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Departamento de Biologia

Filipe Miguel Grave Laranjeiro

Abordagem integrada para a avaliação da poluição por TBT em áreas estuarinas

Integrative approach for the assessment of TBT pollution in estuarine areas

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Integrative approach for the assessment of TBT pollution in estuarine areas

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

o júri

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agradecimentos

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palavras-chave Imposexo, sedimento, tocixidade aguda, toxicidade crónica, TBT, *Nassarius reticulatus*, *Nucella lapillus*, *Hydrobia ulvae*, *Potamopyrgus antipodarum*, *Paracentrotus lividus*

Resumo As áreas estuarinas são geralmente focos de poluição por estarem sujeitas a uma intensa actividade antropogénica. Um exemplo disso é a poluição por tributilestanho (TBT) - um agente biocida presente nas tintas anti-incrustantes aplicadas nos cascos de embarcações. Como os estuários albergam frequentemente portos comerciais, marinas e estaleiros navais, existe aqui um intenso tráfego naval e uma elevada libertação de TBT para a água, contaminando assim todo o ambiente estuarino. Apesar da utilização do TBT em tintas anti-incrustantes de embarcações ter sido proibida em Setembro de 2008, a persistência deste composto nos sedimentos poderá causar um declínio lento dos níveis de poluição ao longo do tempo. Utilizando a Ria de Aveiro como caso de estudo, pretendeu-se neste trabalho desenvolver uma metodologia que pudesse, numa abordagem integrada, avaliar a qualidade dos sedimentos em zonas estuarinas sujeitas a intensa actividade portuária, em particular os efeitos biológicos da contaminação por TBT associada aos sedimentos. Para tal, realizaram-se campanhas de monitorização de *imposex* em algumas espécies de gastrópodes na Ria de Aveiro - *Nassarius reticulatus* e *Nucella lapillus* - assim como a monitorização química de TBT nos sedimentos. No laboratório foi efectuada a exposição dos gastrópodes *Nassarius reticulatus* e *Hydrobia ulvae* a sedimento colhido em alguns locais da Ria de Aveiro. Nestes bioensaios mediu-se o desenvolvimento do imposexo como resposta específica à presença de TBT nos sedimentos. Outros bioensaios foram utilizados com o objectivo de conhecer a toxicidade global dos sedimentos para outros organismos, nomeadamente, bioensaios com o gastrópode *Potamopyrgus antipodarum* exposto a sedimento e bioensaios com larvas do ouriço do mar, *Paracentrotus lividus,* expostas a “elutriados” de sedimentos.

Propõe-se neste trabalho uma abordagem holística para a avaliação da poluição por TBT em zonas estuarinas, combinando a monitorização biológica (*imposex)*, a monitorização química de TBT nos sedimentos e bioensaios laboratoriais com vista à avaliação da toxicidade dos sedimentos.

keywords Imposex, sediment, acute toxicity, chronic toxicity, TBT, *Nassarius reticulatus*, *Nucella lapillus*, *Hydrobia ulvae*, *Potamopyrgus antipodarum*, *Paracentrotus lividus*

abstract Due to intense anthropogenic activities, the estuarine areas are often outbreaks of pollution. An example is the pollution by tributyltin (TBT) - a biocide present in antifouling paints applied to boat hulls. Estuaries typically harbour commercial ports, marinas and shipyards, and consequently there is an intense naval traffic and intense release of TBT into the water, thus contaminating the entire estuarine environment. Despite the use of TBT in antifouling paints for vessels had been banned in September 2008, the persistence of this compound in sediments may cause a slow decline in pollution levels over time. Using the Ria de Aveiro as a case study, it was intended in this work to develop a methodology that could make an integrated assessment of sediment quality in estuarine areas subject to intense naval traffic, particularly the biological effects caused by the presence of TBT in sediments. To accomplish this objective, there were monitoring surveys of imposex in some species of gastropods in the Ria de Aveiro - *Nassarius reticulatus* and *Nucella lapillus* - as well as chemical monitoring of TBT in sediments. Additionally, in the laboratory, the gastropods *Nassarius reticulatus* and *Hydrobia ulvae* were exposed to sediment collected from several sites of Ria de Aveiro. These bioassays measured the development of imposex as a specific response to the presence of TBT in sediments. Other bioassays were used in order to evaluate the overall sediment toxicity to other organisms, including bioassays with the gastropod *Potamopyrgus antipodarum* exposed to sediment and bioassays with larvae of sea urchin, *Paracentrotus lividus*, exposed to sediment elutriates.

