillus* presented a more pronounced decrease than in *N. reticulatus*, but levels seem to have stabilized in recent years. The temporal/spatial evolution of imposex suggests an apparent shift of TBT hotspots, being now restricted to fishing ports and marinas in detriment of large commercial harbours where TBT levels fell rapidly. Butyltins were measured in the whole tissues of *N. lapillus* females collected in 2014: monobutyltin (MBT) varied from < DL (detection limit: 1 ng Sn/g) to 13 ng Sn/g dw, dibutyltin (DBT) from 2.2 to 27 ng Sn/g dw and TBT from 1.5 to 55 ng Sn/g dw. Although TBT body burden has declined over time, the butyltin degradation index ([MBT]+[DBT])/[TBT]) exhibited values < 1 in c.a. 90% of the sites assessed, suggesting that recent TBT inputs are still widespread in the Portuguese coast probably due to illegal use of TBT antifouling systems and also to TBT remobilization from sediments.

3.1.2. Introduction

Tributyltin (TBT) is an organotin compound of anthropogenic origin that was used as a biocide in antifouling systems (AFS) since the 60's. TBT release to the aquatic environment caused well-documented adverse effects in non-target biota around the world (Alzieu, 1991; Fent, 1996; Hoch, 2001) leading to legal restrictions of organotin (OT) based AFS. In Europe the use of TBT-AFS on small boats (< 25 m) was banned by the Directive 89/677/EEC. More recently, the European Union (EU) implemented the Regulation 782/2003/EC that banned the application of TBT-AFS in all vessels by July 2003 and prohibited the sailing of ships bearing these coatings from the 1st January 2008. Additionally, the International Maritime Organization (IMO) implemented a global ban of organotin-AFS since September 2008 through the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention).

One major impact of TBT pollution on the biota is known as "imposex" – the superimposition of male sexual characters onto females (Smith, 1971) – which has affected more than 260 species of gastropods worldwide (Titley-O'Neal et al., 2011). Imposex is one of the best examples of a specific and dose-dependent biological effect caused by a pollutant and so it has been used as a biomarker for TBT pollution monitoring. In Portugal, imposex monitoring studies started in the 80's (Peña et al., 1988; Spence et al., 1990) and continued throughout the 90's, revealing concerning levels of TBT pollution (Gibbs et al., 1997; Barroso et al., 2000; Santos et al., 2000; Pessoa et al., 2001). The inefficacy of Directive 89/677/EEC to reduce pollution was evidenced in Santos et al. (2000) and Barroso and Moreira (2002), but subsequent studies along the Portuguese coast registered a recovery of imposex and a decrease of TBT environmental levels as a consequence of the more extensive ban imposed by the Regulation 782/2003/EC (Sousa et al., 2009; Vasconcelos et al., 2010; Galante-Oliveira et al., 2011). The objective of the current work is to analyse the temporal trend after 2008, when the AFS Convention entered into force. For this purpose, imposex levels were assessed in 2011 and 2014 for the netted-whelk (*Nassarius reticulatus*) and in 2014 for the dog-whelk (*Nucella lapillus*). Additionally, the concentration of butyltins – TBT and its metabolites,

dibutyltin (DBT) and monobutyltin (MBT) – were determined in the whole tissues of dog-whelks for 2014. The data gathered in these surveys was compared with the data reported in previous assessments within a time-lapse of 15 years.

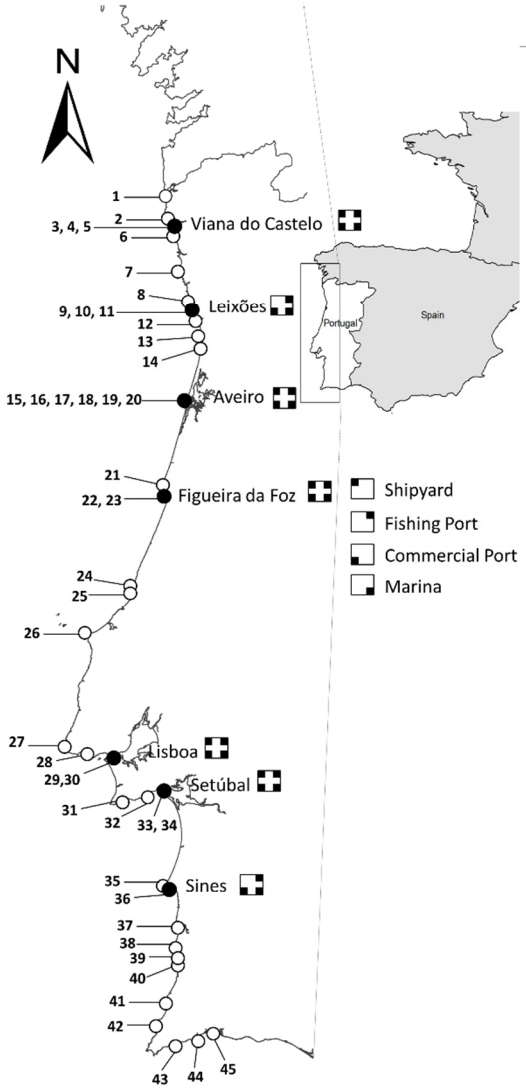
3.1.3. Methods

Sampling and imposex analysis

Nucella lapillus and *Nassarius reticulatus* were sampled at 45 sampling stations along the Portuguese coast in 2014 and 2011/2014, respectively (Fig. 3.1). Due to the natural differences regarding habitat and spatial distribution, these two species share only 13 of those 45 stations. *N. lapillus* was collected at 19 stations in 2014; *N. reticulatus* was sampled at 16 stations in 2011 and 36 in 2014 (see table 3.1). Sampling was carried out between May and July during low tides. Dog-whelks were collected by hand in intertidal rocky shores while netted-whelks were collected by hand in intertidal areas or with baited hoop nets in sublittoral areas. Specimens were transported to the laboratory and maintained in aquaria filled with artificial seawater (35 psu and 18 ± 1 ºC) under constant aeration until further processing (no more than 4 days) for depuration of the digestive tract. Only mature/adult specimens were selected for imposex analysis: dog-whelks with shell height ≥17.5 mm (Galante-Oliveira et al., 2011) and netted-whelks exhibiting a complete white columellar callus and teeth on the outer lip of the shell. Imposex analysis in *N. reticulatus* was preceded by a narcotization period of 60 min in 7% MgCl2 solution in distilled water, while *N. lapillus* was observed without narcotization. Shell height was measured with Vernier callipers to the nearest 0.1 mm. Animals were removed from shells, sexed and dissected for imposex classification under a stereo microscope. Parasitized specimens were discarded from the analysis. Penis length was measured to the nearest 0.14 mm using a graduated eyepiece.

The following imposex parameters were determined for *N. lapillus* (Gibbs et al., 1987): the percentage of imposex-affected females (%I), the mean female penis length (FPL), the mean male penis length (MPL), the relative penis size index (RPSI = FPL3/MPL3 x 100), the vas deferens sequence index (VDSI) and the percentage of sterile females

(%S). The VDSI was classified according to the scoring system proposed by Gibbs et al., (1987). The following parameters were determined for *N. reticulatus*: %I, FPL, MPL, the relative penis length (RPL = FPL/MPL x 100), VDSI, %S and the average oviduct stage (AOS) according to Barreiro et al., (2001). In this species, the VDSI was classified according to the scoring system developed by Stroben et al. (1992b) with minor alterations proposed by (Barroso et al., 2002). Briefly, Stroben et al. (1992b) proposed VDSI stages varying from 0 to 4+, giving the same numerical value of 4 for two different VDS stages, 4 and 4+. Barroso et al. (2002) proposed that value 4+ should be computed with the numerical value of 5 to allow a better discrimination between stations. The presence of aphallic females was registered in both species. In the case of *N. reticulatus* they were computed as b-type VDS development according to Stroben et al. (1992b). Aphallic dog-whelk females were discarded from the analysis of imposex parameters, since this rare phenomenon is not included in the VDS scheme proposed by Gibbs et al. (1987), thus providing reliable comparisons with other works (see Sánchez-Marín et al. (2015)).



Site Nº	Name	Geographical coordinates
1	Vila Praia de Âncora	41° 48.861'N/8° 52.020'W
2	Praia Norte	41° 41.850'N/8° 51.130'W
3	Viana do Castelo - Shipyard	41° 41.259'N/8° 50.346'W
4	Viana do Castelo - Fishing Port	41° 41.262'N/8° 50.132'W
5	Viana do Castelo - Marina	41° 41.597'N/8° 49.350'W
6	Praia da Amorosa	41° 38.497'N/8° 49.403'W
7	Povoa de Varzim	41° 23.103'N/8° 46.501'W
8	Praia de Leça	41° 12.103'N/8° 42.872'W
9	Leixões - Commercial Port, Plat.1	41° 11.342'N/8° 41.572'W
10	Leixões - Commercial Port, Plat.2	41° 11.194'N/8° 41.969'W
11	Leixões - Marina	41° 11.190'N/8° 42.324'W
12	Praia da Foz	41° 9.479'N/8° 41.054'W
13	Aguda	41° 2.691'N/8° 39.174'W
14	Espinho	41° 0.367'N/8° 38.808'W
15	Aveiro - Barra	40° 38.651'N/8° 44.674'W
16	Aveiro - Magalhães Mira	40° 38.570'N/8° 44.109'W
17	Aveiro - Forte da Barra	40° 38.850'N/8° 44.066'W
18	Aveiro - North Comercial Port	40° 38.942'N/8° 43.758'W
19	Aveiro - High Sea Fishing Port	40° 38.388'N/8° 41.639'W
20	Aveiro - S. Jacinto	40° 39.566'N/8° 43.923'W
21	Figueira da Foz, Buarcos	40° 10.417'N/8° 53.762'W
22	Figueira da Foz - Fishing Port	40° 8.355'N/8° 51.602'W
23	Figueira da Foz - Comercial Port	40° 8.816'N/8° 51.022'W
24	Nazaré	39° 36.196'N/9° 4.650'W
25	Nazaré - Fishing Port	39° 34.975'N/9° 4.420'W
26	Peniche	39° 21.014'N/9° 22.584'W
27	Guincho	38° 43.668'N/9° 28.594'W
28	Praia das Avencas	38° 41.557'N/9° 22.061'W
29	Trafaria	38° 40.439'N/9° 14.112'W
30	Porto Brandão	38° 40.680'N/9° 12.421'W
31	Sesimbra - Fishing Port	38° 26.310'N/9° 6.894'W
32	Portinho da Arrabida	38° 28.550'N/8° 59.042'W
33	Setúbal - Fishing Port	38° 31.153'N/8° 53.833'W
34	Troia	38° 29.674'N/8° 54.055'W
35	Sines - Fishing Port	37° 57.202'N/8° 52.282'W
36	Sines Comercial Port	37° 56.217'N/8° 50.897'W
37	Vila Nova de Milfontes	37° 43.216'N/8° 47.296'W
38	Cabo Sardão	37° 36.362'N/8° 48.970'W
39	Zambujeira do Mar, Porto das Barcas	37° 33.100'N/8° 47.569'W
40	Zambujeira do Mar	37° 31.453'N/8° 47.271'W
41	Arrifana	37° 17.720'N/8° 52.176'W
42	Amado	37°15.220'N/8°38.450'W
43	Praia Luz	37° 5.176'N/8° 43.651'W
44	Forte de Lagos	37° 5.888'N/8° 40.064'W
45	Alvor	37° 7.592'N/8° 35.797'W

Figure 3.1 - Map of the Portuguese coast indicating sampling stations (1-45) and location details. Main harbours are indicated by black dots and square symbols are used to identify vessel-related activities.

Since this work aims at making a retrospective analysis of imposex levels in the Portuguese coast, data from previous studies were also considered. Data on *N. lapillus* imposex levels were obtained from monitoring campaigns performed in 2000 (Barroso and Moreira, 2002), 2003 (Galante-Oliveira et al., 2006), 2006 and 2008 (Galante-Oliveira et al., 2011), and also in 2011 (Oliveira et al., 2012). Data on *N. reticulatus* were obtained from monitoring campaigns performed in the same years by the following authors: in 2000 by Barroso et al. (2002), in 2003 by Sousa et al. (2005), in 2006 by Rato et al. (2009)

and in 2008 by Sousa et al. (2009). For a better interpretation of imposex evolution trends, our data was compared with commercial naval traffic registered in main Portuguese harbours (INE, 2015)(Fig. 3.2).

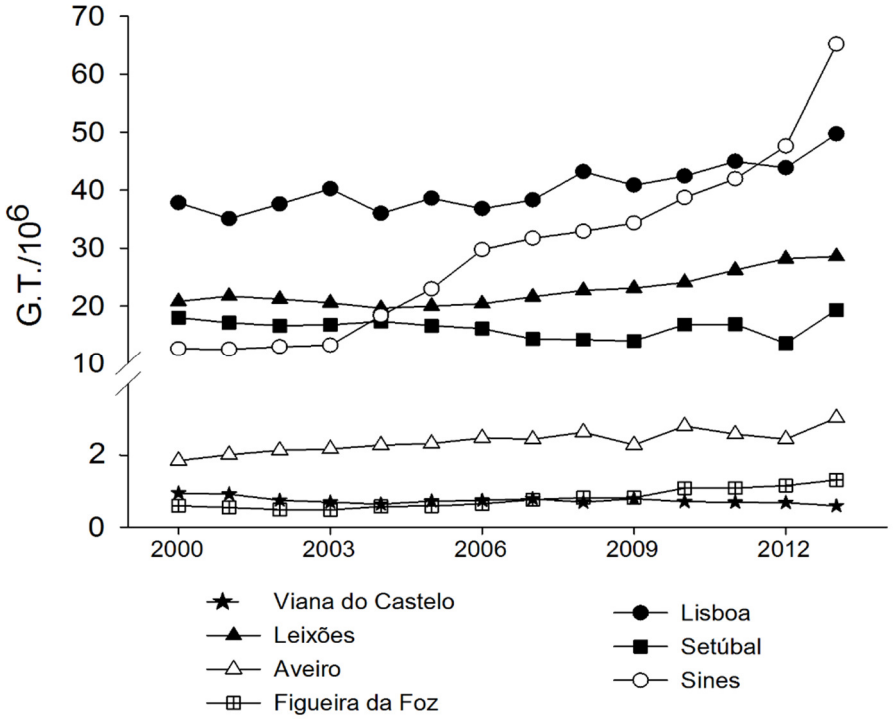


Figure 3.2 - Data on commercial ship traffic in main Portuguese harbours from 2000 to 2013 (Instituto Nacional de Estatística - www.ine.pt). G.T. – Gross Tonnage.

Butyltin analysis

Butyltins (monobutyltin - MBT, dibutyltin - DBT and tributyltin - TBT) were quantified in *N. lapillus* tissue from 18 stations surveyed in 2014 in order to understand if fresh TBT inputs are still occurring along the Portuguese coast. Each sample consisted of a pool of whole soft tissues of 10 - 15 females per station. The analyses were performed in the certified laboratory UT2A – Ultra Trace Analyses Aquitaine, using the methods described by Lopes-dos-Santos et al. (2014). The detection limit (DL), expressed in ng Sn/g dry weight (dw), was 1 for MBT and 0.5 for both DBT and TBT.

Temporal evolution of TBT in *N. lapillus* tissues is also presented, compiling data published for 2000 (Barroso and Moreira, 2002), 2003 (Galante-Oliveira et al., 2006), 2006 (Sánchez-Marín et al., 2015) and 2011 (Oliveira et al., 2012).

Statistical analysis

To detect trends on imposex parameters over the 15 years we used Mann-Kendall correlation test (τ) and Sen's slope estimator (*Q*). Mann-Kendall is a suitable non-parametric test widely used to identify significant trends along time series. If a trend exists and is statistically significant, its magnitude is evaluated through the non-parametric Sen's slope estimator. This procedure was applied to each species separately considering only the sampling stations that have been revisited in the 6 imposex monitoring campaigns. It was also applied individually to each station in case it was surveyed at least 4 times. For comparison of imposex parameters it is important to take into account variations in specimens' size, since there may be a dependent relationship between shell height and penis size or VDS expression (Galante-Oliveira et al. 2011). Therefore, statistical differences on shell heights between the 6 monitoring campaigns abovementioned were performed through two different statistical procedures: the non-parametric Kruskal-Wallis test to determine differences considering all stations, and the non-parametric Friedman test for comparisons only between revisited stations. Other correlations presented in this study refer to the non-parametric Spearman rank correlation. Statistical analyses were performed using the statistical software XLSTAT 2015.

3.1.4. Results

Imposex levels in 2011 and 2014

Imposex levels for both *Nucella lapillus* and *Nassarius reticulatus* are summarized in table 3.1. Imposex incidence in the 2014 campaign for *N. lapillus* ranged between 13% and 100%; RPSI and VDSI varied from 0 to 3.44 and from 0.13 to 3.74, respectively. Dog-

whelks from stn. 39 (Zambujeira do Mar, Porto das Barcas) presented the highest level of imposex, with 8% of females presenting a VDS stage 5 and therefore being sterile. Female aphally was only observed in 3 organisms at stn. 24 (Nazaré) and these were discarded from the analysis.

Regarding *N. reticulatus*, in 2011 the imposex incidence varied between 0 and 100%, RPL varied from 0 to 75 and VDSI from 0 to 3.4, while AOS ranged from 0 to 0.3. Despite no sterile females were observed, the highest imposex levels were still found at historic TBT hotspots: stn. 3 (a shipyard; VDSI = 2.67), stn. 4 and 26 (fishing ports; VDSI = 3.42 & 2.67 respectively), and stn. 11 (a marina; VDSI = 3.13). In 2014, the imposex incidence maintained the 2011 range (0 - 100%) but the highest RPL and VDSI were superior due to the fact that more stations were assessed, varying respectively from 0 to 82 and from 0 to 4.6. The AOS ranged between 0 and 0.5 and sterile females were registered at 3 stations: 2.9% at stn. 4 (Viana do Castelo - Fishing Port), 24% at stn. 25 (Nazaré - Fishing port) and 40% at stn. 31 (Sesimbra - Fishing Port); the last value should, however, be considered with caution, as only 5 females were found at this station. Female aphally (b-type VDS) was observed in 18% of imposex-affected females, and considered widespread on the study area as they were found in 56% of the sites surveyed.

Table 3.1 –*Nucella lapillus* and *Nassarius reticulatus* imposex levels registered along the Portuguese coast. Levels for 2011 and 2014 are presented in the format "2011 value/2014 value". Number of males and females analysed (N ♂, N ♀, respectively), mean male and female shell height (SH ♂, SH ♀, respectively), mean male and female penis length (MPL, FPL, respectively), RPSI (relative penis size index), RPL (relative penis length), VDSI (vas deferens sequence index), %I (Percentage of imposex-affected females) and AOS (average oviduct stage). Along with VDSI values, the symbol "*" indicate the occurrence of sterile females. Mean values are displayed with respective standard deviation (s.d.) rounded to the unit and represented as "mean (s.d.)". The absence of organisms, or stations not assessed, is represented by the symbol "-".

Nucella lapillus

Station	*N* ♂	SH ♂	MPL	*N* ♀	SH ♀	FPL	RPSI	VDSI	%I
1	20	22.2$^{(2)}$	4.26	40	22.0$^{(3)}$	0.06	0	1.0	88
2	17	20.4$^{(1)}$	2.61	39	19.9$^{(2)}$	0.13	0	1.3	87
6	12	19.2$^{(2)}$	3.50	21	19.5$^{(3)}$	0.00	0	0.4	43
7	21	21.4$^{(1)}$	4.01	42	21.2$^{(2)}$	0.01	0	0.9	86
8	20	20.0$^{(3)}$	4.40	40	20.4$^{(2)}$	0.01	0	0.8	75
12	17	19.8$^{(2)}$	4.42	34	20.8$^{(2)}$	0.00	0	0.8	76
13	20	19.8$^{(1)}$	3.29	35	21.0$^{(2)}$	0.00	0	0.8	80
14	20	22.1$^{(2)}$	3.97	40	21.6$^{(2)}$	0.03	0	0.8	75
15	23	26.8$^{(3)}$	3.22	24	28.2$^{(3)}$	0.00	0	0.5	50
17	30	26.3$^{(2)}$	2.97	23	27.1$^{(2)}$	0.00	0	0.1	13
21	20	21.1$^{(2)}$	3.27	42	22.4$^{(2)}$	0.00	0	0.7	68
24	24	19.1$^{(1)}$	3.41	40	20.1$^{(2)}$	0.02	0	1.0	80
27	26	19.4$^{(2)}$	2.69	41	21.9$^{(2)}$	0.01	0	0.7	66
28	9	19.2$^{(1)}$	1.57	22	20.2$^{(2)}$	0.00	0	0.4	41
29	5	24.5$^{(2)}$	4.19	3	24.5$^{(1)}$	0.00	0	1.3	100
30	20	23.1$^{(3)}$	3.75	35	25.0$^{(4)}$	0.10	0	1.1	91
38	10	19.4$^{(2)}$	4.05	29	19.7$^{(2)}$	0.00	0	0.6	62
39	21	19.7$^{(2)}$	3.97	39	20.3$^{(2)}$	1.29	3.4	3.7*	100
40	23	23.1$^{(1)}$	4.59	39	22.8$^{(2)}$	0.03	0	1.1	95

Nassarius reticulatus

Station	*N* ♂	SH ♂	MPL	*N* ♀	SH ♀	FPL	RPL	VDSI	AOS
1	- /30	- /24.6$^{(3)}$	- / 12.44	- /40	- /26.0$^{(2)}$	- /0.02	- /0.1	- /0.08	- /0.0
2	30/30	20.7$^{(2)}$/19.7$^{(2)}$	9.18/11.02	30/21	23.5$^{(3)}$/23.3$^{(2)}$	0.06/0.00	0.65/0.0	0.30/0.05	0.2/0.0
3	27/29	24.1$^{(3)}$23.2$^{(2)}$	9.70/10.34	30/42	26.1$^{(3)}$/25.7$^{(2)}$	2.42/5.22	24.93/50.5	2.67/3.45	0.4/0.5
4	30/30	22.4$^{(2)}$/21.8$^{(2)}$	10.27/9.09	27/34	23.9$^{(3)}$/21.4$^{(2)}$	7.65/6.27	74.53/69.0	3.42/4.00*	0.0/0.4
5	25/20	24.4$^{(2)}$/23.3$^{(3)}$	10.18/11.47	30/42	26.4$^{(2)}$/25.7$^{(3)}$	0.58/0.92	5.65/8.0	1.37/1.07	0.0/0.0
6	30/30	22.3$^{(1)}$/21.4$^{(2)}$	11.12/12.11	23/30	24.4$^{(2)}$/23.2$^{(3)}$	0/0.03	0.00/0.3	0.00/0.17	0.0/0.0
7	- /30	- /22.3$^{(2)}$	- /13.45	- /22	- /23.7$^{(2)}$	- /0.02	- /0.2	- /0.18	- /0.0
8	- /17	- /22.6$^{(2)}$	- /12.04	- /30	- /23.7$^{(2)}$	- /0.02	- /0.2	- /0.10	- /0.0
9	- /23	- /24.5$^{(2)}$	- /11.37	- /42	- /26.0$^{(2)}$	- /0.28	- /2.5	- /0.54	- /0.0
10	26/29	26.6$^{(2)}$/24.1$^{(2)}$	10.41/11.42	30/38	27.6$^{(2)}$/25.0$^{(2)}$	0.73/0.05	7.03/0.4	1.37/0.39	0.0/0.0