It is proposed in this work a holistic approach to the assessment of pollution by TBT in estuarine areas, combining biological monitoring (imposex), chemical monitoring of TBT in sediments and laboratory bioassays for the assessment of sediment quality.

“L´essentiel est invisible pour les yeux”

in, Le Petit Prince

Antoine de Saint-Exupéry

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. General Introduction

1.1. Organotin compounds – an overview

Organotin compounds are organometals characterized by the presence of a Sn (tin) atom covalently bounded to one or more organic substituents. It is chemically represented by general formula *R*Sn*X* where *R* is organic, alkyl or aryl, group and *X* is an organic or sometimes inorganic ligand. All are of anthropogenic origin except methyltins, which can also be produced by biomethylation. The bond between Sn and organic substituent is stable in the present of water, atmospheric oxigen and heat but rapidly cleaved in the presence of UV radiation, strong acids and electrophilic agents (Hoch, 2001). Organotin compounds have different applications such as, PVC (polyvinylchloride) stabilizers, catalysts and biocides in antifoulings, agrochemicals, wood preservation and diverse materials protection against biofouling (Takahashi et al., 1999a; Hoch, 2001).

1.1.1. Organotin toxicity

Inorganic tin is recognized as a non toxic metal but on the other hand organic forms of tin have several toxicological effects that are widely described (see for example (Fent et al., 2006)). The toxicity of these compounds depends on the nature and the number of the organic groups. Trisubstituted compounds, in general, have shown to be the most hazardous towards organisms (Fent, 1996). Tributyltin (TBT) compounds are considered to be the most hazardous compound of this group, being considered by Goldberg (1986) the most toxic compound deliberated released and introduced into the environment. Shell malformations of oysters, imposex in marine snails, retardation of growth in mussels and immunological dysfunction in fishes are described as some chronic effects caused by TBT (Hoch, 2001). Triphenyltin (TPT) is also very toxic and seems to cause some of the effects reported to TBT, such as the induction of imposex in prosobranch gastropods (Horiguchi et al., 1997; Barroso and Moreira, 2002). A general overview of toxicity for TBT and TPT is presented in Table 1.1.

Table 1.1- Acute toxicity values of TBT and TPT for some organisms.

Species	Test Type	Compound	Concentrations	Reference
Mytilus galloprovincialis	Embryogenesis success	TBT	0.16 µg/L (EC10, 48h) 0.38 µg/L (EC50, 48h)	(Beiras and Bellas, 2008)
Sparus aurata	Acute toxicity in embryos	TBTCL TPTCL	28.3 µg/L (LC50, 24h) 34,2 µg/L (LC50, 24h)	(Dimitriou et al., 2003)
Paracentrotus lividus	Embryogenesis success	TBT	0.31 µg/L (EC50, 48h)	(Bellas et al., 2005)
Thais clavigera	Acute toxicity in larvae	TBT TPT	8.4 µg/L (LC50, 24h) 5.6 µg/L (LC50, 48h) 8.6 µg/L (LC50, 24h) 5.4 µg/L (LC50, 48h)	(Horiguchi et al., 1998)
Haliotis madaka	Acute toxicity in larvae	TBT TPT	3.9 µg/L (LC50, 24h) 1.2 µg/L (LC50, 48h) 2.4 µg/L (LC50, 24h) 1.5 µg/L (LC50, 48h)	(Horiguchi et al., 1998)
Haliotis discus discus	Acute toxicity in larvae	TBT TPT	5.4 µg/L (LC50, 48h) 1.4 µg/L (LC50, 48h)	(Horiguchi et al., 1998)
Acartia tonsa	Acute toxicity in adults	TBT (18‰) TBT (28‰)	0.47 µg/L (LC50, 48h) 0.24 µg/L (LC50, 48h)	(Kusk and Petersen, 1997)
Nassarius reticulatus	Acute toxicity in larvae	TBT	4.87 µg/L (LC50, 24h) 1.78 µg/L (LC50, 96h)	(Sousa et al., 2005a)
Eurytemora affinis	Acute toxicity	TBT	2.2 µg/L (LC50, 48h) 0.6 µg/L (LC50, 72h)	(Hall et al., 1988)
Nile tilapia	Acute toxicity	TBT	3.8 µg/L (LC50, 96h)	(Lei et al., 1998)