11	30/25	20.5$^{(3)}$/20.6$^{(2)}$	8.65/11.46	23/33	21.4$^{(2)}$/22.8$^{(2)}$	4.36/1.25	50.50/10.9	3.13/2.12	0.2/0.0
12	21/15	21.0$^{(1)}$/22.4$^{(2)}$	10.76/12.33	20/42	24.1$^{(1)}$/24.3$^{(2)}$	0.03/0.04	0.31/0.3	0.15/0.17	0.0/0.0
15	25/23	25.7$^{(2)}$/24.0$^{(2)}$	12.24/13.37	30/37	27.0$^{(2)}$/25.2$^{(2)}$	0.15/0.09	1.21/0.7	0.15/0.22	0.0/0.0
16	24/11	24.0$^{(3)}$/26.4$^{(2)}$	9.40/10.76	23/42	25.1$^{(4)}$/26.7$^{(2)}$	0.20/0.03	2.22/0.2	0.21/0.17	0.0/0.0
17	28/30	22.2$^{(2)}$/22.2$^{(2)}$	11.16/12.02	30/25	24.6$^{(3)}$/24.4$^{(3)}$	0.16/0.10	1.48/0.8	0.16/0.32	0.0/0.0
18	24/27	20.1$^{(1)}$/21.5$^{(2)}$	9.52/9.98	14/42	22.4$^{(2)}$/23.5$^{(2)}$	0.15/0.00	1.55/0.0	0.29/0.00	0.0/0.0
19	- /25	- /25.6$^{(2)}$	- /11.89	- /41	- /26.7$^{(2)}$	- /0.87	- /7.3	- /2.12	- /0.0
20	- /26	- /21.0$^{(2)}$	- /10.47	- /42	- /23.8$^{(2)}$	- /0.02	- /0.2	- /0.07	- /0.0
22	- /20	- /24.5$^{(2)}$	- /7.74	- /24	- /26.4$^{(2)}$	- /0.10	- /1.3	- /0.33	- /0.0
23	- /18	- /25.1$^{(2)}$	- /11.76	- /42	- /27.8$^{(2)}$	- /0.02	- /0.2	- /0.05	- /0.0
25	- /16	- /20.3$^{(2)}$	- /8.79	- /21	- /21.0$^{(3)}$	- /6.08	- /69.2	- /4.19*	- /0.1
26	12/14	18.7$^{(1)}$/18.7$^{(2)}$	8.33/7.81	18/20	20.4$^{(2)}$/19.7$^{(2)}$	1.73/0.58	20.72/7.4	2.67/1.65	0.1/0.0
28	20/13	18.2$^{(2)}$/18.6$^{(2)}$	7.49/11.38	30/16	21.1$^{(2)}$/20.5$^{(2)}$	0.11/0.04	1.45/0.4	0.47/0.06	0.0/0.0
30	- /16	- /22.7$^{(2)}$	- /12.22	- /36	- /23.3$^{(3)}$	- /0.10	- /0.8	- /0.25	- /0.0
31	- /11	- /21.7$^{(3)}$	- /8.51	- /5	- /19.4$^{(4)}$	- /6.97	- /81.9	- /4.60*	- /0.0
32	- /20	- /22.9$^{(3)}$	- /12.05	- /35	- /24.5$^{(2)}$	- /0.00	- /0.0	- /0.14	- /0.0
33	- /22	- /20.5$^{(3)}$	- /9.44	- /22	- /21.1$^{(2)}$	- /1.89	- /20.0	- /3.14	- /0.0
34	- /3	- /21.2$^{(2)}$	- /8.60	- /4	- /19.6$^{(1)}$	- /0.47	- /5.4	- /1.50	- /0.0
35	- /29	- /21.5$^{(2)}$	- /8.60	- /32	- /21.9$^{(2)}$	- /1.78	- /20.7	- /2.59	- /0.0
36	- /20	- /20.1$^{(1)}$	- /10.26	- /24	- /21.2$^{(2)}$	- /0.00	- /0.0	- /0.04	- /0.0
37	21/21	18.9$^{(3)}$/21.8$^{(2)}$	10.57/11.49	30/41	20.2$^{(2)}$/21.7$^{(2)}$	20.15/0.01	0.25/0.1	0.10/0.02	0.0/0.0
39	18/13	19.2$^{(3)}$/19.8$^{(2)}$	10.10/10.44	30/14	21.9$^{(2)}$/21.3$^{(2)}$	21.92/0.03	0.28/0.3	0.17/0.79	0.0/0.0
41	- /15	- /21.5$^{(2)}$	- /11.30	- /18	- /24.1$^{(2)}$	- /0.15	- /1.4	- /0.44	- /0.0
43	- /4	- /18.1$^{(1)}$	- /8.98	- /10	- /18.5$^{(2)}$	- /0.00	- /0.0	- /0.00	- /0.0
44	- /5	- /20.5$^{(2)}$	- /10.23	- /8	- /22.1$^{(2)}$	- /0.13	- /1.3	- /0.63	- /0.0
45	- /17	- /23.0$^{(2)}$	- /9.37	- /29	- /24.4$^{(2)}$	- /0.00	- /0.0	- /0.00	- /0.0

Butyltin levels in 2014

Butyltin (MBT, DBT and TBT) concentrations in *N. lapillus* tissues are shown in table 3.2. MBT varied from < DL (detection limit: 1 ng Sn/g) to 13 ng Sn/g dw, DBT varied from 2.2 to 27 ng Sn/g dw and TBT from 1.5 to 55 ng Sn/g dw. TBT represents the major fraction of the quantified butyltins, accounting, in average, for 60% of the butyltins sum (ΣBT's = MBT+DBT+TBT). The butyltin degradation index (BDI = ([MBT]+[DBT])/[TBT]) proposed by Díez et al. (2002), indicates that recent TBT inputs (BDI < 1) are probably still widespread in the Portuguese coast, occurring in 16 out of the 18 sites assessed.

Table 3.2 - Butyltin concentrations (expressed as ng Sn/g dw) in *Nucella lapillus* whole female tissues along the Portuguese coast. Recent TBT inputs are expected at stations where BDI <1 (Díez et al., 2002). BDI: Butyltin Degradation Index; DL: Detection Limit.

Sites	MBT	DBT	TBT	BDI
1	2.0 ± 0.5	4.7 ± 0.7	8 ± 1	0.84
2	2.3 ± 0.2	7 ± 1	11 ± 1	0.85
6	2.5 ± 0.5	5.9 ± 0.7	11 ± 1	0.76
7	1.5 ± 0.6	3.7 ± 0.8	6.9 ± 0.7	0.75
8	2.4 ± 0.2	5.9 ± 0.1	10.5 ± 0.2	0.79
12	3.6 ± 0.6	8 ± 1	16 ± 2	0.73
13	2.6 ± 0.3	7 ± 1	18.7 ± 0.9	0.51
14	1.5 ± 0.1	5.3 ± 0.2	17 ± 2	0.40
15	1.5 ± 0.3	4.9 ± 0.2	14.6 ± 0.6	0.44
17	1.7 ± 0.5	4.5 ± 0.8	12 ± 1	0.52
21	1.2 ± 0.2	4.0 ± 0.2	12 ± 1	0.43
24	< LOQ	2.2 ± 0.2	7.7 ± 0.6	0.35
27	2.9 ± 0.5	2.4 ± 0.2	1.5 ± 0.2	3.53
28	1.7 ± 0.1	5.4 ± 0.3	10.5 ± 0.2	0.68
30	13 ± 3	26 ± 5	48 ± 5	0.81
38	< LOQ	2.4 ± 0.4	2.4 ± 0.1	1.21
39	7.3 ± 0.5	27 ± 1	55 ± 4	0.62
40	1.5 ± 0.2	6.1 ± 0.4	7.7 ± 0.5	0.99
LOQ	1	0.5	0.5	

Imposex evolution between 2000 and 2014

Imposex evolution in both species over the last 15 years is presented in figure 3.3. As previously stated, an analysis of the organisms' size variation throughout this period allows a proper comparison of imposex parameters between monitoring campaigns (Galante-Oliveira et al., 2011). For the comparisons including all assessed stations (Fig. 3.3A), the non-parametric Kruskall-Wallis test revealed no significant statistical differences between shell heights throughout the monitoring campaigns (*N. lapillus*: H = 7.090, p = 0.214; *N. reticulatus*: H = 4.243, p = 0.515). No significant differences were also observed with the non-parametric Friedman test when comparing only the revisited stations (*N. lapillus*: s = 7.310, p = 0.199; *N. reticulatus*: s = 5.571, p = 0.350).

Trend analysis for all stations showed a significant decreasing tendency, given by the negative correlation of imposex levels with time along the Portuguese coast in both species: *N. lapillus* (τ = -0.589; p < 0.001); *N. reticulatus* (τ = -0.370; p < 0.001) (Fig. 3.3A). The decrease is more pronounced in *N. lapillus* as shown by Sen's slope (Q = -0.040) contrasting to the less pronounced slope observed in *N. reticulatus* (Q = -0.016). In order to identify a more realistic temporal trend throughout the years, the analysis was restricted to the stations that are common to all sampling campaigns: 10 stations for the dog-whelk and 13 for the netted-whelk (Fig 3.3B). Whilst for *N. lapillus* these stations are located along the shoreline, for *N. reticulatus* they are also distributed within estuaries where naval activity is more intense. Mann-Kendall test reveal again, for both species, highly significant (p < 0.001) decreasing trends in VDSI, %I and RPSI/RPL during this period. VDSI registered a significant decreasing trend in both species: *N. lapillus* (τ = -0.693; p < 0.001), *N. reticulatus* (τ = -0.459; p < 0.001). However, dog-whelks' VDSI values observed in 2014 are about the same as those registered in 2011, showing that the decreasing trend slowed down (almost stopped) in the last triennium. Even so, the decreasing slope is higher in this species (Q = -0.060) indicating a faster imposex recovery than *N. reticulatus* (Q = -0.040). The percentage of imposex-affected females also significantly declined during the 15 years in *N. lapillus* (τ = -0.585; p < 0.001) and *N. reticulatus* (τ = -0.510; p < 0.001). However, while the %I median value in 2014 in the nassariid stands below 20%, in *N. lapillus* it is around 75%. *N. lapillus* RPSI showed also a significant decreasing trend (τ = -0.559; p < 0.001) but this parameter is almost zero since 2006 and so becomes redundant to detect any decreasing trend afterwards. Similarly, *N. reticulatus* RPL revealed a significant decline (τ = -0.401; p < 0.001) with high slope (Q = -0.210) due to the steady decrease observed over the whole 15 years period, reaching the lowest values in 2014.

The imposex data for *N. reticulatus* presents higher dispersion when compared to the dog-whelk. This outcome is influenced by these species wider distribution that extends from the cleaner open coastal shore to the sheltered harbours where the major sources of pollution are located. Therefore, we further analysed the VDSI values for this species after pooling the sampling stations according to the main maritime activity there

located, i.e., “commercial ports” (big commercial vessels), “fishing ports and marinas” (small boats), and “coastal shore” (diffuse sources). The VDSI decreasing trend was statistically significant in all groups of stations (Fig. 3.3C) but some differences must be noted. Commercial harbours presented very low VDSI levels in 2014 and the steepest decrease (Q = -0.276), contrasting with the slower decreases observed in fishing ports and marinas (Q = -0.039) as well as the coastal shore (Q = -0.034). At fishing ports and marinas the VDSI values were high in 2000 and declined very slowly over the 15 years period under analysis so that moderate levels still persisted in 2014. Hence, a more resilient recovery is apparent in areas associated to small/local boats, contrasting with the rapid decline observed at commercial ports (Fig. 3.3C).

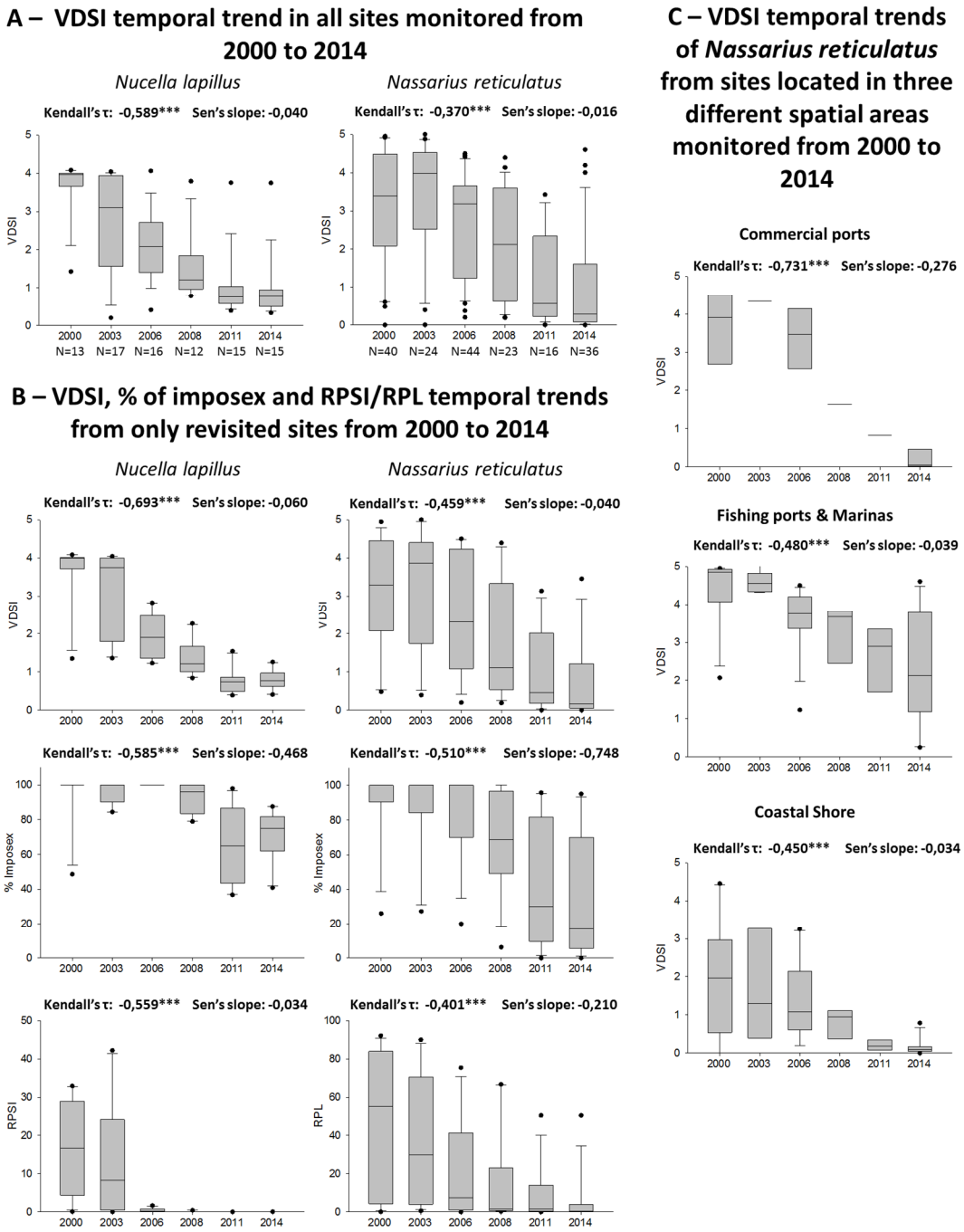


Figure 3.3 - Imposex levels evolution in *Nucella lapillus* and *Nassarius reticulatus* from 2000 to 2014. Results for the Mann-Kendall correlation analysis is presented above each chart. Data from 2000 to 2008 were previously published: for *N. lapillus* (Barroso and Moreira, 2002; Galante-Oliveira et al., 2006; Galante-Oliveira et al., 2011; Oliveira et al., 2012) and for *N. reticulatus* (Barroso et al., 2002; Sousa et al., 2005; Rato et al., 2009; Sousa et al., 2009). A) VDSI temporal evolution in both species for the whole set of stations monitored each given year (number of stations - N - indicated next to the x axis). B) VDSI, %I, RPSI and RPL temporal evolution in both species only for stations common to all campaigns from 2000 to 2014 (N = 10 for *N. lapillus* and N = 13 for *N. reticulatus*). C) VDSI evolution in *N. reticulatus* collected at stations located at "Commercial Ports", "Fishing Ports and Marinas" and "Coastal Shore". The box plots represent the median (line), the range between 25% and 75% of the values observed (grey box), the lowest and highest values observed (whiskers), and the outliers represented by the dots. Due to the reduced number of stations used in the plots represented in C) the quartiles, the lowest and highest values or the outliers could not always be calculated (n=2-5). Statistically significant correlation is given by (*** $p < 0.001$).

Analysing each sampling station separately, the correlation between VDSI and time shows a significant decreasing trend (negative correlation) in both species for most of the stations along the surveyed area (Fig. 3.4). However, some exceptions were detected along the 15-year's time frame: whenever the VDSI is low it fails to give a significant correlation (as observed in *N. lapillus* at stn. 42 and *N. reticulatus* at stn. 39). Moreover, in one case (*N. reticulatus* at stn. 35), a decreasing trend is observed but the power of the statistical analysis was insufficient to detect any trend due to low number of sampling campaigns included. Nonetheless, there are cases where VDSI rose and then dropped (*N. reticulatus* at stn. 41), or did not decrease at all (*N. lapillus* at stn. 39 and *N. reticulatus* at stn. 4 and 25) (Fig. 3.4). These later stations (Stn. 39, 4, 25) share the common feature of being located at fishing ports, which raises the question mentioned above of whether the evolution trends may depend on the typology of the TBT source (i.e. type of vessels) present.

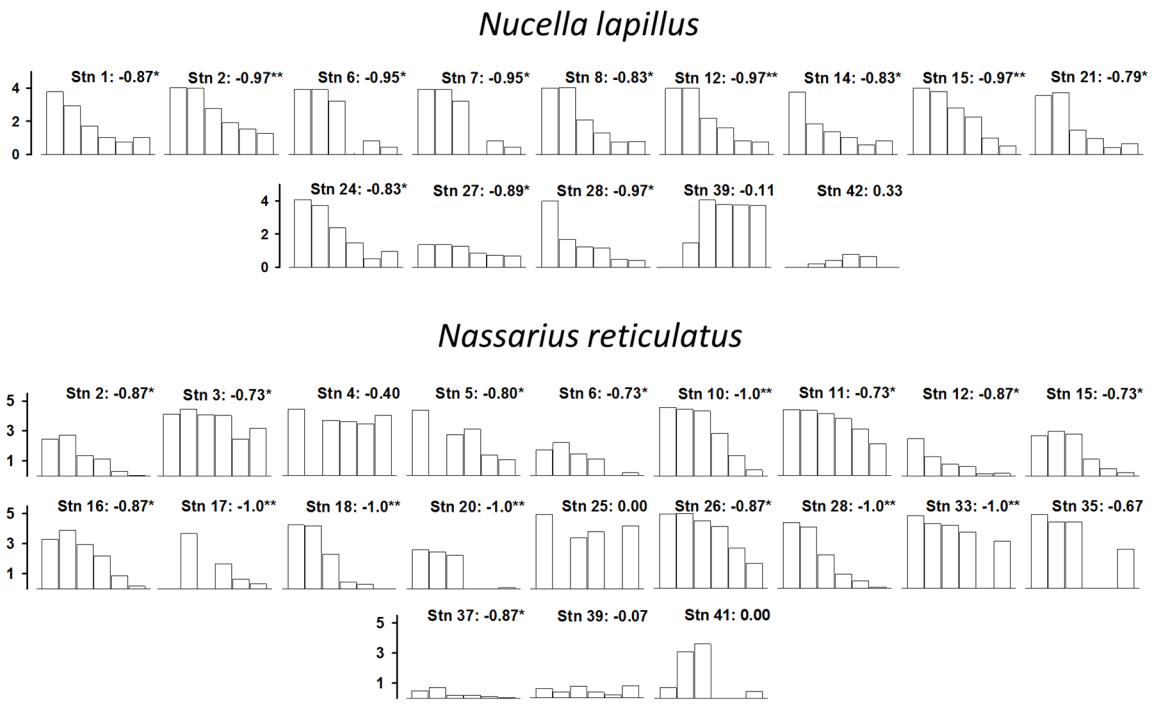


Figure 3.4 - VDSI level evolution at each station with respective Kendall's τ correlation coefficient from 2000 to 2014 (each bar corresponds to one year, increasing from left to right – 2000; 2003; 2006; 2008; 2011; 2014). Only stations visited at least for 4 out of 6 times were analysed. Statistically significant correlation is given by *($p < 0.05$) and **($p < 0.01$).

Figure 3.5 represents the imposex relationship between both species, collected at the same sites along the Portuguese coast during the different monitoring surveys. This data is compared with the relationship VDSI $_{N.\ reticulatus}$ = 0.087*e^(0.793*VDSI $_{N.\ lapillus}$), *r* = 0.731, reported by (Stroben et al., 1992a) for populations collected between 1988 and 1991 in Brittany and Normandy (France). For this figure, the VDSI values of *N. reticulatus* were calculated according to the VDS scale proposed by Stroben et al. (1992b) to be in accordance with the regression (i.e., VDS of 4+ was computed as 4 for VDSI calculation). Since *N. lapillus* populations from stn. 27 and 28 have been identified as presenting signs of lower sensitivity to TBT pollution, as well as aphally (see Sánchez-Marín et al. (2015)), these stations were excluded from this analysis. We can perceive from all surveys that most of the points in figure 3.5 do not deviate much from the regression curve defined by Stroben et al. (1992a), despite the natural random dispersion. Furthermore, our data lie below the 1:1 line, confirming that dog-whelks are generally more sensitive than netted-whelks, i.e., generally present higher levels of imposex at a same sampling station.

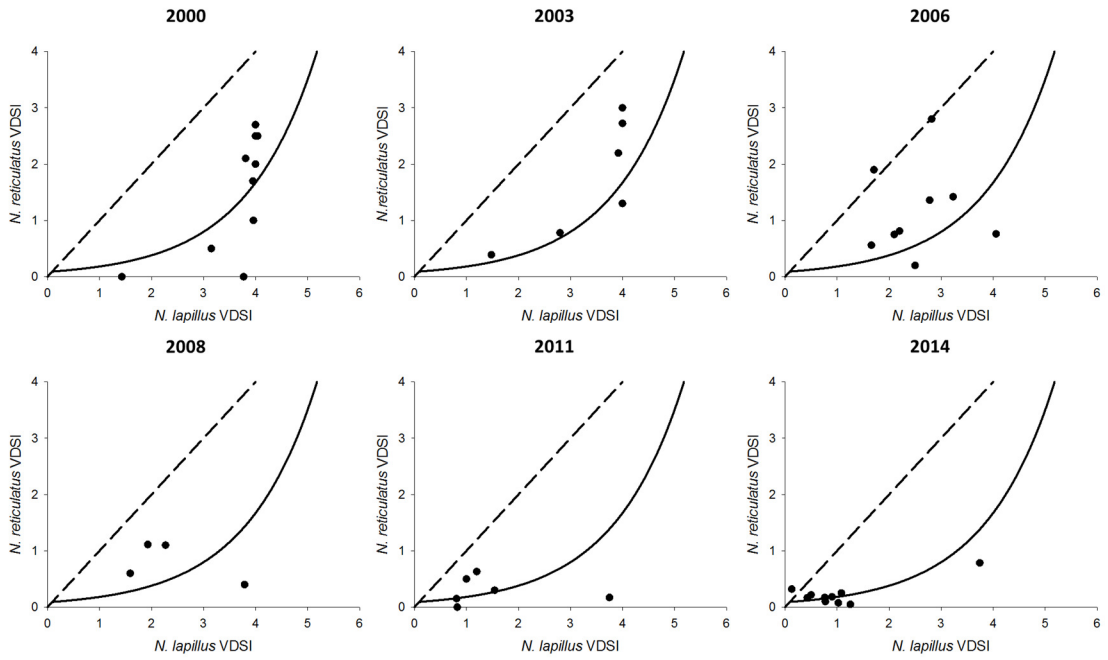


Figure 3.5 - Relationship between VDSI from both bioindicator species collected at common stations in 2000, 2003, 2006, 2008, 2011 and 2014. Each point represents one station. Curve line represents the regression VDSI$_{N.\ reticulatus}$ = 0.087*e^(0.793*VDSI$_{N.\ lapillus}$) defined by Stroben et al. (1992a) that was used for comparison with the current work.

Evolution of TBT levels in *N. lapillus* over 15 years

The decline of the VDSI levels was accompanied by a reduction of TBT content in the whole tissues of dog-whelks during the 15 years period (Fig. 3.6). The same trend is observed if we select only the stations that are common to all monitoring campaigns within this time-lapse (n = 6; not shown). In fifteen years the pollution levels decreased considerably. In 2000 the TBT body burden ranged between 30 - 147 ng TBT-Sn/g dry wt while in 2014 it dropped to less than 20 ng TBT-Sn/g dry wt (except stn. 39). TBT tissue concentrations in 2014 are similar to the ones observed in 2011 and 2006, showing that the decreasing trend slowed down in the latter years.

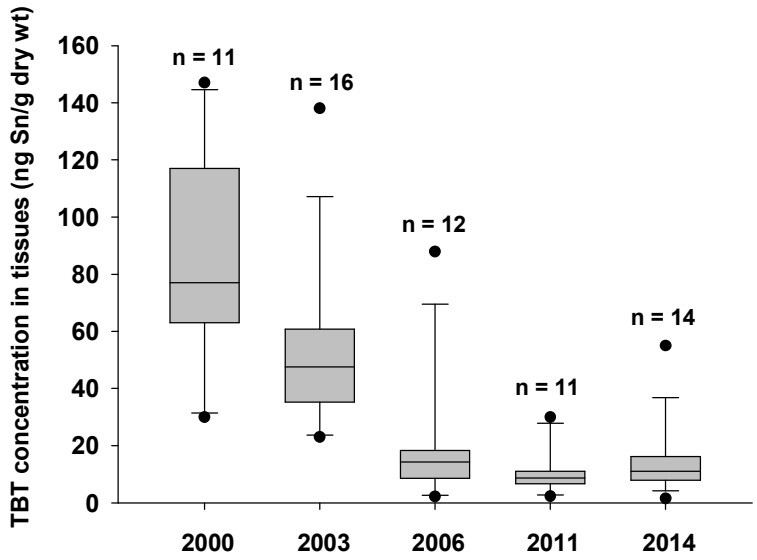


Figure 3.6 - Box-plot showing TBT concentration found in the tissues of *N. lapillus* collected along the Portuguese coast. Data from 2000 to 2011 was taken from the previously published studies (Barroso and Moreira, 2002; Galante-Oliveira et al., 2006; Oliveira et al., 2012; Sánchez-Marín et al., 2015). Number of samples analysed each year are shown above each box.

Figure 3.7 shows the relationship between the TBT body burden and the corresponding VDSI values of dog-whelks over the years. In this plot populations from stns. 27 and 28 were excluded from the analysis due to their lower TBT sensitivity (see Sánchez-Marín et al., 2015). Correlations between these two variables were positive and statistically significant in the monitoring campaigns performed in 2003 ($\rho = 0.665$; $p < 0.05$) and 2006 ($\rho = 0.842$; $p < 0.01$), reflecting the well established cause-effect relationship between TBT contamination and imposex. Stn. 24 appears in 2006 as an outlier for reasons that we cannot assertively justify. In the other years the correlation

was lost ($p > 0.05$) since the VDSI distribution was truncated around the value 4 (in 2000) or 1 (2011 and 2014). Again, figure 3.7 shows a gradual and concomitant decrease of TBT contamination and VDSI along the years, with the exception of stn. 39 where illegal use of TBT antifouling paints may probably occur (see discussion).

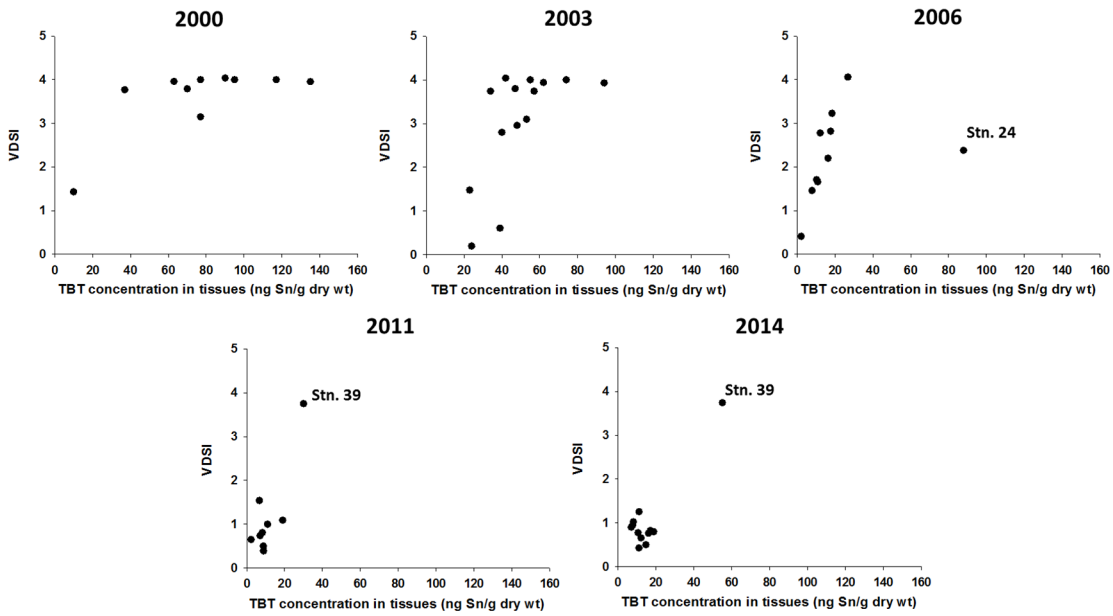


Figure 3.7 - Relationship between VDSI and TBT concentration in *Nucella lapillus* female tissues (ng Sn/g dry wt) for the monitoring campaigns performed along the Portuguese coast using the same bioindicator species.

3.1.5. Discussion

Between 2000 and 2014 occurred a general decrease of imposex levels along the Portuguese coast (Fig. 3.3, 3.4). The first signs of such recovery were observed in 2003 as depicted by the decline of VDSI and RPSI in *Nucella lapillus* or RPL in *Nassarius reticulatus* (Fig. 3.3) and by the lessening of TBT concentration in *N. lapillus* tissues (Fig. 3.6). This situation might be hypothetically related with a reducing trade and consumption of TBT-AFS before 2003 in anticipation to the entry into force of the Regulation 782/2003/EC. A strong reduction on the use of TBT-AFS after 2003 is also suggested by the considerable decrease of imposex levels in both species registered successively in 2006, 2008 and 2011.

When we compare the temporal evolution of TBT body burden and imposex levels in *N. lapillus*, it is interesting to note the delay that may occur between these two parameters in pollution decreasing scenarios. For example, when comparing the plots corresponding to 2006 and 2014 in figure 3.7, it is evident that although the majority of points correspond to TBT concentrations between 0 and 20 ng Sn / g dw, the VDSI values are different, with higher imposex levels being observed in 2006. This is justified by the fact that predicted half-life of TBT in *N. lapillus* tissues ranges between 50 and more than 100 days while these organisms can live up to 5 to 10 years or more (Crothers, 1985; Gibbs and Bryan, 1996). Therefore, and due to the fact that imposex is irreversible (Bryan et al., 1987; Gibbs et al., 1987; Oehlmann et al., 1998), VDSI values are a result of TBT accumulation throughout organisms life and may not represent current environmental concentrations (Barroso et al., 2011). This aspect should always be considered in TBT pollution monitoring programs.

Currently, as denoted by the *N. lapillus* survey performed in 2014, there seems to occur a stabilization trend of imposex intensity at levels around VDSI = 1 in this species. Even so, this phenomenon still affects more than 50% of the females in the majority of stations assessed (see table 3.1). Despite the pronounced decline of imposex levels over the last 15 years, our results indicate that TBT is still occurring in Portuguese coastal waters because in 2014 we found quantifiable concentrations of TBT in all dog-whelks' samples analysed. Moreover, a BDI < 1 was recorded in 89% of the stations (table 3.2) suggesting that fresh inputs of TBT are still occurring in the study area. These "fresh inputs" may not come exclusively from the illegal use of antifouling paints (which seems to happen at stn 39; see below), since TBT remobilization from sediments to the water column may occur and has been widely reported (Santos et al., 2004; Ruiz et al., 2008; Castro et al., 2012). In fact, the sediments may probably constitute the major source of pollution nowadays and this might perpetuate the imposex occurrence during the upcoming years, although at levels that will not pose an effective ecological risk to gastropod populations as female sterility is unlikely to occur (Laranjeiro et al., 2015).

The decreasing trend of imposex levels in *N. lapillus* was also observed in other European countries. The first reports arose in the 90's in England and Scotland (Evans et

al., 1996; Harding et al., 1997) and in France (Huet et al., 2004), where a recovery from very high VDSI values was observed. This recovery was related with the implementation of regulatory actions that forbad the use of TBT in small vessels (< 25 m). However, in other regions, such as Portugal, a similar legislative restriction (Decreto-Lei nº54/93) was ineffective (Barroso and Moreira, 2002; Santos et al., 2002). Years later, after the adoption of more restrictive measures (EU's Regulation 782/2003/EC and IMO's AFS Convention), a definite decreasing trend of imposex levels was reported, namely in Wales (Oliveira et al., 2009; Nicolaus and Barry, 2015), Iceland (Guðmundsdóttir et al., 2011) and England (Langston et al., 2015; Nicolaus and Barry, 2015). In these studies the majority of the assessed sites presented a VDSI < 3, granting a good ecological status regarding TBT pollution, according to the classification proposed by Laranjeiro et al. (2015). In many cases, the decline of imposex levels lead to the recovery of dog-whelk populations, as for example, in south-eastern England, where it was reported up to a 20-fold increase in the number of individuals within a population (Morton, 2009).

We observed a consistent lessening of dog-whelks imposex levels in the Portuguese coast between 2000 and 2014. However, there was a population at Zambujeira do Mar-Porto das Barcas (stn. 39; SW Portugal) that presented a counter current trend. This site is a very small fishing port with a fleet of up to 12 small boats with < 7 m length located in a pristine area of the open coast. It is positioned in a natural embayment of about 700 m2 with sandy bottoms, surrounded by rocky shore. At this location *N. lapillus* imposex levels increased until 2006 and remained high, with sterile females occurring in all monitoring campaigns from then on. Figure 3.4 evidences that this station did not follow the decreasing trend in imposex levels observed in the majority of stations along the coast. From all Portuguese coast, this is the only station still showing high VDSI levels (3.75 and 3.74 in 2011 and 2014, respectively (table 3.1)) and highest TBT female body burden (55.0 ng Sn/g dw in 2014 (table 3.2)). To better understand this exceptional case, we assessed imposex levels at two nearby stations (stn. 38 located 5 km north; stn. 40 located 3 km south) in 2014. Both the imposex levels and TBT female body burdens were lower at these stations (stn. 38, VDSI = 0.62, TBT = 2.4 ng Sn/g dw; stn. 40, VDSI = 1.05, TBT= 7.7 ng Sn/g dw) (table 3.1) clearly showing that TBT pollution is

constrained to stn. 39. This scenario points to a local input of TBT and the only plausible source seems to be the small fishing boats there anchored. So we hypothesize that the illegal use of TBT-based AF paints may occur at this location. Although this seems to be an isolated occurrence in an extensive geographic area (the Portuguese coast), it constitutes an example that highlights the need to strengthen the control over TBT-based AF paints marketing and use.

Similarly to *N. lapillus*, imposex levels in *N. reticulatus* generally decreased over the 15 years' period under analysis. However, when looking at the VDSI temporal evolution at each sampling station individually, significant decreases were not observed in all of them. Moreover, sterile females were still observed at some stations in 2014 (table 3.1 and Fig. 3.4). As indicated by *N. lapillus*, some sites with resilient TBT contamination can still be found. Typically, imposex levels observed along the open coast were generally lower than in sheltered/estuarine areas where ports and marinas (historical hotspots of TBT) are located. This was registered in the monitoring campaigns from 2000 to 2006. However, the scenario has changed after 2006 with the sharp decline of imposex in commercial ports, reaching VDSI values close to zero in 2014 (Fig. 3.3C). Since the commercial naval traffic did not decrease over the same period (INE, 2015) (Fig. 3.2), we conclude that large commercial vessels are no longer the main source of TBT. On the contrary, at fishing ports and marinas, imposex decreased very slowly in the same period, with high VDSI levels and sterile females still being reported in 2014. Several hypothesis already suggested by other authors may explain this resilience in TBT contamination in fishing ports and marinas: (i) illegal use of TBT-based AFS in small boats (Borges et al., 2013; Ruiz et al., 2015) and (ii) poor water exchange with external waterbodies due to a constricted connection, that would contribute for a high TBT residence time and accumulation in sediments (Rodríguez et al., 2009). We also admit the possibility that a less frequent dredging in marinas and fishing ports, in comparison to what happens in commercial ports, would retain TBT in the sediments at these sites. This last hypothesis may appear at a first sight controversial, since an inverse effect might be observed due to the resuspension of contaminated sediments that increases TBT bioavailability, as observed by Cuevas et al. (2014). These authors reported the rise of imposex values at

sites influenced by dredging activities. However, in a long term perspective, places subjected to a frequent dredging in the past should recover more rapidly considering that great part of TBT was already removed.

Both *N. lapillus* and *N. reticulatus* have been used to monitor TBT pollution in Europe since the 80's, with evident benefits coming from their combined use (Gibbs et al., 1987; Stroben et al., 1992a; Bryan et al., 1993; Barroso and Moreira, 2002). Early studies described good correlations between imposex levels in both species, when collected at common sites. These relationships could be described using non-linear regressions (Stroben et al., 1992a; Huet et al., 1995) that could grant, for instance, the estimation of imposex levels for a species that is absent from a given site, if the other species is present. However, most of these relationships were established in the end of the millennium, when TBT-based AFS were still in use. It is now important to understand how these relationships may have been affected after the implementation of the most recent bans on TBT-based AFS (i.e. the AFS Convention entered into force in September 2008). Despite our data (Fig. 3.5) do not deviate much from the regression curve defined by Stroben et al. (1992a) in all the monitoring campaigns, in 2006 two stations substantially deviate from the previously referred relationship, and their data points lie along the 1:1 line that describes an equal sensitivity to TBT pollution by both species. Analysing figure 3.5 we also understand that VDSI levels in *N. lapillus* start to decrease before *N. reticulatus* and, therefore, at some stations might occur that at a given time both species present similar VDSI. This deviation from the early relationship defined by Stroben et al. (1992a) was already reported in an Portuguese estuarine area (Laranjeiro et al., 2015) and can be justified by the fact that, in a TBT-declining scenario, the decrease in TBT concentration in the water column will be faster than its decrease in sediments, since the sediment acts as a sink for TBT (Sarradin et al., 1995; Langston et al., 2015). *N. reticulatus*, as a burrower, can be longer exposed to TBT and, consequently, express higher VDSI levels than those expected by its sensitivity when compared with imposex expression in *N. lapillus* specimens collected at the same location. Nevertheless, figure 3.5 also depicts that, for our set of stations where both species occur, *N. lapillus* is currently (in 2014) the

only species with adequate sensitivity to monitor the evolution of TBT pollution in the open coastal shore where imposex levels in the netted-whelk are currently close to zero.

3.1.6. Conclusions

This work shows a general decrease of imposex in *N. reticulatus* and *N. lapillus* and also in *N. lapillus* TBT body burden lalong the Portuguese coast between 2000 and 2014. This proves the effectiveness of the most recent and definite EU restriction on the application of TBT-based AFS on ships: the Regulation 782/2003/EC. Given the efficacy of this measure on the reduction of TBT pollution since 2003, the effectiveness of IMO's AFS Convention is difficult to perceive in this study area, as imposex and TBT levels were already decreasing before its entry into force. Despite the general decline, a total recovery of imposex was still not achieved, with high levels being still observed at some locations characterized by the presence of small boats. This may result from illegal use of TBT-AFS and/or high TBT persistency in sediments. Therefore, monitoring is still required to follow TBT pollution trends for the upcoming years.

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CHAPTER 3.2 – ECOLOGICAL QUALITY STATUS OF THE PORTUGUESE COAST REGARDING TBT POLLUTION

3.2. Ecological Quality Status of the Portuguese coast regarding TBT pollution

3.2.1. Abstract

The recent years witnessed an increasing consciousness by the society on subjects concerning aquatic pollution. In Europe, several legislative pieces have been recently implemented in order to improve and protect the aquatic environment like the Water Framework Directive (WFD), the Marine Strategy Framework Directive (MSFD) and Regional Sea Conventions. Therefore the objective of the current work is to assess the ecological quality status of the Portuguese coastal and transitional waters regarding TBT pollution, based on the data described in chapter 3.1. The imposex classification developed for WFD (proposed in chapter 2.1) and the OSPAR Assessment Criteria are here applied to interpret the imposex levels of the two gastropod bioindicator species *Nucella lapillus* and *Nassarius reticulatus* in order to assess the ecological status of the study area. A good ecological status was achieved in the majority of the stations located in coastal waters while worse ecological status was perceived in stations located in transitional water bodies, due to the proximity of TBT pollution sources. The comparison between the WFD and the OSPAR Assessment Criteria for imposex show a similar temporal trend, however slight differences can be perceived and are discussed. Both WFD and OSPAR are considered and should be respected on the MSFD implementation but they differ in their objectives regarding TBT pollution. Therefore this work also focuses on the integration of imposex monitoring within MSFD and recommends the use of the WFD assessment criteria in this directive.

3.2.2. Introduction

In Europe, the implementation of an integrated policy for the protection of marine environment has been attempted over the last decades. Four regional sea conventions were created during the second half of the last century - the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention), the Convention on the Protection of the Marine Environment of the Baltic Sea Area (HELCOM

Convention), the Convention for the Protection of the Mediterranean Sea Against Pollution (Barcelona Convention), and the Convention on the Protection of the Black Sea Against Pollution (Bucharest Convention; only since 1992). These conventions aim to protect the marine environment involving countries that share a same region, which should work together and converge in the same environmental objectives. At the European Union (EU) policy level, marine environment was only considered in directives such as Habitats Directive, Birds Directive and Water Framework Directive (WFD) (Directive 2000/60/EC). This last directive was implemented in 2000 and was a vital step for the protection of EU water bodies including transitional and coastal waters. Finally, in 2008, the implementation of the Marine Strategy Framework Directive (MSFD) gave an integrated policy regarding the protection of the marine environment to EU member states. This directive aims to achieve and preserve a good environmental status in the European marine environment by 2020, based on the fulfilment of 11 qualitative descriptors, detailed in its Annex I. However, and in contrast with other forums, such as OSPAR and WFD, there are no specific guidelines or targets on how the good environmental status should be achieved (see European Commission decision 2010/477/EU) and should be member states to adopt necessary measures to accomplish this goal. Also, a good environmental status achieved under MSFD should always be in agreement with other directives' demands (e.g. WFD's EQS) and performed in coordination with member states sharing the same marine region, with regional sea conventions playing a central role for this purpose. Still, not always exists a total agreement between EU directives and regional sea conventions regarding the temporal milestones and the precise objectives to be achieved, or even the strategy to follow.

TBT is a well-known substance due to its use as a biocide in antifouling paints, which contributed in the past for an overall spread of this substance throughout the aquatic environment, causing a variety of adverse effects to non-target organisms at very low concentrations (Matthiessen and Gibbs, 1998; Alzieu, 2000). Probably the most known adverse effect of TBT is imposex, a female masculinization phenomenon already described for more than 260 gastropod species worldwide (Titley-O'Neal et al., 2011). Due to the high toxicity of TBT, this chemical is included in the list of priority substances in

WFD and in the Regional Sea Conventions OSPAR and HELCOM. A global ban of TBT antifouling paints was implemented in 2008 by the International Maritime Organization (IMO) through the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention), which was preceded by a similar action initiated in 2003 in the EU (Regulation 782/2003/EC).

As previously referred, TBT compounds make part of the priority substances listed in WFD. This directive requires member states to perform a holistic monitoring assessment integrating several elements of quality (biological, hydromorphological and physico-chemical). The biological elements assessment is based on the population and community condition and evaluated by means of Ecological Quality Ratios (EQR), while a good chemical status is achieved when Environmental Quality Standards (EQS) defined for each priority substance are respected. However, there is an increasing interest for the integration of aquatic effect-based monitoring tools as an essential bridge between the chemical and biological elements assessment (Hagger et al., 2008; Martinez-Haro et al., 2015). The need for this approach is evidenced in a recent technical report on aquatic effect-based monitoring tools produced by the European Commission (Wernersson et al., 2015). This opening was already foreseen in the later MSFD, especially on its descriptor 8 "Concentrations of contaminants are at levels not giving rise to pollution effects" (Law et al., 2010; Lyons et al., 2010; Wernersson et al., 2015). Imposex, due to its high specificity and sensitivity to TBT pollution, should be therefore considered as an useful tool in monitoring programs under WFD or MSFD, as it is already mandatory under OSPAR Coordinated Environmental Monitoring Programme (CEMP). OSPAR developed specific guidelines for imposex monitoring based on a number of bioindicators, including the dog-whelk *Nucella lapillus* and the netted-whelk *Nassarius reticulatus*, and defined six classes of ecological quality based on the Vas Deferens Sequence Index (VDSI; parameter used to grade imposex expression, see Gibbs et al. (1987) for *N. lapillus* and Stroben et al. (1992) for *N. reticulatus* (OSPAR 2004). Through this imposex-based classification is possible to understand if a site reaches the Ecological Quality Objective (EcoQO), which indicates if organisms are exposed to TBT concentrations in water below the Environmental Assessment Criteria (EAC) set at 0.1 ng/L. HELCOM also consider imposex as a core

indicator for this hazardous substance under their Baltic Sea Action Plan (BSAP) adopting the OSPAR referred classification scheme (HELCOM, 2007). However, HELCOM adopted an EQS for TBT of 0.2 ng/L (the same as WFD), which is different from the EAC set by OSPAR (Nyberg et al., 2013).

In order to commit imposex monitoring within WFD objectives, it was recommended in Section 2.1 the adoption of the same OSPAR CEMP strategy into this directive. This would allow a harmonization of environmental policies in Europe and would provide the WFD with robust and resourceful instruments to assess the ecological status of water bodies. However, considering that the main focus of the WFD for the general protection of the aquatic ecosystems is to keep the diversity and abundance of species undisturbed, in Section 2.1 it was proposed different assessment classes based on VDSI levels (Laranjeiro et al., 2015). In fact, these authors suggest that the VDSI boundaries should better reflect the risk of population decline or extinction due to TBT pollution, and so they proposed five ecological classes - as required by the WFD - that are slightly different from OSPAR. As a consequence, the quality objectives of OSPAR and WFD are met at different VDSI values. As previously referred, regional sea conventions and EU directives have a fundamental role on the MSFD with their experience, dataset, and tools granting the basis for the directive implementation (Borja et al., 2013; van Hoof et al., 2014). Since OSPAR and HELCOM implemented imposex-based monitoring programs, this could also be extended to the MSFD (besides the WFD) to grant a good policy harmonization.

The aim of this work is to assess the temporal evolution of the ecological status of the Portuguese coast, regarding TBT pollution, under two different perspectives: one based on the imposex assessment criteria defined by OSPAR, and the other based on the imposex assessment criteria suggested by Laranjeiro et al. (2015) for the WFD. This analysis provides a better evaluation of the new proposal of Laranjeiro et al. (2015) and allows addressing some recommendations to be included in the MSFD. The data set used for this analysis corresponds to imposex monitoring surveys performed with the bioindicators *N. lapillus* and *N. reticulatus* in the Portuguese coast in 2000, the year of WFD implementation, and 2014, a crucial year that precedes the date by which WFD

environmental objectives must be met (2015). The Portuguese coast represents a good scenario for this study because it is subjected to intense naval traffic related to commercial, fishing and shipyard activities and, to a lesser extent, recreational navigation. On the other hand, some of the world's major maritime shipping routes cross Portuguese jurisdictional waters, namely the ones connecting Africa and North America with Northern and Southern Europe (through the Mediterranean). Hence, coastal ecosystems in this country are frequently exposed to contamination derived from this anthropogenic activity, making this a good example to track TBT pollution trends along the years and to compare OSPAR and WFD classifications.

3.2.3. Methods

Study area

The study area extends along the Portuguese mainland coast, from station 1 - Vila Praia de Âncora - in the North, to station 45 - Alvor - in the South-East (Fig. 3.8). As Portugal is a signing member of the OSPAR Convention, this sampling area is included in the OSPAR region IV (Bay of Biscay and Iberian Peninsula). This geographical range belongs to the same sub-region defined by the MSFD as part of the wider marine region of North-East Atlantic Ocean, and is also under the jurisdiction of the WFD. The sampling of gastropods for imposex assessment was performed along the coastline in sandy and rocky shores covering 11 out of 15 coastal water bodies defined by WFD in Portugal. Sampling also occurred inside estuarine areas with 7 transitional water bodies being monitored. Historically, the major hotspots of TBT pollution are located inside these transitional waters as they harbour commercial and fishing ports, shipyards, marinas and moorings (Smith, 1981; Bryan et al., 1986; Ten Hallers-Tjabbes et al., 1994).

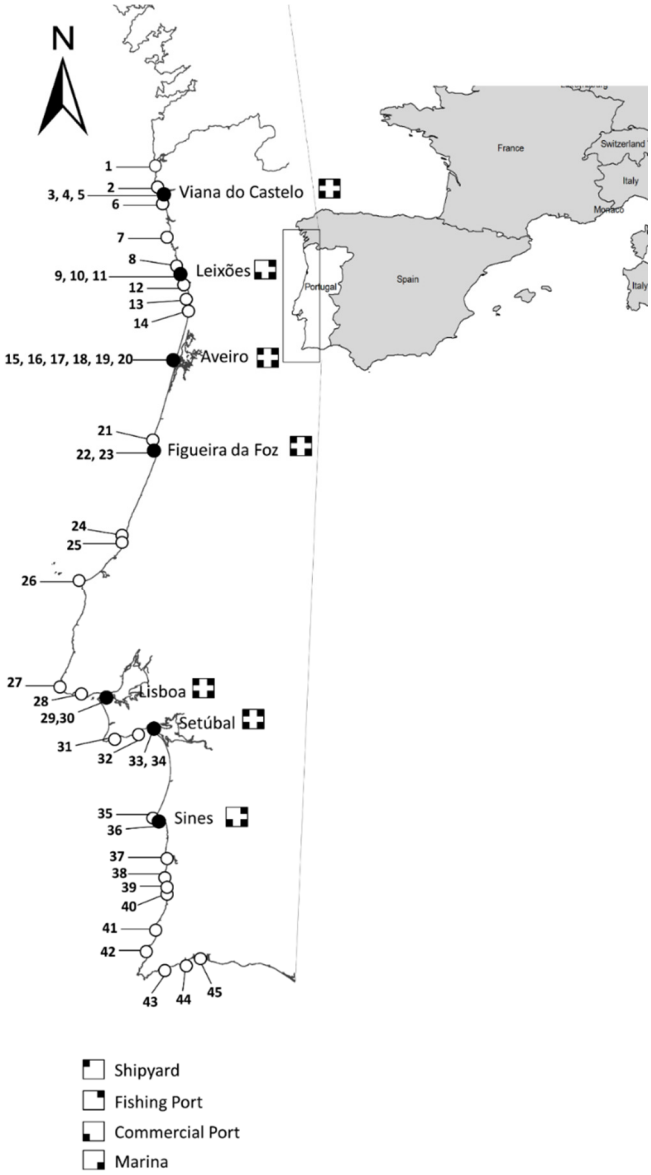

Figure 3.8 - Map of the Portuguese coast showing the sampling sites location (1 to 45), main harbors (black dots) and the respective vessel-related activities (indicated by square symbols according to the bottom legend).

Imposex data

Since this work aims to analyse the evolution of the Ecological Quality Status along the Portuguese coast between 2000 and 2014, regarding TBT pollution, the imposex values from the previous subchapter (section 3.1.) are here analysed. The data for the year 2000 was obtained from Barroso et al. (2002). For the current work it was selected only the data regarding the Vas Deferens Sequence Index (VDSI) to grade the masculinization level of females and to assess the impact of TBT pollution on the ability of

females to reproduce, as a proxy to predict population abundance and perpetuity. The VDSI was classified according to the scoring system proposed by (i) Gibbs et al. (1987) for *N. lapillus* and (ii) Stroben et al. (1992) for *N. reticulatus*.

Ecological Quality Classification

In this study we assess the temporal evolution of the ecological quality status of the Portuguese coast, regarding TBT pollution, while simultaneously compare two different imposex-based criteria to classify this quality status. Both classification systems are presented in figure 3.9. One was established by OSPAR after imposex was considered a mandatory element under OSPAR's CEMP (OSPAR, 2004). Assessment Classes were defined based on intervals of VDSI values observed in *N. lapillus*, i.e., sites are classified according to the degree of imposex severity evidenced, integrating the biological effects with predicted TBT concentrations in the environment. According to OSPAR, EcoQO is achieved when *N. lapillus* presents VDSI < 2.0 (classes A or B), which indicates exposure to TBT concentrations below the EAC defined as 0.1 ng TBT/L. This means that adverse effects in the more sensitive taxa caused by chronic exposure to TBT are unlikely to occur if concentrations are below the EAC. The EcoQO is not achieved for VDSI ≥ 2.0, a situation that comprises the following classes: Class C (2.0 ≤ VDSI < 4.0) represents gastropod exposure to environmental levels of TBT higher than the defined EAC, which means that there is a risk of adverse effects in the more sensitive taxa; a dog-whelk population with VDSI levels belonging to Class D (4.0 ≤ VDSI < 5.0) have their reproductive capacity severely affected indicating high TBT levels in the environment; Class E (VDSI ≥ 5.0) indicates that *N. lapillus* populations are unable to reproduce due to female sterility while Class F classifies areas where the more sensitive gastropod species are absent due to TBT pollution (OSPAR, 2004). These classes were also developed for other species (ex.: *N. reticulatus*), taking into account VDSI regressions between these species and the dog-whelk collected at the same locations (see figure 3.9). This allows a more robust assessment as *N. lapillus* might be absent at some sites either due to pollution, as it is a very sensitive organism, or to habitat characteristics.

The second criteria was proposed by Laranjeiro et al. (2015) in order to integrate imposex monitoring assessment within the WFD, since TBT is also regarded as a priority hazardous substance under this directive. This proposal is based on the previous criteria for *N. lapillus* but with some alterations to meet WFD demands and objectives. Therefore, to proceed according to the WFD, imposex values are computed to EQR through the equation: $EQR_{\text{imposex}} = \frac{(M-O)}{M}$, where M is the maximum VDSI score that a population may attain and O is the observed VDSI value for a population at one given site. This will render a value between 0 and 1 as demanded by the WFD to guarantee comparability between EU member states in all biological monitoring results. According to Laranjeiro et al. (2015) the VDSI criteria for the WFD should be centred in the impact of pollution on population abundance and community diversity, and be regarded as operational criteria to decide if a member state action is required or not. Therefore, a "High" status is achieved for VDSI < 0.3 whilst a “Good” ecological status under WFD may be reached for VDSI as high as 3.0 in *N. lapillus*, which means that TBT has a negligible impact on the population abundance due to the absence of sterile females. According to these authors, a VDSI of 4.5, which may relate to about 50% sterile females, could distinguish a “Moderate” status where the population faces a lower risk of extinction (VDSI < 4.5) and a “Poor” status where the risk of extinction is higher (VDSI > 4.5). Finally, the “Bad” ecological status refers to a situation where *N. lapillus* and other most sensitive species became extinct due to TBT pollution. As can be seen, the number of Ecological Quality classes are reduced to five, instead of six proposed by OSPAR (see figure 3.9). Laranjeiro et al. (2015) also propose the use of other bioindicators besides *N. lapillus* in order to cover a wider diversity of habitats and to expand the monitoring area. Therefore, a correspondence between *N. lapillus* and *N. reticulatus* VDSIs was established through the same methods used by OSPAR (see figure 3.9). For an extended description of the rationale used to set each Ecological Class limits consult Laranjeiro et al. (2015).

3.2.4. Results

Table 3.3 presents VDSI levels in *N. lapillus* and *N. reticulatus* observed along the Portuguese coast and the correspondent EQRs. It is evident that imposex levels decreased in both species between 2000 and the latest monitoring assessment performed in 2014. In 2000, *N. lapillus* presented VDSI values ranging between 1.35 and 4.08, with the majority of populations showing VDSI > 3. Two populations (stations 2 and 24) presented 3.7 and 7.7% of sterile females, respectively. In 2014, the VDSI dropped to a range of 0.13 - 1.33, excepting a single case of a population (stn. 39) presenting VDSI = 3.74, with 8.0% of sterile females.

The imposex evidenced by *N. reticulatus* in the year 2000 showed a great variability: VDSI ranged from 0.0 to 4.0 but the majority of the populations analysed exhibited VDSI > 3.0 and many (stn. 3, 4, 9, 11, 25, 26, 29 and 30) presented sterile females with a prevalence of 4.4 to 50.0% across sites. In 2014, VDSI values dropped in the majority of the surveyed sites to values below 1. Still, stations 3, 4, 25, 31 and 33 evidenced high VDSI values (VDSI > 3) with sterilization being also observed at stn. 4, 25 and 31 affecting 2.9, 23.8 and 40.0% of the females. These are located essentially near hotspots of TBT pollution (most in transitional waters), such as fishing ports and marinas.

Table 3.3 - *Nucella lapillus* and *Nassarius reticulatus* VDSI levels registered along the Portuguese coast in 2000 and 2014, and correspondent EQRs. The percentage of sterile females are shown, when present, between parentheses. 1 - Data from Barroso and Moreira (2002); 2 - Data from Section 3.1; 3 - Data from Barroso et al. (2002).

		Nucella lapillus				***Nassarius reticulatus***			
		2000 [1]		**2014 [2]**		**2000 [3]**		**2014 [2]**	
Site	**Name**	**VDSI**	**EQR**	**VDSI**	**EQR**	**VDSI**	**EQR**	**VDSI**	**EQR**
1	Vila Praia de Âncora	3.79	0.37	1.03	0.83	2.14	0.47	0.08	0.98
2	Praia Norte	4.04 (3.7%)	0.33	1.26	0.79	2.46	0.38	0.05	0.99
3	Viana do Castelo - Shipyard	-	-	-	-	3.90 (14.3%)	0.13	3.19	0.20
4	Viana Castelo - Fishing Port	-	-	-	-	3.60 (13.4%)	0.03	3.76 (2.9%)	0.06
5	Viana Castelo - Marina	-	-	-	-	3.90	0.10	1.07	0.73
6	Praia da Amorosa	3.95	0.34	0.43	0.93	1.70	0.57	0.17	0.96
7	Póvoa de Varzim	3.96	0.34	0.90	0.85	1.00	0.75	0.18	0.95
8	Praia de Leça	4.00	0.33	0.78	0.87	1.96	0.51	0.10	0.98
9	Leixões - Commercial Port, Plat.1	-	-	-	-	3.30 (4.4%)	0.18	0.54	0.87
10	Leixões - Commercial Port, Plat.2	-	-	-	-	3.90	0.03	0.39	0.90
11	Leixões - Marina	-	-	-	-	3.90 (4.5%)	0.03	2.12	0.47
12	Praia da Foz	4.00	0.33	0.76	0.87	2.50	0.38	0.17	0.96
13	Aguda	-	-	0.80	0.87	-	-	-	-
14	Espinho	3.77	0.37	0.83	0.86	0.00	1.00	-	-
15	Aveiro – Barra	4.00	0.33	0.50	0.92	2.67	0.33	0.22	0.95
16	Aveiro - Magalhães Mira	-	-	-	-	3.20	0.20	0.17	0.96
17	Aveiro - Forte da Barra	-	-	0.13	0.98	-	-	0.32	0.92
18	Aveiro - North Comercial Port	-	-	-	-	3.70	0.08	0.00	1.00
19	Aveiro - High Sea Fishing Port	-	-	-	-	-	-	2.12	0.47
20	Aveiro - S. Jacinto	-	-	-	-	2.60	0.35	0.07	0.98
21	Figueira da Foz, Buarcos	3.55	0.41	0.66	0.89	-	-	-	-
22	Figueira da Foz - Fishing Port	-	-	-	-	2.07	0.48	0.33	0.92
23	Figueira da Foz - Comercial Port	-	-	-	-	2.40	0.40	0.05	0.99
24	Nazaré	4.08 (7.7%)	0.32	0.95	0.84	-	-	-	-
25	Nazaré - Fishing Port	-	-	-	-	4.00 (4.8%)	0.00	3.76 (23.8%)	0.06
26	Peniche	-	-	-	-	4.00 (27.8%)	0.00	1.65	0.59
27	Guincho	1.35	0.78	0.68	0.89	3.30	0.18	-	-
28	Praia das Avencas	4.00	0.33	0.41	0.93	4.00	0.00	0.06	0.98
29	Trafaria	-	-	1.33	0.78	4.00 (5.6%)	0.00	-	-
30	Porto Brandão	-	-	1.08	0.82	4.00 (50.0%)	0.00	0.25	0.94
31	Sesimbra	-	-	-	-	4.00	0.00	4.00 (40.0%)	0.00
32	Portinho da Arrabida	-	-	-	-	3.80	0.05	0.14	0.96
33	Setúbal - Fishing Port	-	-	-	-	4.00	0.00	3.14	0.22
34	Troia	-	-	-	-	4.00	0.00	1.50	0.63
35	Sines - Fishing Port	-	-	-	-	4.00	0.00	2.59	0.35

36	Sines Comercial Port	-	-	-	-			0.04	0.99
37	Vila Nova de Milfontes	3.15	0.48	-	-	0.48	0.88	0.02	0.99
38	Cabo Sardão	-	-	0.62	0.90	-	-	-	-
39	Zambujeira do Mar, Porto das Barcas	-	-	3.74 (8.0%)	0.38	0.60	0.85	0.79	0.80
40	Zambujeira do Mar	-	-	1.05	0.83	-	-	-	-
41	Arrifana	-	-			0.80	0.80	0.44	0.89
42	Praia do Amado	-	-	0.33	0.94	-	-	-	-
43	Praia Luz	1.43	0.76	-	-	0.00	1.00	0.00	1.00
44	Forte de Lagos	-	-	-	-	-	-	0.63	0.84
45	Alvor	-	-	-	-	-	-	0.00	1.00

The imposex decreasing trend between 2000 and 2014 indicates an improvement of the quality status along the Portuguese coast. However, when comparing the two ecological quality criteria - OSPAR and WFD - some differences are depicted, as seen in figure 3.9. For *N. lapillus*, in 2000 the OSPAR's EcoQO is only registered in 2 stations (stn. 27 and 43) with others varying between assessment classes C (6 sites) and D (6 sites). In 2014 the EcoQO is reached for all stations, except in stn. 39 classified as C. According to the WFD proposal by Laranjeiro et al. (2015), most EQRs determined for *N. lapillus* in 2000 are below 0.5 (table 3.3), indicating a major deviation from undisturbed conditions caused by TBT. Similarly to the OSPAR classification, only two stations (stn. 27 and 43) reached the "Good" ecological status with the remainder presenting a "Moderate" ecological status. In 2014 the EQR values are close to 1 in all surveyed sites implying a "Good" status condition (high status at stn. 17), except stn. 39 that maintains the "Moderate" status.

Regarding *N. reticulatus* in 2000, and according to the OSPAR classification, only 2 stations (stn. 14 and 43) reached the EcoQO, with the other stations portraying a worse classification: 5 sites with class C; 11 sites with class D; 16 sites with class E. An improvement in the populations' condition was registered in 2014, as 18 stations reached the EcoQO while the other stations were classified as class C (10 sites), D (5 sites) and E (3 sites). A similar picture is obtained with the WFD classification though, in 2000, 4 stations (stn. 14, 37, 39 and 43) had already met WFD objectives by presenting a "Good" or "at Least Good" ecological status. The "at Best Poor" ecological status, the worse ecological

classification given by this species, was found at 24 stations, whilst the “Moderate” status was diagnosed at other 6 sites. An improvement in the ecological status is observed in 2014: a “Good” or “at Least Good” ecological status is found at 28 stations while 8 stations failed to reach this objective, with 6 of them still presenting the “at Best Poor” ecological status.

Both criteria used in this study are in general accordance regarding the objectives achievement, nevertheless some differences are perceived. The fact that OSPAR Assessment Criteria has 6 quality classes while WFD’s proposal has only 5, produces a mismatch between quality classes. For instance, the imposex levels in 2000 for *N. lapillus* reveals that some stations are classified with OSPAR’s class C or class D while according to WFD the same stations solely present a Moderate ecological status. Other difference between classifications can be well perceived in 2014 with the quality status retrieved by the imposex levels in *N. reticulatus*: 18 sites reach the EcoQO proposed by OSPAR contrasting to the 28 sites that presented a Good Ecological Quality status according to WFD proposal.

OSPAR			Proposal for the Water Framework Directive					
Imposex assessment class	VDSI		*Nucella lapillus*			*Nassarius reticulatus*		
	Nucella lapillus	*Nassarius reticulatus*	Ecological status class	Proposed EQR classes	Corresponding VDSI	Ecological status class	Proposed EQR classes	Corresponding VDSI
A	<0.3	<0.3	High	[1.00 - 0.95[	[0.00 - 0.30[	At Least Good	[1.00 - 0.93[	[0.00 - 0.28[
B	[0.3 - 2.0[		Good	[0.95 - 0.50[	[0.30 - 3.00[			
C	[2.0 - 4.0[	[0.3 - 2.0[				Good	[0.93 - 0.80[	[0.28 - 0.80[
D	[4.0 - 5.0]	[2.0 - 3.5]	Moderate	[0.50 - 0.25[	[3.00 - 4.50[	Moderate	[0.80 - 0.40[	[0.80 - 2.40[
E	]5.0 - 6.0]	]3.5 - 4.0]	Poor	[0.25 - 0.00]	[4.50 - 6.00]	At best	[0.40 - 0.00]	[2.40 - 4.00]
F	Pop. Abs.		Bad	Population Absent		Poor		

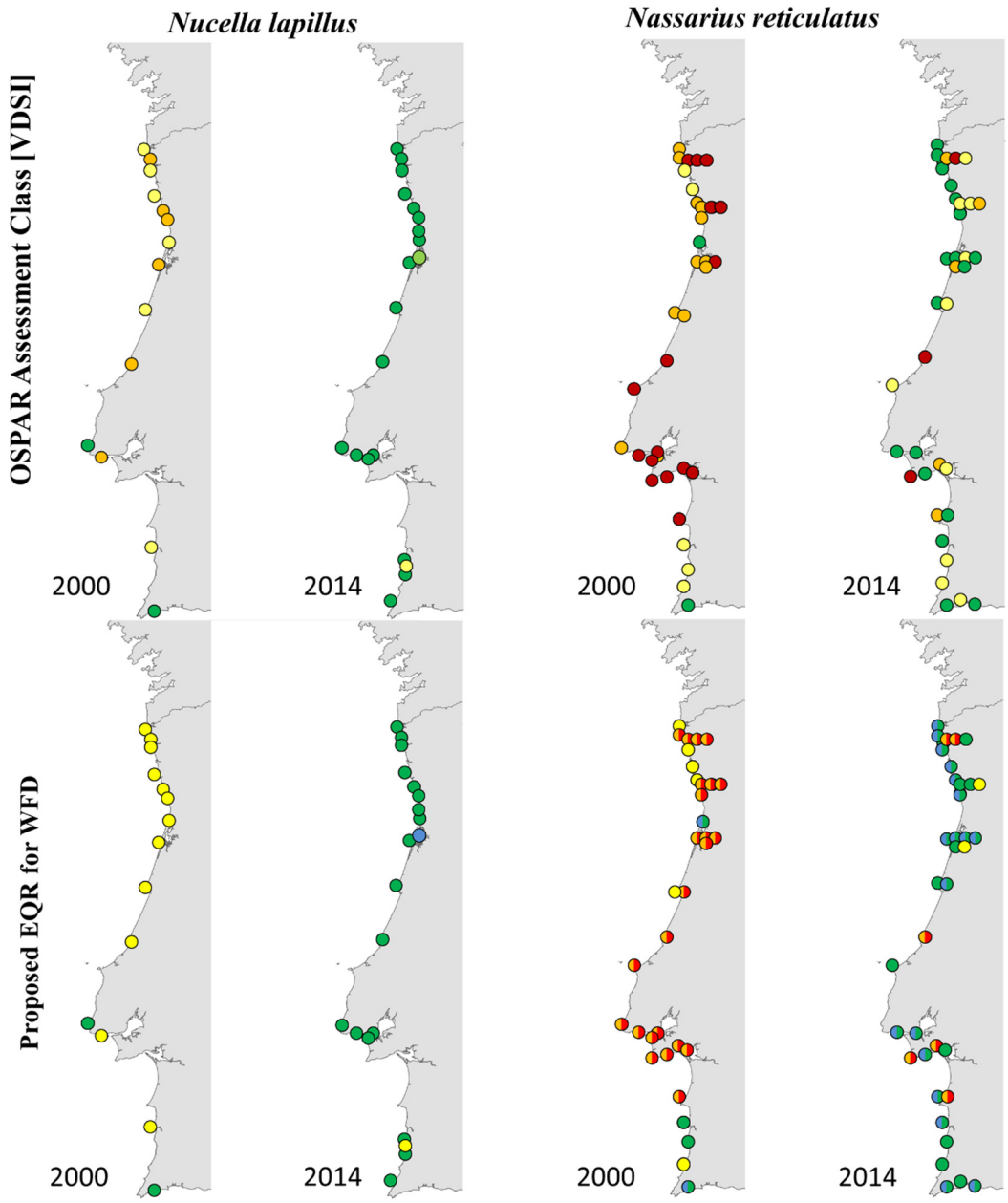

Figure 3.9 - Classification of the ecological quality status of the Portuguese coast, regarding TBT pollution, using two different criteria, the OSPAR Assessment Criteria and the WFD Proposed Ecological Quality classification by Laranjeiro et al. (2015) (see table at the top). This classification is based on the imposex levels of two gastropod species: *Nucella lapillus* and *Nassarius reticulatus*.

In order to better understand the temporal evolution in terms of the quality classification at each site, we used results previously published for the Portuguese coast, for *N. lapillus* (Galante-Oliveira et al., 2006; Galante-Oliveira et al., 2011; Oliveira et al., 2012) and *N. reticulatus* (Sousa et al., 2005; Rato et al., 2009; Sousa et al., 2009), and also data listed in the previous subchapter (section 3.1.). Figure 3.10 shows the distribution of ecological classes according to OSPAR and WFD defined by each species at sites where both species live sympatrically. By the analysis of this figure it is possible to observe that OSPAR criteria is more conservative than the WFD proposal, since the WFD Good Ecological status is reached before the OSPAR EcoQO along the years. Besides, the netted-whelks indicate a WFD Good ecological status in 2014 for all sites analysed, whilst some sites fail to reach the OSPAR EcoQO. Moreover, it is also perceived some differences between species classification: the imposex levels in *N. lapillus* decrease before *N. reticulatus*; at the end, in 2014, all stations reach the Good WFD according to the nassariid, but some fail this objective according to the muricid. Considering all the years, both species retrieve the same Ecological classification for a given site 53% of the times with OSPAR classification and 56% of the times with WFD classification (in this analysis the two upper WFD classes - “High” and “At Least Good” - were grouped as “Good”).

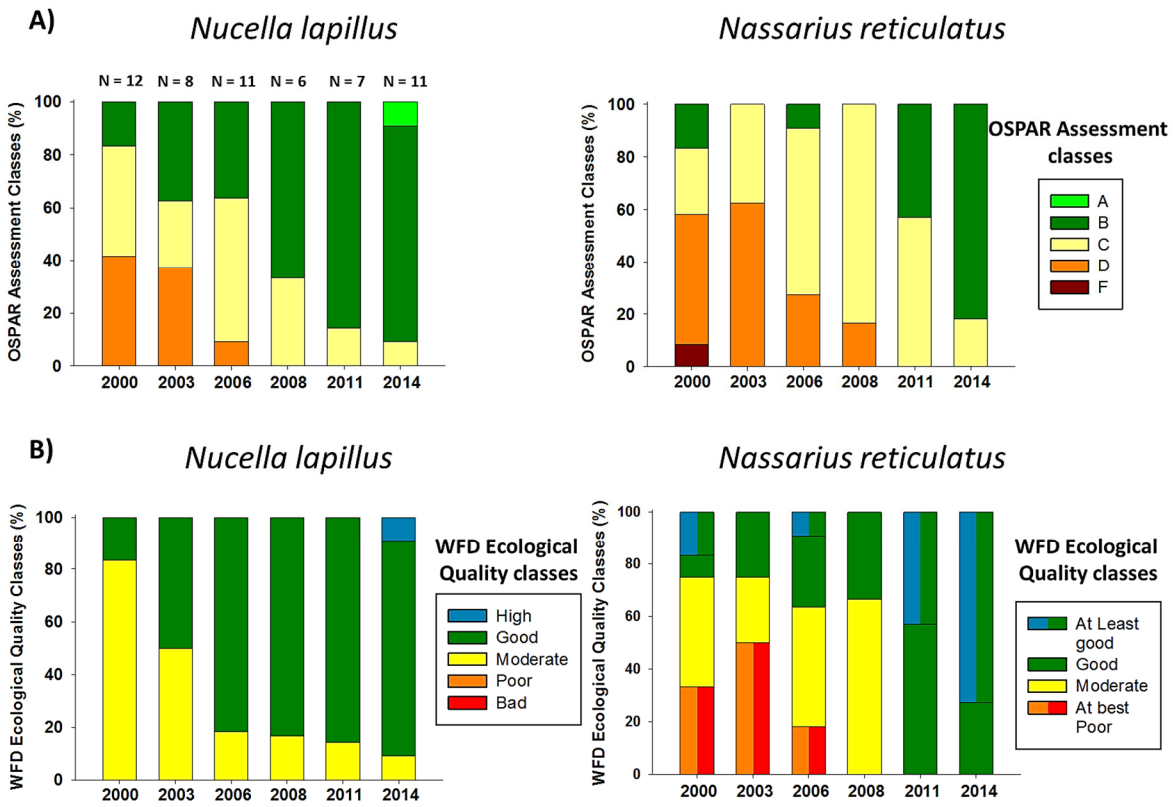

Figure 3.10 - Distribution of the classifications given by *N. lapillus* and *N. reticulatus* for sites where they live sympatrically, according to (A) OSPAR Assessment Criteria and (B) WFD proposal by Laranjeiro et al. (2015). N = stations analysed.

3.2.5. Discussion

TBT pollution has been a concerning issue all over the world (Alzieu, 1991; Horiguchi et al., 1995; Gibbs and Bryan, 1996), a portrait that can be perceived from the Portuguese coast in the year 2000, as seen in figure 3.9. By then, both *N. lapillus* and *N. reticulatus* evidenced a wide dissemination of TBT pollution throughout the Portuguese coast, at concentrations that generally produced low quality status. This situation was facilitated by the ineffective legal restrictions on TBT usage along the Portuguese coast as this chemical could still be used in ships > 25 m in 2000 (Barroso and Moreira, 2002; Santos et al., 2002). However, later restrictions banning TBT in all kind of ships seemed to finally cause a reduction of TBT pollution (Sousa et al., 2009; Galante-Oliveira et al., 2011), which is confirmed by the imposex levels decrease registered in both species till 2014,

accompanied by an improvement of the quality status according to both OSPAR and WFD classifications. The data obtained for *N. lapillus* - mainly distributed along the open coast - indicate that in 2014 only one station (stn. 39) did not reach the OSPAR EcoQO or the WFD Good Ecological Quality Status regarding TBT pollution. The data obtained for *N. reticulatus* retrieve a slightly different picture of the Portuguese coast. Even if different, this picture does not contradict the status evidenced by *N. lapillus*, since the *N. reticulatus* imposex levels registered at the open coast also reached the EcoQO/Good Ecological Quality Status. However, due to its distribution, the netted-whelk can be found near the hotspots of TBT pollution identified along the Portuguese coast (subchapter 3.1) where *N. lapillus* does not occur, which allows the assessment of a wider geographical area. In these areas, the EcoQO/Good Ecological Quality Status is not achieved in several stations in 2014, namely in Lima River (stn. 3 and 4), in fishing ports in Aveiro (stn. 19), Nazaré (stn. 25), Sesimbra (stn. 31), Setúbal (stn.33) and Sines (stn. 35) and marinas like Leixões (stn. 11).

The WFD demands that waters reach a minimum of "Good status" by the end of 2015, otherwise a member state action is required to improve the condition of water bodies through 'River Basin Management Plans'. The current work identified a number of sites along the Portuguese coast where bioindicators exhibit high levels of imposex, indicating that the Good status was not reached at all sampled locations, according to Laranjeiro et al. (2015). These sites, particularly fishing ports and marinas, still register unacceptable levels of TBT pollution, and so compulsory measures are needed to achieve the "Good status" targets quickly. Fresh inputs due to the illegal use of TBT-AFP may occur - as it is probably the case of Zambujeira do Mar, Porto das Barcas (stn. 39) - and must be inspected and controlled by national authorities. However, the main ubiquitous source of TBT seems to be the sediments. As sediments act as a long-term sink of TBT and represent a source of pollution, new methods for pollution control must target this compartment clean-up. These may include: (i) mapping TBT levels in sediments by chemical methods and evaluate bioavailability by specific bioassays (such as the one proposed in previous chapters); (ii) localization of "hotspots" of contamination; (iii) removal of contaminated sediments by dredging; (iv) decontamination of sediments by

physical/biological processes. Following these remedial dredging operations, monitoring must continue to assess the effectiveness of the clean-up action. This clean-up procedure could be harmonised with conventional dredging operations that are frequently performed in ports and marinas to maintain or improve navigable depths.

It seems thus evident that the imposex monitoring assessment will be more robust when more species are considered due to their different distribution. However, it is observed that, even when collected at the same sites, both bioindicators may not retrieve the same classification. Nevertheless, what can be seen as a flaw in these imposex-based classifications might be, in fact, a reflection of the natural variability induced by multiple environmental and biological factors. For instance, the regressions used to convert VDSI levels of one species into another species, which are the basis for the definition of the quality classes VDSI boundaries in *N. reticulatus*, comprise residual errors. Besides, TBT concentration in the water column, where *N. lapillus* inhabits, may evolve along the years at different rates than in sediments, where the netted-whelk lives, since this matrix can act as a sink for TBT (Sarradin et al., 1995; Langston et al., 2015), and this produces inter-species discrepancies. Even so, more than 50% of the sites retrieve the same classification using both species and, thus, the multispecies monitoring still seem to be the best approach for monitoring vast marine areas.

When comparing the two imposex-based quality classifications, the assessment criteria by OSPAR and the ecological quality classification proposed for WFD, is important to understand their main differences. As previously written, the major differences rely on the number of classes and on the premises and objectives that are behind the quality classes definition. Regarding the number of classes, OSPAR criteria for *N. lapillus* comprises six assessment classes while for the WFD only five are defined. This fact will consequently grant OSPAR classification more power to discriminate TBT pollution levels among stations, as we can see in figure 3.9. For instance, in the year 2000, when using *N. lapillus*, several sites are classified as C or D according to OSPAR but only as Moderate according to the WFD. Another interesting difference between both quality classifications, as seen in figure 3.10, is that a WFD's Good Ecological Status is generally achieved before the OSPAR EcoQO when using both species. This makes OSPAR

classification more conservative than WFD, regarding TBT pollution, which is in good agreement to the TBT chemical quality objectives by OSPAR (EAC = 0.1 ng/L) and WFD (EQS = 0.2 ng/L).

The integration of TBT pollution monitoring into MSFD is one of the proposals of the current work, and for that reason it is better to first understand the similarities and divergences between the different legislative pieces, regarding TBT pollution. The OSPAR Convention defines TBT as a priority substance and requires a comprehensive TBT monitoring assessment as it defines TBT's EACs for water, sediment and biota, and uses imposex as a mandatory monitoring tool under its CEMP; moreover this monitoring should be performed in transitional waters and territorial seas of the Contracting Parties, as well as the sea beyond and adjacent to the territorial sea under the jurisdiction of the coastal state, and the high seas. WFD also defines TBT as a priority substance. This directive sets an EQS for water only, and does not require any effect-based monitoring tool; therefore, TBT monitoring is only mandatory under the chemical quality elements in surface waters, which comprises rivers, lakes, transitional and coastal waters (up to a distance of 1 nautical mile), and underground waters. Finally, MSFD does not explicitly define an objective regarding the chemical evaluation of TBT, being member states responsible to determine which hazardous substances (and its monitoring approach) should be considered in order to characterize the environmental quality status. Nevertheless, the hazardous substances assessment should be performed respecting the descriptors 8 "*Concentrations of contaminants are at levels not giving rise to pollution effects*" and 9 "*Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards*". This directive is to be applied in waters from the seaward side of the territorial waters baseline to the outmost area where a member state exercises jurisdictional rights, including the coastal waters defined by the WFD. MSFD is, therefore, the least objective directive regarding this kind of pollution, but it is open to an integration of the regional sea conventions and EU legislation requirements (e.g. WFD). Moreover, due to the spatial overlap between WFD and MSFD regarding coastal waters, and as already suggested by other authors, some tools and targets from WFD could be adopted in MSFD (Borja et al.,

2010). On this basis, member states can adopt the already defined WFD's EQS for TBT to avoid any inconsistency between directives. This does not preclude, however, that also the effect-based monitoring tools applied by regional sea conventions cannot be used. In fact, regarding the OSPAR Convention and the MSFD, similar objectives are shared, in a common area of action, and it is intended that MSFD uses the mechanisms already applied by regional sea conventions. Therefore, imposex monitoring (a mandatory element in OSPAR or HELCOM monitoring programs) has the best predicates to be used in the definition of the good environmental status under MSFD. The ultimate goal of MSFD is that contaminants are at levels not giving rise to pollution effects, which corresponds to undisturbed conditions defined by the Class "High" of the WFD or Class "A" of OSPAR. Still, the main question should be whether to apply the OSPAR assessment criteria or the WFD criteria proposed by Laranjeiro et al. (2015). Analysing both proposals it is possible to observe that there is a critical VDSI range (2.0 - 3.0 for *N. lapillus* and 0.3 - 0.8 for *N. reticulatus*) where both species define the Good Ecological Quality Status/EcoQO differently. This is of foremost importance since economic and environmental issues may arise if member states do not comply with the same ecological objectives, which differ in this specific VDSI range. Therefore, accounting the spatial overlap between WFD and MSDF in coastal areas and the already initiated effort by OSPAR to align their assessment methodologies and objectives with WFD, like the OSPAR's EAC with WFD's EQS (OSPAR, 2012), the application of a more WFD-oriented classification would be more practical and desirable for a better accordance between EU directives. Therefore, we recommend that a multi-species approach using the imposex or intersex as a biomarker of TBT pollution should be mandatory under the MSDF as well, applying the criteria proposed by Laranjeiro et al. (2015) for the classification of the ecological quality status. This would not suppose any major changes on the methods employed by OSPAR, on which the proposal of these authors is based, the only difference would be the criteria for the classification of the ecological status and the EQS for TBT to be aligned with WFD's. Still, it is important to note that, considering a literal assumption of descriptor 8, MSDF share the fundamental objective of all legislative frameworks to finally eradicate TBT pollution. However, it is important to understand that there is the need to define more holistic and

realistic ecological objectives. With this in mind, one should not relax on the intent to eradicate TBT pollution, but as it seems that this contaminant may still remain in the environment for decades (Langston et al., 2015) more practical objectives are needed. In fact, a VDSI < 3.0 in *N. lapillus* as here proposed to be used in MSFD seems to be protective, since it corresponds to a TBT concentration in water that would not utterly affect the marine ecosystems (Laranjeiro et al., 2015).

The implementation of the MSFD has been seen as a difficult challenge, mainly due to the great dissimilarities between marine regions that will probably make member states to differ on their environmental objectives (Bertram and Rehdanz, 2013; Bellas 2014; Crise et al., 2015). The case here proposed is not an exception since TBT pollution monitoring has been implemented for years in the marine regions Baltic Sea (HELCOM) and North East Atlantic (OSPAR), but are only now recommended to be used in the Mediterranean Sea (MEDPOL) (UNEP/MAP 2015; UNEP/MAP 2016), while no information is available for the monitoring programs involving TBT in the Black Sea, under the Black Sea Commission. For these regions, the implementation of imposex monitoring under the MSFD would require additional work at the regional sea conventions level to find adequate bioindicators. There are several reports of imposex in the Mediterranean, with the most important species being *Hexaplex trunculus* (Axiak et al., 1995; Terlizzi et al., 1999), *Bolinus brandaris* (Solé et al., 1998; Chiavarini et al., 2003), *Stramonita haemastoma* (Rilov et al., 2000; Chiavarini et al., 2003) and *Nassarius nitidus* (Berto et al., 2007; Lahbib et al., 2011). For the Black Sea, *Rapana venosa* seems to be one of the few bioindicator species available (Micu et al., 2009). However, no intercalibration between these species and the ones used by OSPAR or HELCOM were found, i.e., the assessment of imposex levels with these species living sympatrically in order to obtain a correlation between their VDSI levels, which would define their TBT sensitivity relationship. Still, some studies evidenced that is possible to find these species sympatrically: *Hexaplex trunculus* and *Nassarius reticulatus* (Gibbs et al., 1997); *Nassarius nitidus* and *Nassarius reticulatus* (Rodríguez et al., 2009). Moreover, Cacciatore et al. (in press) has recently proposed a classification to be used under the WFD based on imposex expression in the bioindicator species *N. nitidus.* Even if this classification is slightly different from the one

proposed by Laranjeiro et al. (2015), the authors report that the obtained EQRs for *N. nitidus* are in accordance with the ones proposed for *N. reticulatus* by Laranjeiro et al. (2015), which would be expected due to their similar sensitivity in terms of the Relative Penis Length (Rodríguez et al., 2009). Nevertheless, it is evident that intercalibration studies are needed to define similar quality objectives between North Atlantic species and those from the Mediterranean and the Black Sea. This last task seems easier since several studies report the presence of several of these gastropod species sympatrically (Rilov et al., 2000; Pavoni et al., 2007; Lahbib et al., 2011).

3.2.6. Conclusions

The ecological status of the Portuguese coast, in what concerns TBT pollution, improved considerably proving the effectiveness of the latest legislation that banned the use of TBT AFS. Nevertheless there are some sites where the good ecological status was still not achieved due to the high imposex levels evidenced, being most likely necessary remediation actions - such as clean-up of TBT contaminated sediments - to achieve WFD objectives. This work again evidences the use of a multi-species imposex assessment as a consistent tool in TBT pollution monitoring, as it retrieves a wider picture of pollution. The already proposed classification by Laranjeiro et al. (2015), to define the ecological status under WFD, is now recommended for MSFD monitoring programs, since both directives share common objectives and geographical area. Still, further work is needed regarding the calibration of imposex values between species from the Atlantic, Mediterranean, Black Sea, and others, in order to find a common ground between all the European marine regions to assess the ecological quality status.

3.2.7. Bibliography

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CHAPTER 4 - IMPOSEX: THE PERFECT BIOMARKER FOR TBT POLLUTION MONITORING?

4. IMPOSEX: THE PERFECT BIOMARKER FOR TBT POLLUTION MONITORING?

"Navegar é preciso,
Viver não é preciso."
in Caetano Veloso's *Os argonautas*, 1969

Imposex is considered one of the best biomarkers in environmental pollution monitoring for being sensitive to a specific class of contaminants, with a well established dose-effect response. Consequently, it is a useful tool to be applied in TBT pollution monitoring under the WFD and MSFD, as proposed in the previous chapters. Nevertheless, the use of this tool for the assessment of the ecological quality status of EU water bodies will only be effective if there is a correct choice of the bioindicators and if imposex is in fact a specific response to TBT, a priority substance under WFD. The objective of this chapter is to evaluate if there are any exceptions that may preclude the general application of imposex as a precise monitoring tool in EU directives.

CHAPTER 4.1 – IS *PERINGIA ULVAE* A SUITABLE TBT POLLUTION BIOINDICATOR?

4.1. Is *Peringia ulvae* a suitable TBT pollution bioindicator?

4.1.1. Abstract

Ria de Aveiro has been intensively studied regarding TBT pollution with first studies reporting high levels of imposex and TBT contamination, in both water and sediment, near the main ports, shipyards and marinas in this area. After TBT was banned from antifouling paints, several studies started to report a general decrease of imposex levels in gastropod species such as *Nucella lapillus* and *Nassarius reticulatus*, and a decline of intersex in *Littorina littorea*. Surprisingly, a different trend was observed in the evolution of *Peringia ulvae* imposex levels in this study area. The present work aims to describe the most recent evolution of imposex in *P. ulvae* and understand the suitability of this species as a bioindicator of TBT pollution. With this purpose, an imposex monitoring campaign was performed in 2013 and 2015 and the results were compared with data obtained in previous campaigns. *P. ulvae* imposex levels did not decrease in the period 1998 - 2015. Several hypothesis are raised in relation to this counter current phenomenon, but what seems clear is that *P. ulvae* may not be a suitable bioindicator for TBT imposex monitoring.

4.1.2. Introduction

The use of tributyltin as a biocide in antifouling paints experienced several restrictions, starting with partial bans in the last century (national efforts in countries like France, United Kingdom, United States of America, Australia or other European states (EU Directive 89/677/CEE)), being followed by a total ban in this century (EU Regulation 782/2003 or IMO's Anti-Fouling System Convention). TBT environmental levels started to decrease (Choi et al., 2008; Sousa et al., 2009; Langston et al., 2015) and its deleterious effect in gastropods, such as the imposex (Oliveira et al., 2009; Cuevas et al., 2014; Laranjeiro et al., 2015) also declined. Imposex is an endocrine disruption phenomenon defined by the superimposition of male sexual characteristics onto gastropod females (Smith, 1971) and is currently reported for more than 260 gastropod species (Titley-O'Neal

et al., 2011). The gastropod *Peringia ulvae* (Pennant, 1777) (current name for *Hydrobia ulvae*) was proposed as a bioindicator species for TBT pollution by Schulte-Oehlmann et al. (1997) and used with this purpose in other scientific studies (Barroso et al., 2000; Galante-Oliveira et al., 2010). Furthermore this species is currently recommended to be used in imposex monitoring in Sweden by the Baltic Marine Environment Protection Commission - Helsinki Commission under their monitoring programs (HELCOM, 2012). However, in Ria de Aveiro (NW Portugal) it was suggested that this species could be acting as a counter-current bioindicator (Galante-Oliveira et al., 2010). In this area, despite the fact that imposex levels in other bioindicator species have been decreasing (Sousa et al., 2007; Galante-Oliveira et al., 2009; Laranjeiro et al., 2010), imposex levels in *P. ulvae* augmented during the 1998–2012 period (Galante-Oliveira et al., 2010; Domingues, 2012). The aim of this study is to assess *P. ulvae* imposex levels in Ria de Aveiro in 2013 and 2015 and understand if this trend persists for these later years. Also with the aim to clarify the role of this species as a TBT pollution bioindicator, imposex values observed are compared with other bioindicator species and TBT environmental levels.

4.1.3. Methods

Study area

Ria de Aveiro (Fig. 4.1) is a bar-built estuary located in the Northwest coast of Portugal, reaching a maximum of 45 km in length, parallel to the coast, and with 10 km width. Water exchange occurs through a narrow lagoon mouth, which controls the hydrographical circulation and grants wide salinity gradients along the three channels that compose this estuarine system (Barroso et al., 2000; Dias et al., 2000). Ria de Aveiro hosts fishing and commercial ports, as well as some dockyards located in the main navigation channel, between the mouth of the lagoon and the city of Aveiro (see Fig. 4.1). Also, several marinas are dispersed throughout the channels, increasing the potential sources of TBT (Fig. 4.1). This area has been object of regular TBT pollution monitoring studies since 1998 (Barroso et al., 2000; Barroso et al., 2005; Sousa et al., 2007; Galante-Oliveira et al., 2009;

Laranjeiro et al., 2010) being therefore a perfect study area to analyse the temporal evolution of TBT pollution and the legislation effectiveness over the years.

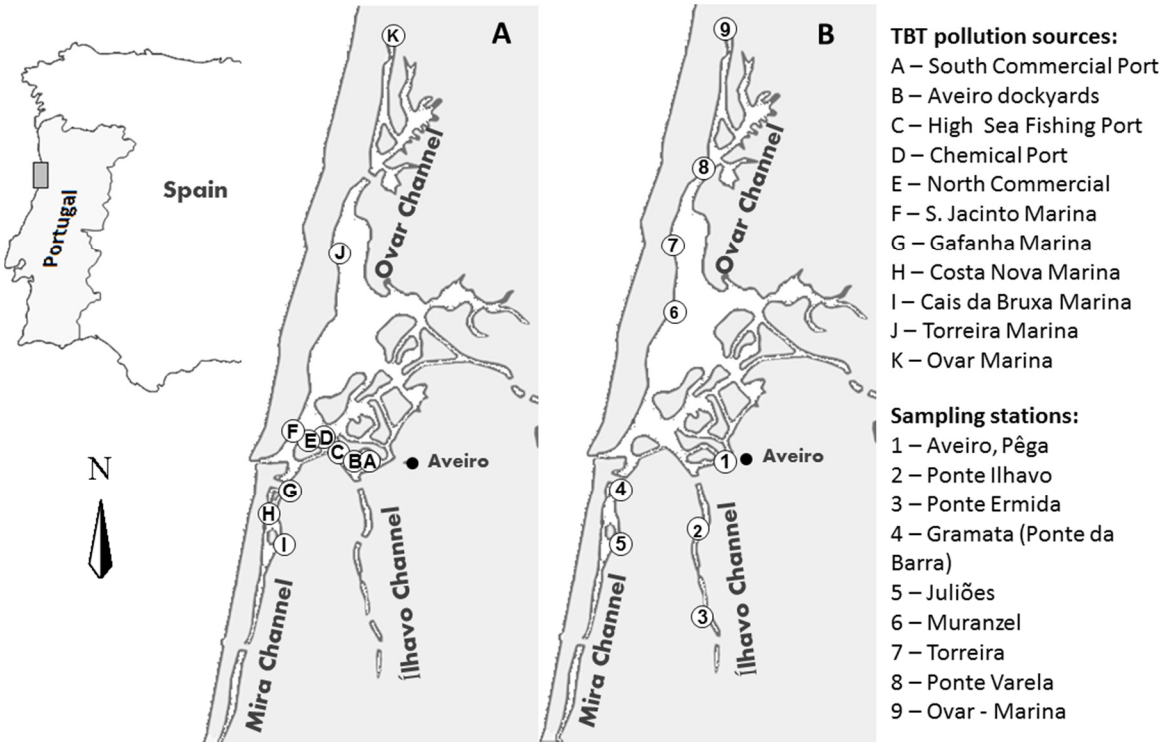

Figure 4.1 - Map of the Ria de Aveiro (NW Portugal) showing the location of the sampling stations (1-9) and the potential sources of TBT pollution identified in the study area (A-K).

Imposex assessment

Based in previous studies (Barroso et al., 2000; Galante-Oliveira et al., 2010; Domingues, 2012), 9 sampling stations (stn.) were selected covering a wide area inside Ria de Aveiro (Fig. 4.1). Whenever present, 60 specimens of *Peringia ulvae* with shell height greater than 4.0 mm were collected by hand during the low tide, between June and August in 2013 and 2015. Once in the laboratory, organisms were kept in constantly aerated artificial seawater (20 ± 1 °C and 25 ± 1 psu) for a maximum of 3 days until imposex analysis. Individuals were narcotized with a 3.5% magnesium chloride ($MgCl_2$) solution in distilled water for about 60 minutes. Afterwards, organism's height was measured, under a stereomicroscope with a graduated eyepiece, to the nearest 0.14 mm and shells were

cracked open with a bench vice. Organisms were then dissected, sexed and whenever possible, 40 adult females and 20 adult males were carefully examined. Percentage of imposex-affected females (%I), the Mean Female Penis Length (FPL), the Mean Male Penis Length (MPL), the Relative Penis Length (RPL) and the Vas Deferens Sequence Index (VDSI) were evaluated for each station. VDSI was determined according to scoring system proposed by (Schulte-Oehlmann et al. (1997). The presence of parasites was checked under a stereo microscope and parasitized organisms snails were discarded from the analysis.

An historical review of *P. ulvae* imposex data in this study area was also performed. The non-parametric Kruskal-Wallis analysis of variance, followed by a pairwise multiple comparison, was performed to evaluate possible statistical differences among stations. Statistical analyses were performed with IBM SPSS Statistics 22 Software.

4.1.4. Results

P. ulvae imposex levels in Ria de Aveiro in 2013 and 2015 are shown in table 4.1. The incidence of imposex in 2013 varied from 50 to 78%; RPL and VDSI varied from 4.76 to 12.13% and from 0.57 to 0.83, respectively. The highest incidence of imposex was observed at stn. 1 with 78% of the females observed being affected by the phenomenon; however the higher imposex intensity was registered in stn. 4 with VDSI = 0.83. In 2015, the imposex levels registered were generally higher than the values recorded in 2013, with %I varying between 50 and 93%, and RPL and VDSI between 4.22 and 13.76% and 0.63 to 1.15, respectively. The highest percentage of imposex-affected females was now observed in stn. 9 (93%), while stn. 4 appear again as the site with the highest VDSI level (VDSI = 1.15).

Table 4.1 - *Peringia ulvae* imposex levels registered in Ria de Aveiro in 2013 and 2015. Number of males and females analysed (N ♂, N ♀, respectively), mean male and female shell height (SH ♂, SH ♀, respectively), mean male and female penis length (MPL, FPL, respectively), RPL (relative penis length), VDSI (vas deferens sequence index) and %I (Percentage of imposex-affected females) are shown.

	Station	*N* ♂	SH ♂(mm)	MPL (mm)	*N* ♀	SH (mm)♀	FPL (mm)	RPL (%)	VDSI	%I
2013	**1**	5	5.20 ± 1.07	1.44 ± 0.36	10	5.07 ± 1.18	0.13 ± 0.13	9.32	0.70 ± 0.48	78
	4	23	3.44 ± 1.01	1.39 ± 0.31	18	2.94 ± 0.77	0.14 ± 0.35	9.99	0.83 ± 0.92	61
	5	18	6.74 ± 1.19	2.69 ± 0.47	26	6.61 ± 1.30	0.13 ± 0.15	4.92	0.62 ± 0.75	50
	6	16	3.62 ± 0.31	1.89 ± 0.26	22	3.76 ± 0.45	0.11 ± 0.09	5.67	0.82 ± 0.85	64
	7	11	4.39 ± 0.14	1.90 ± 0.24	15	4.63 ± 0.32	0.09 ± 0.09	4.99	0.73 ± 0.80	60
	8	7	3.42 ± 0.32	0.96 ± 0.27	19	3.46 ± 0.59	0.12 ± 0.12	12.13	0.58 ± 0.60	53
	9	1	3.49	1.12	7	3.32 ± 0.65	0.05 ± 0.08	4.76	0.57 ± 0.61	57
2015	**2**	15	3.73 ± 0.23	1.81 ± 0.17	16	3.71 ± 0.26	0.08 ± 0.15	4.22	0.63 ± 0.81	50
	3	17	4.52 ± 0.33	1.89 ± 0.22	26	4.60 ± 0.38	0.14 ± 0.09	7.36	0.85 ± 0.37	85
	4	24	5.62 ± 0.38	2.44 ± 0.40	26	5.57 ± 0.53	0.33 ± 1.20	13.76	1.15 ± 0.88	81
	5	17	5.54 ± 0.46	2.05 ± 0.28	28	5.76 ± 0.54	0.18 ± 0.21	8.91	1.08 ± 1.00	64
	6	25	4.73 ± 0.41	1.88 ± 0.28	25	4.75 ± 0.54	0.13 ± 0.14	6.99	1.00 ± 1.04	64
	7	18	4.24 ± 0.19	1.85 ± 0.20	19	4.25 ± 0.21	0.08 ± 0.06	4.52	0.74 ± 0.45	74
	8	6	4.36 ± 0.30	1.70 ± 0.34	11	4.33 ± 0.31	0.09 ± 0.07	5.38	0.73 ± 0.47	73
	9	9	4.54 ± 0.29	2.00 ± 0.12	43	4.75 ± 0.32	0.19 ± 0.11	9.34	1.10 ± 0.70	93

Imposex historical data reported by other authors for *P. ulvae* was also included in the present work, comprehending the period between 1998 and 2015 (Fig. 4.2). In their study on the use of this species as TBT pollution bioindicator in Ria de Aveiro, Galante-Oliveira et al. (2010) pointed out the positive correlation between animal size and imposex intensity. Therefore, before discussing imposex levels variation over the 17 years' period, it is important to understand shell height variations in the analysed organisms. Data demonstrate a considerable shell height variation throughout the years, with a significant decrease being observed in 2007, 2013 and 2015, in relation to the first study performed in 1998. Therefore, imposex levels comparisons between these years should be taken carefully.

A constant increase in %I until 2012 is perceived, being this year's value statistically different from previous imposex monitoring campaigns (1998, 2003 and 2004). However, an abrupt decrease is observed in the following years to levels comparable to the first study performed in 1998. This might be justified by the smaller organisms sampled in 2013, since

a decline is registered in shell height values. The variation in shell height from 2013 to 2015 should again be the reason for the %I increase in 2015 to levels comparable to those of 2007. Regarding RPL, the levels observed are now lower than those observed in the first 2 monitoring campaigns (1998 and 2003), except again for the year 2012 where a high increase in this parameter was registered. Statistically significant differences to 2012 were observed for the years 2007, 2013 and 2015 where lower FPL levels were observed. VDSI levels in 2012 were again the highest, being significantly higher than those observed in the first monitoring campaign and in 2013, a year when a significant decrease is registered in all assessed imposex parameters.

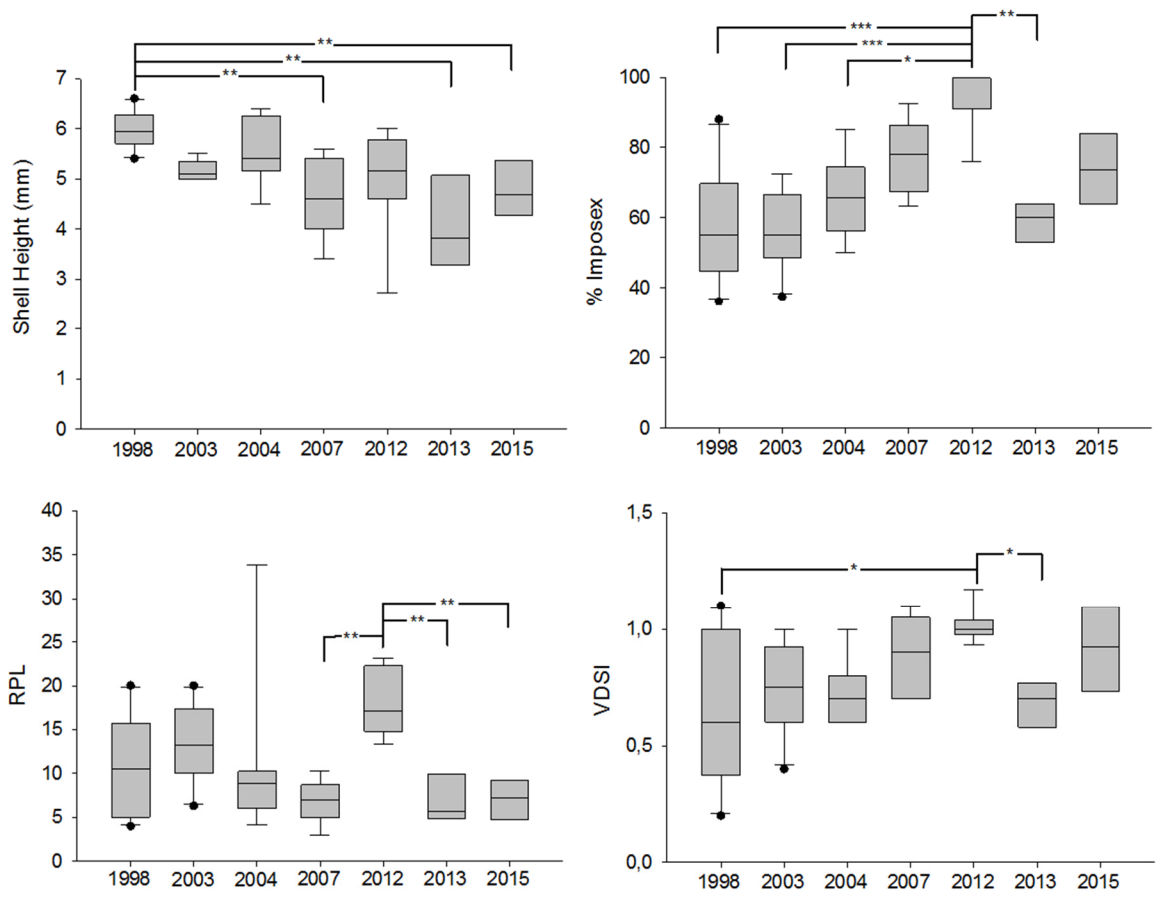

Figure 4.2 - Evolution of shell height, percentage of imposex-affected females (%I), RPL and VDSI in *Peringia ulvae* collected in Ria de Aveiro from 1998 to 2015. Data from 1998 to 2012 were previously published: 1998 by Barroso et al. (2000); 2003, 2004 and 2007 by Galante-Oliveira et al. (2010); and 2012 by Domingues (2012). Box plots represent: the median (line), the range between 25% and 75% of the values observed (grey box); the lowest and highest values observed (whiskers); and the outliers (dots). Statistically significant differences between years are shown (*$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$).

4.1.5. Discussion

The results obtained in this study show the ubiquitous presence of *P. ulvae* females with signs of masculinization in Ria de Aveiro. Is this, in fact, reporting TBT contamination?

TBT pollution and imposex have been intensively studied in Ria de Aveiro for a period of 17 years (1998 - 2015). First studies reported high imposex levels inside the lagoon (Barroso et al., 2000; Barroso et al., 2005). However, the entry into force of legislation banning TBT from antifouling paints contributed to a decrease of TBT inputs leading to a drop in imposex levels. In Ria de Aveiro, several studies indicated that a TBT levels' reduction was evident in diverse environmental matrices such as water (Galante-Oliveira et al., 2009), sediments (Laranjeiro et al., 2010; Domingues 2012), biota (Sousa et al., 2007; Sousa et al., 2009) and in the imposex levels reported by several bioindicator species like *Nucella lapillus* (Galante-Oliveira et al., 2009; Laranjeiro et al., 2010; Laranjeiro et al., 2015), *Nassarius reticulatus* (Sousa et al., 2007; Laranjeiro et al., 2010; Laranjeiro et al., 2015) and *Littorina littorea* (Laranjeiro et al., 2015). Despite all these evidences of an imposex recovery to values closer to zero (Laranjeiro et al., 2015), the mud snail *P. ulvae* shows a contradictory trend in this study area. While in other species imposex levels started to decrease in 2003, %I and VDSI levels in the mud snail continuously increased until 2012, independently of the shell height of the analysed specimens. Then, after they have dropped in 2013, here probably accompanying the reduction in the size of the specimens used in imposex assessment, %I and VDSI increased again in 2015 to values that are even higher than the ones registered in the TBT pre-ban period of 1998 and 2003.

Galante-Oliveira et al. (2010) had already observed this counter-current trend for the period between 1998 and 2007. These authors suggested that TBT persistence in the sediment and the *P. ulvae* feeding habits, based on sediment particles ingestion, could be the responsible cause for this trend. However, after 8 years, our data keep revealing that imposex levels in *P. ulvae* show a completely different pattern from other bioindicator species in Ria de Aveiro. For example, imposex levels observed in *N. reticulatus*, another sediment-dweller, register an evident decreasing trend in this area, with recent data from 2013 showing VDSI levels close to zero (maximum observed VDSI = 0.36) and the %I ranging

3 - 36% (Laranjeiro et al., 2015). Furthermore, recent data on TBT concentration in sediments (1.5 - 29 ng Sn/g dw), collected at the same stations as the mud snails, show a clear reduction in relation to TBT levels reported in 1998 (< 6 - 88 ng Sn/g dw), (Domingues, 2012). These observations challenge the imposex increasing trend observed in the mud snail (until 2012) as a response to TBT pollution, since it is clear that the levels of this contaminant decreased significantly.

The work by Domingues (2012) proposes that initial and undetectable signs of parasitism, as well as an increase of parasitism incidence in natural populations over the years, can influence the imposex levels observed in this area. It was described, even before TBT pollution exists, that female masculinization in this species could also be related with parasites infestation (Schulte-Oehlmann et al., 1997). These authors suggested that even if parasitism play some role in female masculinization, TBT contamination would be the major causative agent for the imposex levels observed in this species. Still, these authors recommend that parasitized specimens should be excluded from analysis in TBT biomonitoring programs, and this has been performed in all studies executed in Ria de Aveiro. Yet, Domingues (2012) questioned if its possible to effectively remove all parasitized organisms from the samples under analysis because these small snails may present initial and undetectable stages of parasitism. Therefore, if other factors affect the well-established relationship between TBT and imposex in *P. ulvae* (Schulte-Oehlmann et al., 1997), then this species might not be a reliable TBT bioindicator.

The conclusions of this study are of major relevance since recently HELCOM have proposed this species to monitor TBT pollution in the eastern parts of the Baltic Sea where other bioindicators do not exist (HELCOM, 2009). This Commission developed imposex assessment classes for this species that, if named according to EU's Water Framework Directive, would give the following classes: Good Ecological status – VDSI < 0.3; Moderate Ecological status - 0.3 ≤ VDSI < 1.0; Poor Ecological status - 1.0 ≤ VDSI < 3.0 (Nyberg et al., 2013). According to this classification, our data would show 4 stations exhibiting a Moderate Ecological status and other 4 exhibiting a Poor Ecological status in 2015. This is in total disagreement with observations by Laranjeiro et al. (2015) that show through the imposex assessment in three different indicator species - *Nucella lapillus, Nassarius*

reticulatus and *Littorina littorea* - that Ria de Aveiro present at least a Good Ecological status in all assessed stations two years before, even considering that the criteria used to define ecological status classes may not be exactly the same. For the above reasons, we recommend that *P. ulvae* should not be selected for TBT monitoring under WFD and MSFD until it is made clear what factors actually influence imposex expression in this snail.

4.1.6. Conclusions

P. ulvae was proposed as a bioindicator of TBT pollution by Schulte-Oehlmann et al. (1997) and presented some advantages comparing to other bioindicator species. The fact that this species lifespan is shorter than other bioindicators allows the assessment of imposex levels that reflect more recent contamination. Also, and as observed for Ria de Aveiro (Barroso et al., 2000), this species tolerates waters with low salinity, which allows monitoring in the inner part of estuarine areas where other bioindicators fail to exist. However, the main property of a bioindicator is to reflect reliably the status of pollution of a given area. If so, why contradictory data is being collected between *P. ulvae* and the rest of the indicator species used to monitor the evolution of TBT pollution in Ria de Aveiro? One possible reason could be a slower recovery from imposex condition in this species because it is particularly exposed to sediment contamination (by food ingestion and burrowing habits) and also because TBT can persist for a longer time in this compartment. However, this seems not to be the case as we confirmed for a longer period of time that the imposex levels for this species did not accompanied the decrease of TBT levels in the environment, being at the present time even higher than in 1998. It seems, therefore, that the imposex in *P. ulvae* vary independently of TBT pollution in this estuarine system, an aspect that should be addressed in future studies, and so this species should not be regarded as an adequate bioindicator.

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CHAPTER 4.2 - TRIPHENYLTIN INDUCES IMPOSEX IN *NUCELLA LAPILLUS* THROUGH AN APHALLIC ROUTE

Laranjeiro, F., P. Sánchez-Marín, A. Barros, S. Galante-Oliveira, C. Moscoso-Pérez, V. Fernández-González and C. Barroso (2016). "Triphenyltin induces imposex in *Nucella lapillus* through an aphallic route." Aquatic Toxicology **175**: 127-131

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4.2. Triphenyltin induces imposex in *Nucella lapillus* through an aphallic route

4.2.1. Abstract

Triphenyltin (TPT) was used until recently as a biocide in antifouling systems and nowadays is still applied as an agriculture pesticide in some countries. This compound is known to cause imposex (the imposition of male characters in females of gastropod molluscs) in a very limited number of species, when compared with tributyltin (TBT), the universally recognized imposex causing agent. In this study, we tested if TPT could induce imposex in females of the dog-whelk *Nucella lapillus*. Experimental groups of 40 females were injected with a volume of 2 µL/g of soft tissue wet weight (ww) of one of the following treatments, using DMSO as a solvent carrier: DMSO (solvent control); 1 µg/g ww of TBT (positive control); 0.2, 1 and 5 µg/g ww of TPT and a non-injected group (negative control). Concentrations were confirmed in the organism tissues by means of chemical analyses of a pool of 10 specimens at T_0 and then after the imposex analysis at T_{56} days. After 8-week trial, results pointed out statistically significant differences between treatments, with both TPT and TBT positively inducing imposex. However, imposex development in TPT-injected females differed from that of TBT, since females that developed imposex presented an aphallic condition (no penis development) while the TBT treated females developed standard imposex (with penis formation). These results suggest that TPT and TBT act differently in the sequential process of female masculinization, casting new insights about the hypothetical pathways underlying imposex development.

4.2.2. Introduction

Triphenyltin (TPT) is an organotin compound used until recent years as a fungicide in agriculture, namely in United States and in some countries in Asia (Higley et al., 2013; Yi et al., 2012). For some decades it was also applied as a biocide in ship antifouling systems (AFS), together with the most common organotin biocide at the time - tributyltin (TBT).

Both compounds were banned from ship AFS in 2008 through the AFS Convention implemented by the International Maritime Organization. The leaching of TPT and TBT from ship hulls led to a wide dispersion of these compounds throughout the marine environment, reaching concentrations that could cause adverse effects in biota (Shim et al., 2000; Yi et al., 2012). One of most notable adverse effects is imposex, an endocrine disruption effect defined as the superimposition of male characteristics (penis and vas deferens) in mollusc gastropod females. This phenomenon has been already observed in more than 260 gastropod species worldwide (Titley-O'Neal et al., 2011) and is generally associated to TBT pollution. Although being known that the main causative agent of imposex is TBT, it has been shown in laboratory that TPT can also induce imposex in the gastropods *Reishia clavigera* (Horiguchi et al., 1997), *Marisa cornuarietis* (Schulte-Oehlmann et al., 2000), *Nassarius reticulatus* (Barroso et al., 2002), *Bolinus brandaris* (Santos et al., 2006) and *Stramonita haemastoma* (Limaverde et al., 2007). However, in the dog-whelk *Nucella lapillus*, one of the most sensitive species to TBT pollution in Atlantic coasts and recommended as a biomonitor by OSPAR (2009), no causative relationship has been obtained until now for TPT and imposex. Moreover, imposex development seems to occur through pathways that involve binding of the agents to nuclear receptors RXR (retinoid X receptor) and PPARγ (peroxisome proliferator-activated receptor gamma) (Castro et al., 2007; Nishikawa et al., 2004; Pascoal et al., 2013). Not only TBT, but also TPT has been shown to be able to bind with high affinity to these receptors (Grun et al., 2006; Kanayama et al., 2005; Nakanishi, 2008). Therefore, since TPT seems to be able to induce in vitro the pathways that may lead to imposex development, we have designed this experiment to understand if TPT is also able to induce imposex in vivo in *Nucella lapillus*.

4.2.3. Methods

Dog-whelks were collected from a previously monitored site (Espinho, NW Portugal, 41°00.44 N; 8°38.71 W), known to have very low imposex levels, with a vas deferens sequence index (VDSI) of 0.4. At the laboratory, specimens were narcotized for a period of 60 min with a solution of 7% $MgCl_2$ in distilled water and analysed under stereo microscope by gently pulling the gastropod foot out of the shell, to make possible the sex identification.

Only females not showing signs of imposex were selected for experimentation (note that VDS stages 0 and 1 cannot be differentiated without cracking the shell of the animal; Gibbs et al., 1987). Selected females were left to recover in aquaria for one week, while the other organisms were returned to environment. Experimental groups of 30 females were again narcotized and injected in the foot with a volume of 2 µL per g of soft tissue wet weight (ww) of one of the following treatments, using DMSO as a carrier solvent: DMSO (solvent control); 1 µg/g ww of TBT; three distinct TPT experimental groups injected with 0.2, 1 and 5 µg/g ww of TPT, respectively, and a non-injected group (negative control). Each group was separated in 6 flasks of 750 mL (5 organisms in each) to avoid possible crossed contamination between treatments, and to avoid propagation of mortality (by fungus for instance). Animals were kept in artificial seawater, constantly aerated, at a temperature of 18 ± 1°C. Additionally, ten individuals were injected with the same treatments as exposed animals, left to recover in artificial seawater and pooled to check for actual concentrations of injected TBT and TPT in tissues at the beginning of experiment (T_0). Tissues were freeze dried and homogenized. The test had the duration of 56 days with water changes performed twice a week. During the bioassay the animals were not fed to avoid possible TBT contamination through diet, which could undermine the aim of this study since *N. lapillus* feeds on mussels, which are known to accumulate TBT (Barroso et al., 2004; Sousa et al., 2009). It is described from field observations that *N. lapillus* can undergo on several days without feeding with no effect on mortality (Burrows and Hughes, 1991), and even periods of 4 months of starving have been reported in natural population (Crothers, 1985). For the test to be valid it was considered as acceptable mortality below 20% in control, as considered in other prosobranch bioassay guidelines (Ducrot et al., 2014; Duft et al., 2007). At the end of this period, the imposex parameters female penis length (FPL) and VDSI were assessed and classified according to Gibbs et al. (1987). Since not all females followed the normal VDS development pathway we classified VDSI based on the vas deferens development as described by Sánchez-Marín et al. (2015). Briefly, females with VDS>1 but no penis, were considered aphallic. A long vas deferens which only starts at the genital papilla is considered a VDS stage 2; an interrupted vas deferens which starts both from the genital papilla and the base of the right tentacle (where it should start the penis

development) is considered a VDS stage 3; and a complete vas deferens from the genital papilla to the base of the right tentacle corresponds to VDS stage 4. After imposex examination, ten individuals of each treatment were pooled (T_{56}) for organotin quantification in tissues.

TPT, TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) were extracted by ultrasonic extraction and determined by gas chromatography followed by mass spectrometry (GC-MS) in SRM mode (Moscoso-Pérez et al., *in preparation*). An appropriate amount of surrogate standard (TBT d_{27}) and 6 mL of a solvent mixture of acetic acid:methanol:water (1:1:1) was added to 0.5 g of sample (dw). The tubes were sonicated for 30 minutes in an ultrasonic bath and centrifuged to obtain a liquid/solid phase separation. Ten mL of acetate buffer solution (pH 5) and a fixed volume of internal standard were added to the liquid phase. The extraction solution was derivatized with $NaBPr_4$ solution and extracted in 2 ml of isooctane and the organic phase was transferred to a chromatography vial., Determination was carried out with a Thermo-Finnigan (Waltham, MA, USA) Trace GC chromatograph equipped with a Triplus autosampler, PTV injector and coupled to a triple quadrupole mass spectrometer (TSQ Quantum XLS). The analytical uncertainty was < 20% and method quantification limit (MQL) was 0.011 µg/g ww for all substances. The analytical method was validated for MBT, DBT and TBT using ERM®-CE 477 Mussel Tissue. For TPT, the method was validated using spiked reference material, because there is no certified reference value in this material. Analytical recoveries of MBT, DBT, TBT and TPT were 50%, 80%, 107% and 90%, respectively.

Statistical analyses were performed with IBM SPSS Statistics 22 Software. Differences between treatments were evaluated through one-way ANOVA after normality and homogeneity were verified, followed by Dunnet's post hoc test. The critical significance level adopted was 0.05.

4.2.4. Results

The mortality during the test ranged between 17% in the control group, which validates the bioassay, and 50% in the highest TPT concentration. Even though the

mortality in some experimental groups were high (for instance, 50%), we could still trust the test, because there is no induction of imposex in the control group. Moreover, at least a sample of 15 females per treatment by the end of the exposure period was analysed that guarantees an acceptable number of observations for the statistical analysis. The results presented in this manuscript are a repetition of a preliminary experiment in which the mortality in the control group did not meet the validity criterion of mortality < 20%. Nevertheless, the organisms exposed to TPT analysed in that previous trial (unpublished data) shown an equal imposex induction, confirming our results.

The results showed a statistically significant increase in VDSI for all TPT concentrations and the TBT control (Fig. 4.3A). TPT seems to have the same effectiveness as TBT on imposex development, since VDSI is similar at the same concentration of 1 µg/g ww. However, clear differences are observed in FPL (Fig. 4.3B). While females injected with 1 µg/g ww of TBT developed a penis with variable length (from 0.62 to 1.30 mm), the majority of the specimens injected with TPT did not show any sign of penis development (Fig. 4.3C) but exhibited a conspicuous vas deferens reaching VDS values of 2 - 4 (Fig. 4.3D). Only 2 TPT-injected females exhibited penis, and this was at VDS advanced stages 4 and 5, while 27 imposex-affected females (VDSI≥2) showed no penis being therefore considered to present an aphallic condition, as described in methods section. TBT-injected individuals developed a penis primordium already at VDS stage 2, which continues its development through the next stages (Fig. 4.3C), being this the normal described pathway in this species (Gibbs et al., 1987).

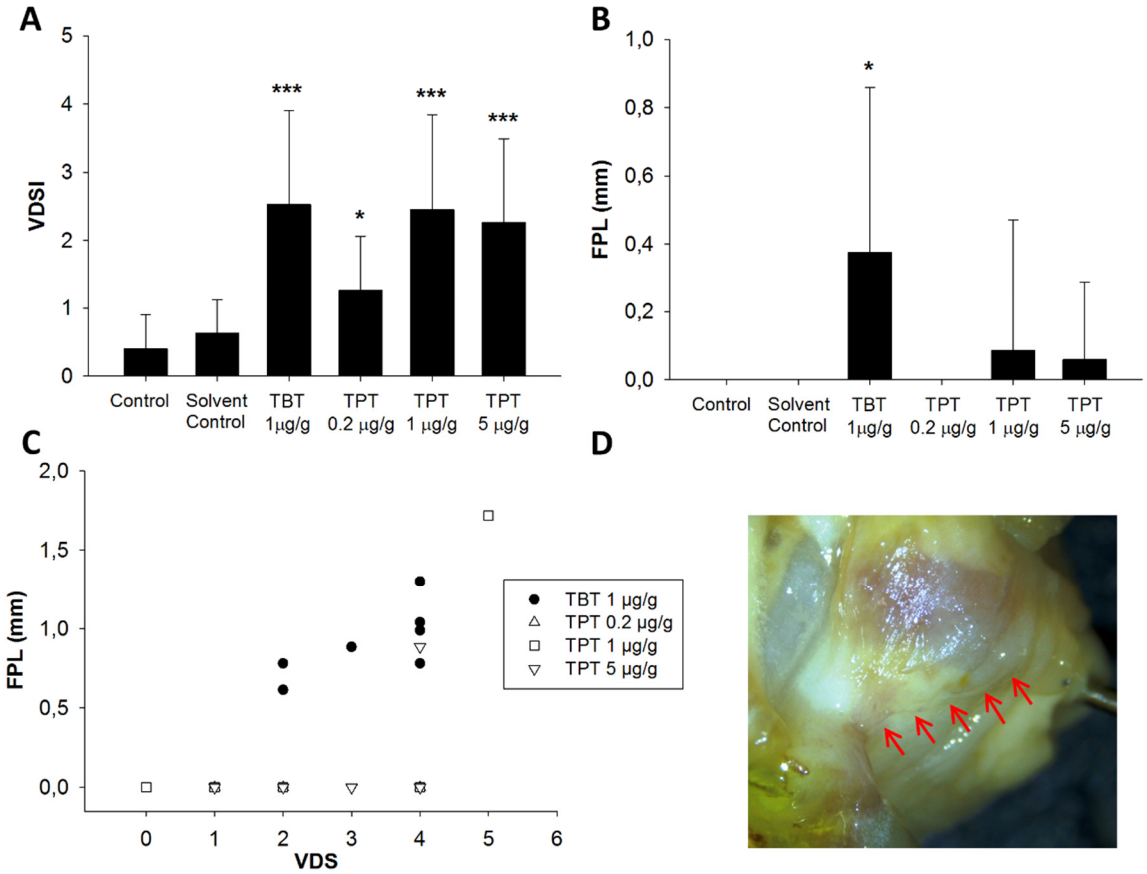

Figure 4.3 - Vas deferens sequence index (VDSI) (A) and mean female penis length (FPL) (B) obtained after 8 weeks of injection to the indicated compounds. Statistically significant differences to control (*$p < 0.05$ and *** $p < 0.001$). In C chart shows the correspondence between penis length and VDS measured in the treatments with TBT and TPT (no penis was oberved in Control and Solvent Control). In D, an aphallic female with a well-developed vas deferens (injected with 1 µg/g ww TPT).

The results of tissues' chemical analysis are shown in table 4.2. At T_0, TBT and TPT concentrations in tissues were near nominal values, varying between 103% (TBT 1 µg/g) and 188% (TPT 0.2 µg/g) of nominal concentrations, while other non-injected compounds were at concentrations below the limit of quantification. At the end of the experiment (T_{56}), the concentrations of TBT and TPT were still high and different among treatments, although some elimination and transformation occurred, as evidenced by the detection of DBT in the TBT-injected treatment.

Table 4.2 - Organotins (TBT and TPT)a concentration measured in tissues just after injection at T0 (T0) and at the end of the experiment (T56), with the indication of nominal concentrations injected. LOQ - limit of quantification.

Treatments and nominal concentrations (µg/g ww)	Measured concentrations (µg/g ww[b])			
	T_0		T_{54}	
	TBT	TPT	TBT	TPT
Control	-	-	< LOQ	< LOQ
Solvent Control	< LOQ	< LOQ	< LOQ	< LOQ
TBT 1	1.03	< LOQ	0.78	< LOQ
TPT 0.2	< LOQ	0.38	< LOQ	0.12
TPT 1	< LOQ	1.05	< LOQ	0.91
TPT 5	< LOQ	6.01	< LOQ	3.55

[a] Degradation products were only detected in TBT treatment at T_{56}. DBT – 0.16 µg/g ww.
[b] For comparison purposes, the relationship dry weight/wet weight was 0.28.

4.2.5. Discussion

Imposex is a phenomenon known to be promoted by TBT and/or TPT, however few species are known to develop imposex after exposure to this last compound. Hence, our results add *N. lapillus* to this list of species, despite other previous studies reported the opposite (Bryan et al., 1988; Schulte-Oehlmann et al., 2000). Bryan et al. (1988) observed that TPT did not have any effect on imposex promotion in the dog-whelk after exposure via water or injection. However, the initial imposex levels of the exposed population were considerably higher than ours, which might affect any comparison. Our results suggest that TPT does not trigger the penis formation in initial phases of imposex development since no penis was observed until the late VDS stage of 4 (females presenting an aphallic condition for VDSI ≤ 4), while Bryan et al. (1988) exposed females with VDS ≥ 2, that already presented a penis at the beginning of the experiment, and TPT did not promote further penis development. Afterwards, experiments by Schulte-Oehlmann et al. (2000) retrieved the same absence of imposex induction. The contrast with our results might be justified by the fact that Schulte-Oehlmann et al (2000) exposed animals via water to relatively lower concentrations of TPT (5 - 100 ng TPT-Sn/L). The bioaccumulation of TPT in their experiment probably lead to TPT concentrations in tissues lower than those in our study.

Another important discovery of the present work is that most of the females exposed to TPT developed imposex through an aphalic route, different to the one observed for TBT. In fact, a continuous and complete development of the vas deferens was observed

in the majority of the females injected with TPT while the occurrence of a penis was only observed in specimens exhibiting high stages of imposex (VDS ≥ 4, n = 2). The standard imposex development in *N. lapillus* starts by the formation of the vas deferens that precedes that of the penis (VDS = 1); afterwards (in VDS = 2, 3 and 4), these sexual characters follow a concomitant continuous development (Gibbs et al., 1987). However, our results show that TPT possibly inhibits penis formation (aphallic condition) up to the complete development of the vas deferens (Fig. 4.3C), at VDS stages for which TBT-injected females clearly exhibit the penis. Even so, and since two TPT-injected females with penis were observed at higher VDS stages, we should remain cautious in stating that the aphallic condition is a permanent state under TPT exposure. Actually, there is still a possibility that TPT injected-females might later form and develop a penis if the condition is dependent on the dose and time of exposure. In addition, a time lag between the development of these structures is also reported in other species (Horiguchi et al., 2012; Oehlmann et al., 1996) and should not be disclosed here. Nevertheless, the results here presented are evidences that the development of the penis and vas deferens in this species might follow different biochemical pathways, as already suggested by Barreiro et al. (1999), based on the work by Féral and Le Gall (1983).

The occurrence of aphally in females of this species is not a novelty but has been related with the occurrence of Dumpton Syndrome (DS), a recessive mutation that affects the development of genital system in males (Gibbs, 1993, 2005). As an advantage it was observed that imposex-affected females from DS populations were more resistant to TBT and exhibited aphally (Barreiro et al., 1999; Gibbs, 1993; Huet et al., 1996). Also several studies detected female aphally in low TBT polluted sites and on TBT pollution decreasing scenarios in *N. lapillus* (Huet et al., 2008; Sánchez-Marín et al., 2015) or in other species as *N. reticulatus* (Sousa, 2009) and *Stramonita haemastoma* (Toste et al., 2013). Our work now shows that female aphally in *N. lapillus* might not always be related with DS. Recent studies on TPT levels in tissues in natural populations of *N. lapillus* show maximum concentrations of 0.002 µg/g dw (Oliveira et al., 2009) or 0.023 µg/g dw (Guðmundsdóttir et al., 2011), much lower than the concentrations injected in this study. Therefore, we cannot establish any causative relationship among TPT and female aphally observed in field

samples. Past TPT concentrations reported by Ruiz et al. (1998) in dog-whelk tissues were within the range of our lowest tested concentration but aphally was not reported in those samples, which is expected given that TBT concentrations exceeded those of TPT. At the present time high TPT concentrations seems to be only found at high polluted sites in Asia with reported maximum concentrations in the gastropod *Reishia clavigera* of 23.23 µg/g dw (Ho and Leung, 2014). Imposex development was associated with this substance for the first time in Japan (Horiguchi et al., 1994) and has been an historical issue in Asia, where the highest concentrations in the marine environment have been reported (Yi et al., 2012). Still, similar experimental results or field observations were not reported for Asian gastropod species.

Concluding, our results show for the first time that TPT, a strong agonist for both RXR and PPARγ, induces imposex in *N. lapillus*. This outcome strengthen the hypothesis that imposex is caused after the disruption of the retinoid signaling pathway by organotins (Nishikawa, 2006) and/or through PPARγ or the heterodimer RXR/PPARγ (Pascoal et al., 2013). Still, given that different imposex expression is induced by two agonists of these nuclear receptors (TBT and TPT) in this species, it is evident that some pieces of the imposex puzzle can still be missing. Therefore, and despite the existence of extensive knowledge on the retinoid signalling pathway in gastropods, it seems that there is still some lack of information and further studies are needed (André et al., 2014; Sternberg et al., 2010). Moreover, our work supports the fact that gastropods can be a good model in toxicology studies since most of its metabolic processes are highly conserved and results obtained can be assumed to be found in more complex organisms like vertebrates (Rittschof and McClellan-Green, 2005). For example, Sternberg et al (2010) suggests that the study of selected organisms with aphally could give a hint to better understand the pathways by which imposex is developed, putting in evidence the biochemical targets of organotins in gastropods and its role in imposex development.

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CHAPTER 4.3 – THE USE OF REPORTER GENE ASSAY AS A TOOL TO DETECT POSSIBLE ENDOCRINE DISRUPTION EFFECTS IN GASTROPODS

4.3. The use of reporter gene assay as a tool to detect possible endocrine disruption effects in gastropods

4.3.1. Abstract

Throughout this thesis it was made clear that the imposex phenomenon is promoted by the organotins TBT and TPT. Nevertheless, it is important to understand that no other substances are able to induce imposex in gastropods, to guarantee that the proposed tools are specific for these organotins' pollution. Since the imposex phenomenon is caused by the activation of the nuclear receptors Retinoid X Receptor (RXR) and Peroxisome Proliferator-Activated Receptor gamma (PPARγ), the fastest approach to test if other substances promote imposex would be through a reporter gene assay involving these receptors. Therefore, in this work a reporter gene assay was performed using the GAL4/UAS system in cells transfected with human receptors with the aim to detect the ability of 28 selected environmental contaminants to activate these receptors. This activation was not only tested in RXR and PPARy but also on the Retinoic Acid receptor (RAR) and Estrogenic Receptor (ER) as they were also already identified in gastropods. Results obtained demonstrates that: i) organotins (TBT and TPT) were the only substances able to activate RXR and PPARy at comparable levels of the specific ligands that were used as positive controls; ii) other substances significantly activated these receptors but to a less extent; iii) several substances had also the capacity to significantly activate ER. Since human cells and receptors were used, the obtained results cannot be directly extrapolated to the gastropods, Even so, they can give a good indication of possible effects in these organisms. The use of this rapid screen method to detect potential endocrine disrupting compounds, as well as the use of gastropods as a model in ecotoxicology tests, is discussed.

4.3.2. Introduction

Gastropods are known to be affected by endocrine disrupting compounds (EDCs) with imposex phenomenon being the most cited and occurring in more than 260 gastropod species (Titley-O'Neal et al., 2011). Imposex is known to be induced by the organotins tribultyltin (TBT) and triphenyltin (TPT) but the pathway by which this phenomenon occurs remained unclear for several years. Some hypotheses emerged as an attempt to describe the process of imposex induction (see the review by Oehlmann et al. (2007)) but it is now widely accepted that this occurs after organotin binding to the nuclear receptor (NR) RXR (Nishikawa et al., 2004; Castro et al., 2007a; Sousa et al., 2010). However, it is still not clear which are the full set of signaling pathways where RXR is involved as homodimer and the interplays between RXR and other NRs. For example, Pascoal et al. (2013) proposed that imposex may also be triggered by activation of the RXR-PPARγ heterodimer through the binding of TBT or TPT to each or both NRs.

NRs are proteins able to regulate gene expression, upon binding to specific ligands (small lipophilic compounds), involved in several body functions (Sladek, 2011). If xenobiotics are able to bind to some of these receptors they may trigger unscheduled cell responses that may induce physiological and morphological changes in the organisms, such as the imposex, making these proteins greater sentinels to predict future harmful effects of xenobiotics to biota (Nishikawa et al., 1999; Janosek et al., 2006). Therefore, screening potentially toxic effects of contaminants can be accomplished by testing the NR activation by the chemicals under test. If extensive knowledge on NRs exists for vertebrates, for the majority of invertebrates, including gastropods, this knowledge is still scarce (Castro and Santos, 2014). Nevertheless, recent studies start to identify a wide diversity of human homologue receptors in gastropods such as RAR (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014), PPAR (Kaur et al., 2015), ER (Bannister et al., 2007; Castro et al., 2007b), RXR (Bouton et al., 2005; Urushitani et al., 2011) and the thyroid hormone receptor (Kaur et al., 2015). This confirms that the widely diverse superfamily of NRs evolved from a common ancestral and is distributed throughout the Metazoa while at the same time their

nature and biological functions are conserved throughout the evolutionary tree (Bridgham et al., 2010).

It is only possible to accurately test the effects of EDCs in gastropod NRs after their proper genetic characterization, which should be regard of major ecological importance (Castro and Santos, 2014). However, the fact that NRs are conserved throughout the evolutionary tree allows the use of vertebrate NRs in *in vitro* assays to provide indications for potential EDCs effects in gastropods, as well as in other invertebrates. In the specific case of imposex phenomenon, it has already been shown in the gastropod *Biomphalaria glabrata* that RXR is highly conserved, both structurally and functionally, with respect to the vertebrate RXR (Bouton et al., 2005). Moreover, rosiglitazone, a ligand for the human PPARγ, was shown to induce imposex in the gastropod *Nucella lapillus* trough the activation of the heterodimer RXR-PPAR (Pascoal et al., 2013), which is a well-established hetero-dimerization in mammals (Kliewer et al., 1992). Regarding other NRs, it has been reported that ER also occurs in gastropods but can be expressed without any estrogen binding (Bannister et al., 2007). Nevertheless, and even if reporter assays confirm that 17-β-Estradiol (a natural estrogen) do not activate gastropod ER, other EDCs might have the capacity to interact with this receptor (Bannister et al., 2013).

Therefore, in order to disclose the possible effects of a wide range of environmental contaminants in gastropods at this level, a reporter gene assay was performed using GAL4/UAS system in human cells transfected with human NRs that were already identified in gastropods (RXR, PPAR, ER and RAR). Substances that are able to activate the reporter gene are identified as having the potential to cause some disruption in gastropods, including, possibly, the imposex. Hence, the main objective of the current work is to perform a rapid screen regarding the potential of many environmental contaminants to act as agonists to the above NRs and, by this way, depict if they have the potential to cause any disruption on gastropods, including imposex induction.

4.3.3. Methods

The substances tested in the reporter gene assay and their concentrations are presented in table 4.3. Group of substances such as the Polycyclic aromatic hydrocarbons (PAHs), Polychlorinated Biphenyl (PCB), Organotins (OTs), Polybrominated diphenyl ethers (PBDEs), Pesticides and others recognized endocrine disruptor compounds (Nonylphenol, Bisphenol A and Perfluorooctanoic acid (PFOA)) are constant presences in the lists of priority substances and therefore were selected to be tested. Moreover, substances like the UV filters were selected due to recent insights on its endocrine disruption potential and the new antifouling paints biocides were selected to understand if these organotin substitutes will not cause similar harmful effects. The toxicity of these substances was previously tested in a cell viability test (data not shown) in order to define a maximum concentration that would allow a good cell viability during the reporter gene assay, and these were the selected concentrations tested (table 4.3). Celltiter-Glo® Luminescent Cell Viability Assay (Promega) was used following the manufacturer's instructions to assess the toxicity of the compounds into HEK-293 cells.

Table 4.3 - Substances and the tested concentrations used in the reported gene assay.

Group	Substance	CAS nº	Tested concentration
Polycyclic Aromatic Hydrocarbon (PAH)	1. Anthracene	120-12-7	0.560 µM
	2. Benzo [a] pyrene	50-32-8	10 µM
	3. Fluoranthene	205-912-4	100 µM
	4. Naphtalene	202-049-5	100 µM
Polychlorinated Biphenyl (PCB)	5. PCB 101	37680-73-2	1 µM
	6. PCB 138	35065-28-2	1 µM
	7. PCB 153	35065-27-1	1 µM
Organotins	8. TBT	1461-22-9	0.1 µM
	9. TPT	7440-06-4	0.1 µM
Polybrominated diphenyl ethers	10. BDE 47	5436-43-1	1 µM
	11. BDE 99	60348-60-9	10 µM
Pesticides	12. Alachlor	15972-60-8	100 µM
	13. Atrazine	1912-24-9	100 µM
	14. Chlorpyrifos	2921-88-2	100 µM
	15. Diuron	330-54-1	100 µM
	16. Endosulfan	115-29-7	0.278 µM
	17. Pentachlorophenol	201-778-6	100 µM
Antifouling Paint Biocides	18. Tralopyril	122454-29-9	100 µM
	19. Capsaicin	404-86-4	1000 µM
	20. Sea Nine 211	64359-81-5	100 µM
	21. Irgarol	28159-98-0	100 µM
UV – Filters	22. 4-Methylbenzylidene camphor (4-MBC)	36861-47-9	100 µM
	23. Benzophenone-3 (BP3)	131-57-7	100 µM
	24. Benzophenone-4 (BP4)	4065-45-6	47.0 µM
	25. 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC)	5466-77-3	100 µM
Others	26. Nonylphenol	84852-15-3	1 µM
	27. Bisphenol A	80-05-7	10 µM
	28. Perfluorooctanoic acid (PFOA)	335-67-1	100 µM

For the reporter gene assay, HEK-293 were seeded in 6-well culture plates with Dulbecco's modified Eagle medium (Gibco® DMEM) containing 1% of streptomycin plus penicillin and seeded at a density of approximately 3 x 10^5 cells by well in a humidified atmosphere at 5% CO_2 and 37 °C for 24 hours. Afterwards, cells were transfected adding a total of 1.4 µg of DNA per well (1 µg of Upstream Activating Sequence plasmid (UAS), 0.3 µg of Renilla (luciferase reporter plasmid) and 0.1 µg of GAL4 expression plasmid containing human RXRα, RAR, PPARγ, ERα or ERβ (depending on the receptor tested) and using 4 µl of JetPEI® by Polyplus Transfection™ as the transfection reagent. After 6 hours, transfection was stopped by washing the cells, fresh medium was added to the culture plate and cells were let to recover overnight. Cells were then transferred to a 96 well-plate and after 8 hours (time considered necessary for the cells to attach the plate), cells were exposed, in triplicates, for 16 hours to culture medium containing the test compounds at the concentrations referred in table 4.3. Specific NRs ligands were used as a positive control at the concentrations showed in table 4.4. Dimethyl sulfoxide (DMSO) was used as a solvent control to a concentration of 0.1% per well. After exposure, cells were lysed and luciferase activity measured with Dual-Luciferase® Reporter assay kit (PROMEGA) in a luminometer, following the manufacturer's instructions. Statistical analysis was performed with the software SPSS 19. One-way ANOVA followed by Dunnet's post hoc test was applied after logarithmic transformation of data. The substances that exhibited a statistically significant transactivation induction were considered as having potential agonistic activity, and discussed accordingly.

Table 4.4 - Substances used as receptor ligands and their concentrations in the assay.

Receptor Ligand	Substance	CAS nº	Tested concentration
RXR ligand	LG1069	153559-49-0	1 µM
RAR ligand	All-Trans Retinoic Acid (ATRA)	302-79-4	1 µM
PPARγ ligand	Rosiglitazone	122320-73-4	1 µM
ERα/ERβ ligand	17-β-Estradiol	50-28-2	0.001 µM

4.3.4. Results

Induced transactivation for all the studied NRs are shown in figure 4.4. Data is represented as fold induction in relation to the DMSO control, meaning that this equal to 1. The ligands LG1069, ATRA, Rosiglitazone and 17-β-Estradiol, acting as positive controls, activated significantly the corresponding NRs for which they have high affinity, attesting the validity of the method. RXRα was significantly transactivated by the organotins TBT (8) and TPT (9), as expected, but also by Chlorpyrifos (14), PFOA (28) to minor scale and less significantly by Nonylphenol (26). Only Chlorpyrifos induced significantly the transactivation function in RAR; in fact, this substance had the same effect in all NRs which might indicate that it interferes with the used transfection system. Then, again the organotins strongly induced the transactivation in PPARγ. In a minor scale, transactivation was again induced by Chlorpyrifos and PFOA, but also by BDE 99 (11), Alachlor (12), Tralopyril (18), Sea Nine 211 (20) and EHMC (25). As for ERα, several substances induced the transactivation in this NR with the expected induction by Bisphenol A (27) and Nonylphenol but also strongly by Benzo [a] pyrene (2) and 4-MBC (22) and, again, Chlorpyrifos. Interestingly all UV filters tested (22 - 25) showed some activity on the transactivation function of this NR. For ERβ, a smaller number of substances were effective in inducing transactivation, still both Bisphenol A and Nonylphenol strongly activated this receptor, along with, at a less extent, Benzo [a] pyrene, Chlorpyrifos and two UV filters, 4-MBC (22) and BP3 (23).

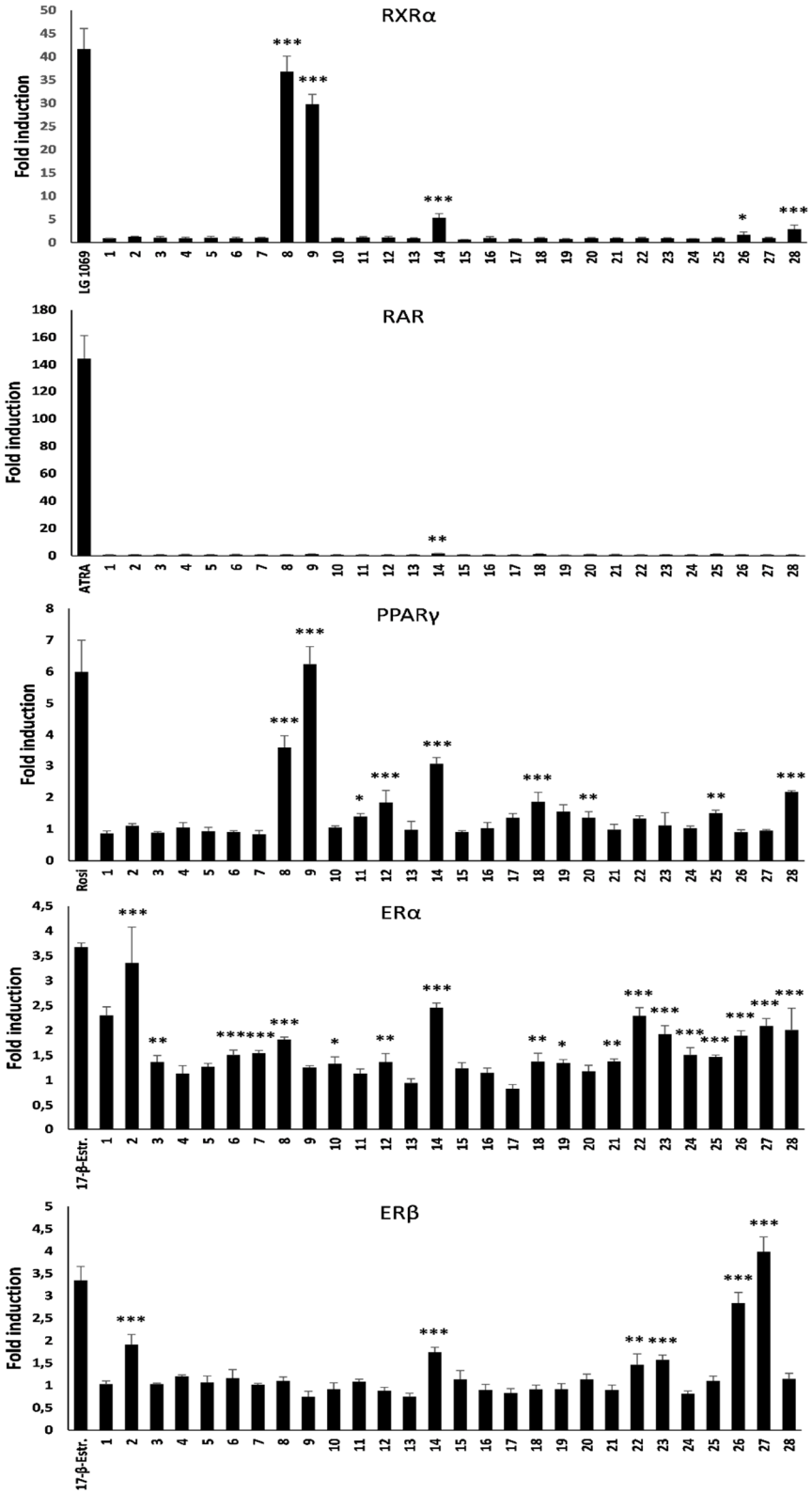

Figure 4.4 - Transactivation induction (as folded induction) by the chemical substances identified in table 4.3 regarding the nuclear receptors RXRα, RAR, PPARγ, and ERα or ERβ. Statistically significant differences to DMSO control (DMSO fold induction = 1) are indicated by asterisks: *($p < 0.05$), **($p < 0.01$) and *** ($p < 0.001$).

4.3.5. Discussion

The results here presented should be regarded as indicative for effects in gastropods (see discussion below) and should be validated with further studies. Even so, we will start by the analysis of the results obtained for RXR and PPARγ, as these receptors are suspected to be directly involved in imposex induction. As it would be expected, the organotins TBT and TPT activated strongly these NRs. This activation had already been demonstrated (Kanayama et al., 2005; le Maire et al., 2009) and considered the pathway by which the imposex phenomenon is induced (Nishikawa et al., 2004; Pascoal et al., 2013) or the reason why these compounds are considered obesogens in both vertebrates (Kanayama et al., 2005; Grun et al., 2006) and invertebrates (Jordão et al., 2015). Moreover, our data also shows a more powerful transactivation function in RXR by TBT than TPT, while the opposite pattern occurred in PPARγ, an induction pattern that had also been observed by Kanayama et al. (2005).

The other substances that have significantly transactivated both RXR and PPARγ have not demonstrated the same effectiveness as the organotins. Nevertheless, and regarding RXR, the data obtained in the current work differ from that of Vanden Heuvel et al. (2006) that reported no effect of PFOA in RXR induction. On the other hand, we confirmed the Nonylphenol weak agonistic activity to RXR reported by Shiizaki et al. (2014). No scientific data was found for the interaction of Chlorpyrifos with RXR. For PPARγ, PFOA showed a weak transactivation activity already reported for human PPARγ (Vanden Heuvel et al., 2006) while other studies have shown that, even if this substance is highly effective on the activation of PPARα, it shows no transactivation effect on PPARγ (Maloney and Waxman, 1999; Takacs and Abbott, 2007). Other studies differ from our results, with Chlorpyrifos (Takeuchi et al., 2006) and Alachlor (Kanayama et al., 2005; Takeuchi et al., 2006) not having any transactivation effect in PPARγ. For the other substances that significantly activated this receptor, no information was found in literature regarding their possible effects. From the tested substances, and apart from TBT and TPT, Nonylphenol is the only described as capable to increase imposex levels and penis length in the gastropod species *Nucella lapillus* (Evans et al., 2000). However, this xenoestrogen was also pointed

as causing a decrease in imposex levels in this species, namely the penis size (Santos et al., 2008). As for the other substances, more tests should be performed to understand their potential on imposex promotion as fold activation is still considerably lower than organotins.

The observation that the retinoic acid (RA) 9-cisRA was able to induce imposex (Nishikawa et al., 2004) suggested that RAR could also be involved on the imposex induction process. However, recent studies point out that even if this receptor do exist in gastropods its transcriptional activity is not activated by common retinoid or organotins, thus suggesting that imposex induction or sexual organs development follow a different signaling pathway that do not implicate RAR (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014). Nevertheless, RAR was only significantly activated by Chlorpyrifos but to a very lower extent (ca. 87 times less) when compared with the specific ligand ATRA. Other scientific publications corroborate our observations, indicating that substances such as Nonylphenol or Endosulfan (also tested), among others, might also interfere with this receptor but with weak activation potency (Kamata et al., 2008; Inoue et al., 2010).

Estrogen receptor orthologues were already identified in several gastropod species (Kajiwara et al., 2006; Bannister et al., 2007; Castro et al., 2007b; Stange et al., 2012; Kaur et al., 2015) but, as observed for RAR, in gastropods 17-β-Estradiol does not seem to bind this receptor (Kajiwara et al., 2006; Bannister et al., 2013). On the other hand, this receptor seems to be constitutively active without the need of a ligand (Kajiwara et al., 2006; Bannister et al., 2013) and possibly modulated in the presence of xenoestrogens (Castro et al., 2007b; Stange et al., 2012; Bannister et al., 2013). Moreover, several studies suggest that gastropods express estrogenic responses to substances, or mixture of substances, that are known to have estrogenic response in vertebrates (Oehlmann et al., 2000; Duft et al., 2003; Segner et al., 2003; Benstead et al., 2011). From those substances known to have estrogenic effect, both Nonylphenol and Bisphenol A activate significantly the tested receptors. Besides, other 16 substances induced the transactivation in ERα what, with the low capacity demonstrated by the specific ligand to induce the transactivation, might indicate that the transfection system was not completely appropriate for this purpose. Worth of mention should be the strong transactivation effect by Benzo [a] pyrene that

confirms the high potential of this substance to disrupt this signaling pathway (Charles et al., 2000; Fertuck et al., 2001). Also, all UV filters tested have significantly induced transactivation activity in this receptor, which is interesting since these substances where already shown to interfere with the gastropod *Potamopyrgus antipodarum* reproduction (Schmitt et al., 2008; Kaiser et al., 2012; unpublished data). For ERβ, both Nonylphenol and Bisphenol A strongly transactivated this receptor along with, to a less extent, Benzo [a] pyrene, Chlorpyrifos and two UV filters, 4-MBC and BP3.

All the reporter gene assays were performed with the same reporter cell line and this can decrease the level of sensitivity and responsiveness of the transfection since it lacks specificity for each NR (Grimaldi et al., 2015). This lack of specificity might also explain the fact that both estrogenic receptors, and PPARγ to some extent, were weakly transactivated by the specific ligand, denoting that transfection system was not totally effective. It should also be taken into consideration the possibility that the plasmid was not well transfected or cell lines were too old, affecting therefore the effectiveness of the transfection system. Interestingly, Chlorpyrifos had a significant effect in all NRs but this substance interference with the transfection system used should not be discarded and, therefore, it is recommended that the results should be again confirmed. Besides this, the obtained data should not be directly extrapolated to the gastropods since the employed method lacks specificity, in both cell lines or NR used, for this group. Still, as many NR are supposed to be ancient and distributed throughout the Eumetazoa (Bouton et al., 2005) these results can be perceived as an indication of possible effects to the invertebrates, namely gastropods. This group has been attracting a growing interest from the scientific community on its use as models in ecotoxicological tests for their high sensitivity to endocrine disruption compounds (Rittschof and McClellan-Green, 2005; Matthiessen, 2008). Therefore, with the indicative results given by the transactivation of some NRs, some gastropod species can be exposed to the substances that are suspicious to cause a deleterious effect. In fact, some individuals of *N. lapillus* and *N. reticulatus* were injected with a concentration of 2 µg/g ww of PFOA and chlorpyrifos, substances that significantly transactivated RXR. The preliminary results indicated no imposex induction but the high mortality observed prevented the confirmation of this result. Hence, more studies are necessary to refine the test protocols

that may rely on the two-step approach: i) rapid *in vitro* screen methods to assess the ability of contaminants to bind NRs and ii) *in vivo* bioassays to test the effects of the selected contaminants on target organisms. These studies are very useful to test the endocrine disruption potential of emergent chemicals and, in this context, gastropods may constitute good invertebrate models for this research.

4.3.6. Conclusions

Invertebrates, like gastropods, were already proposed as good models for environmental toxicology tests, namely in the research regarding endocrine disruptor substances (Rittschof and McClellan-Green, 2005; Matthiessen, 2008). This group share some conserved regulatory pathways with the vertebrates, vertebrate-type steroids or neuropeptides, nuclear receptors and, besides, they are highly sensitive to environmental contaminants as they lack effective metabolic mechanisms to biotransform these xenobiotics (Oehlmann et al., 2007). Still, there is a long way to go in order to fully perceive the gastropod endocrine system (André et al., 2014; Kaur et al., 2015). Therefore, the results obtained in the current work should always be regarded as an indication of possible effects, even if this tool has proved to be effective and rapid to identify substances which are already known to interfere with NRs in gastropods, as the case of organotins for RXRα and PPARγ, or Nonylphenol, Bisphenol A and some UV filters for ER. It is desirable - to perform this type of assays, more ecologically relevant - to develop *in vitro* bioassays with gastropod cell lines and respective NRs to better foresee possible effects of xenobiotics in this group (Castro and Santos, 2014).

4.3.7. Bibliography

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CHAPTER 5 - GENERAL DISCUSSION

5. GENERAL DISCUSSION

"As the present now will later be past
The order is rapidly fadin'
And the first one now will later be last
For the times they are a-changin'."

in Bob Dylan's The times they are a-changin', 1969

The growing society awareness on ecological issues constantly demands effective legislative actions for the improvement of environmental quality, which consequently requires the scientific community to constantly and rapidly develop tools that can answer the political demands imposed. In the European Union (EU) those demands, regarding the aquatic environmental quality, came with the implementation of Water Framework Directive (WFD) in 2000, followed by the introduction of the Marine Strategy Framework Directive (MSFD) in 2008. These directives require EU member states to protect aquatic environments and, when necessary, improve their ecological status.

TBT is a priority substance under the WFD but this directive does not mention any guidelines regarding the biological effects monitoring of this pollutant that could be useful to classify the ecological status of water bodies. Similarly, there is a complete lack of guidance in the MSFD regarding the assessment and classification of TBT pollution levels. Hence, and in accordance with recent recommendations for the use of biological effect based tools under the European directives (Allan et al., 2006; Martinez-Haro et al., 2015; Wernersson et al., 2015) one of the main objectives of this thesis was to develop and apply these tools to WFD and MSFD. Therefore, in chapter 2 an *in situ* biomonitoring tool (Chapter 2.1) and a laboratory bioassay tool (Chapter 2.2 and 2.3) are proposed to assess the status of water bodies, using the imposex and/or intersex as biomarkers.

Imposex and intersex are simple and reliable biomarkers for TBT pollution monitoring that give an indication of the average long-term levels of TBT environmental concentration and the deleterious impact of this hazardous chemical on gastropod

populations (Matthiessen and Gibbs, 1998; Barroso et al., 2000; Horiguchi, 2009). Their value as a monitoring tool is acknowledged by OSPAR as a mandatory element of the CEMP (OSPAR 2015), and specific guidelines for monitoring TBT-specific biological effects (imposex/intersex) were developed for several gastropods species. Taking advantage of this already developed tool, this thesis proposes that the OSPAR assessment criteria should be transposed to WFD with appropriate adjustments. In this proposal (Chapter 2.1) the gastropod *N. lapillus* is considered the key bioindicator and the remainder gastropod species are complementary, i.e. useful at sites where *N. lapillus* is absent or when more adequate species sensitivity is needed. As seen in Chapter 1, WFD and OSPAR may differ in their objectives with WFD being more operational and focused on the ecological impacts of pollution in terms of population abundance and community diversity. Accordingly, a discrepancy between the WFD proposed classification and OSPAR assessment criteria occurs with the good ecological status being accepted in the WFD for VDSI<3.0 while OSPAR defines an EcoQO of VDSI<2.0 for the key bioindicator *N. lapillus*. A VDSI<3.0 indicates that the dog-whelk breeding is normal, which complies with the WFD requirements by not affecting population abundance and community structure. So, the presented proposal is less ambitious than the OSPAR EcoQO regarding TBT pollution, what can be sustained by the chemical objectives since the WFD AA-EQS (0.2 ng TBT/L) is slightly higher than the OSPAR Environmental Assessment Criteria (EAC) (0.1 ng TBT/L).

In order to achieve a sufficient level of harmonization regarding the environmental monitoring in the EU, and to avoid unnecessary duplication of environmental efforts since there is a spatial overlap between European directives and Regional sea conventions, we propose that the imposex/intersex tool developed for WFD in chapter 2.1 should also be employed in MSFD (chapter 3.2). The fact that under this directive member states should use the tools and structures already applied by the regional sea conventions makes this proposal easy to implement, since imposex is a mandatory monitoring element in regional sea conventions like OSPAR or HELCOM.

One of the strength of this proposal is based on its multi-species approach. Due to differences in the gastropod species distribution, their biological characteristics and sensitivity to TBT pollution, it is obtained a more robust and complete evaluation of the

Ecological Quality status of the water bodies when more indicator species are used. This is evidenced principally by the dichotomy of two species, *N. lapillus* and *N. reticulatus* (Chapter 2.3 and Chapter 3). Even if they can be found sympatrically, the dog-whelk is mainly found in rocky shores along the open coast, while the netted-whelk is a sediment dweller that is very abundant in estuaries, where the main TBT hotspots of pollution are located in Portugal (Barroso et al., 2002a; Sousa et al., 2005; Sousa et al., 2009). Consequently, both species will cover a wider diversity of habitats allowing for a broader assessment area. *N. lapillus* is considered the key bioindicator to define the ecological quality status under WFD since it is a sensitive species that will better demonstrate the impact of TBT on benthic communities. Nevertheless, the use of other bioindicators, such as *N. reticulatus*, *L. littorea* or others, is of paramount importance to better reflect the actual impact of TBT pollution on gastropod populations and community structure as a whole.

The imposex/intersex tool proposal will only be effective to assess the ecological quality status under the WFD or MSFD if there is a good choice of the bioindicators and if imposex/intersex is specific to TBT pollution. Regarding the first point, the species employed in this thesis for monitoring the Portuguese coast - *N. lapillus*, *N. reticulatus* and *L. littorea* - seem to be adequate as they have the capacity to develop a response that is positively correlated with TBT environmental levels, as already proved in this thesis and by several laboratory and field studies (Bryan et al., 1987; Gibbs et al., 1987; Stroben et al., 1992; Bauer et al., 1997; Barroso et al., 2000). However, if these premises for a good bioindicator species are also referred to the gastropod *P. ulvae* (Schulte-Oehlmann et al., 1997; Schulte-Oehlmann et al., 1998), the current work shows that this species failed to track TBT environmental variations at Ria de Aveiro, presenting imposex levels that are in disagreement with the TBT pollution trends observed. The reasons for this incongruity are unknown and must be studied, but the major conclusion to retain is that it is required a precise knowledge of the bioindicator properties for its correct application in monitoring programs.

Regarding the specificity of the biomarkers response, it is widely known that triphenyltin can induce imposex in some gastropod species, including *N. reticulatus*. This

thesis shows, for the first time, that this organotin also causes the development of imposex in *N. lapillus*. This could be a major obstacle to the WFD proposal since this species plays a key role as a bioindicator. However, the concentrations used in the experiment are not environmentally relevant in Europe and this substance plays a less important role on imposex development since its environmental levels are generally lower than those of TBT (Barroso et al., 2002b). Nevertheless, it is recommended that both organotins should be quantified in the tissues of the bioindicators (besides other environmental compartments) in order to better understand the contribution of each compound to the overall imposex response. Apart from the TPT, other contaminants should also be tested in order to evaluate their capacity to induce imposex/intersex. A rapid and preliminary screen test was performed in this thesis with human nuclear receptors (NR) to see if other contaminants could bind important NRs known to be involved or interfere with the imposex/intersex response; if a given contaminant would present a strong affinity for these NRs it should be injected in live animals to check the real ability to induce or interfere with the development of imposex/intersex. This thesis shows that no other tested substances were able to activate RXR and PPARγ (receptors involved on imposex development) with similar strength than the organotins. The reporter gene assay seems a very interesting tool to rapidly screen the potential of a vast diversity of substances to induce imposex or intersex in gastropods. However, to make this test more ecologically relevant there is the need to develop *in vitro* bioassays using cell lines and NRs of gastropods.

The imposex and intersex assessment tool proposed in Chapter 2.1 evidenced a TBT pollution decline and an ecological status improvement in Ria de Aveiro (Chapter 2.1) and other parts of the Portuguese coast (Chapters 3.1. and 3.2) over the last years. Globally, the results indicate that legislation has been effective to reduce TBT pollution and imposex levels. Still, alarming imposex levels are reported in 2014 around a number of fishing ports and marinas, evidencing a moderate or lower ecological status. In particular, a population of *N. lapillus* at Zambujeira do Mar (SW Portugal) exhibited high imposex levels with sterilized females, being the only dog-whelk population indicating a Moderate ecological status. In this station, as well as in others throughout the Portuguese coast, fresh TBT inputs were detected, which may still occur due to the illegal application of TBT-based AF paints

or due to natural processes like TBT desorption from sediments. Probably, most of these sites that were monitored in 2014 did not achieve the good environmental status in 2015 as demanded by WFD and so an action from the Portuguese government is required according to the WFD. As sediments act as a long-term sink for TBT and represent a source of pollution, there is a necessity for the removal and transport of the contaminated sediment off-sites followed by their decontamination by physical and/or biological processes. After the conclusion of the sediment dredging operations, monitoring must continue to assess the long-term effectiveness of the sediment cleanup action.

In conclusion, TBT pollution is clearly decreasing in Portuguese coastal and transitional waters, however deleterious effects associated with this type of pollution is still observed in the environment, which confirms the need for a continuous monitoring of this substance. Therefore the tools proposed in this thesis can be of extreme utility in TBT pollution monitoring, creating at the same time a good opportunity to find a common ground between WFD, MSFD and regional sea conventions. The use of gastropods as an ecotoxicological model is well evidenced throughout this thesis because they were useful as bioindicators for *in situ* monitoring and for laboratory bioassays, but they can also be of interest for other toxicological studies aiming to understand the impact of environmental contaminants at the individual, population or higher levels of organization. The fact that this group share with the vertebrates some conserved regulatory pathways, vertebrate-type steroids or neuropeptides, makes them also an interesting non-vertebrate model in toxicology.

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“When you've understood this scripture, throw it away.
If you can’t understand this scripture, throw it away.
I insist on your freedom.”

Jack Kerouac