1.1.2. Fate of TBT in the environment

The role of chemical speciation in water and sediment provide important information on toxicity and bioavailability of organotin compounds. In aqueous solutions, TBT ionic form (TBT+) is in equilibrium with hydro- (TBTOH) and chloro-species (TBTCl). The uptake of TBT by organisms occurs if dissolved organotin species are uncharged. Thus, TBTOH and TBTCl forms increase bioavailability of TBT (Fent, 1996; Alzieu, 1998). In marine ecosystems under natural conditions of pH and salinity, TBT is found in TBTOH form, this is important because TBT bioavailability increases when it is found in TBTOH form (Fent, 1996; Alzieu, 1998). In sediments, TBT behaviour seems to be different from the one reported to water. However published studies on TBT partitioning simulating natural conditions are scarce, then, present knowledge on this subject is yet restricted. Physicochemical sediment properties, such as organic matter, particle size, salinity and pH, control TBT partitioning behaviour and consequently bioavailability (Burton et al., 2004).

Bioavailability in sediments depends of several parameters, such as pH and organic matter. For example, bioavailability increases at a neutral or slightly basic pH or low levels of organic matter due to the high capacity of absorption to it. Once released in environment, TBT is absorbed by bacteria, algae or suspended matter in water column (Burton et al., 2004).

TBT is quickly integrated in tissues of filter-feeding or grazer animals and, ultimately, in superior predators as fishes, birds or mammals. This leads to an accumulation in tissues that is carried throughout the food chain (Takahashi et al., 1999b; Antizar-Ladislao, 2008). This accumulation could have impacts on humans trough the ingestion of contaminated fish and crustaceans. Some studies already reported the presence of TBT in human blood samples (Takahashi et al., 1999a).

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

$$Bu_3Sn^{+} \longrightarrow Bu_2Sn^{2+} \longrightarrow BuSn^{3+} \longrightarrow Sn^{4+}$$

Degradation of TBT produces DBT, MBT and inorganic tin that are less toxic than TBT. Natural degradation can be, this way, an important process in sediment remediation (Fent, 1996). TBT half-lives in water generally range from a few days to a few weeks. In sediment degradation rates are lower with half-lives from days to decades (Dowson et al., 1996; Hoch, 2001). In the literature half-lives for TBT in environment are mainly related to laboratory experiments and are thus not comparable to real circumstances where rates of breakdown depends on numerous biogeochemical factors. Therefore, TBT persistence in natural sediments is often underestimated and half-lives of TBT, estimated from in situ measurements, can be stable for at least 2 decades in anoxic sediments (normally the deeper sediments) (Dowson et al., 1993). Due to low rates of degradation and high persistence in sediments, sediment appears to act as a sink for TBT and represents a permanent threat for water contamination via desorption and remobilization processes or directly for benthic organisms trough contact or ingestion of particles (Hoch, 2001).

1.1.2.1. Regulatory actions on TBT

Regulatory actions for TBT were first adopted in France in 1982 followed by United Kingdom in 1985. In France the use of TBT was restricted in boats smaller than 25m while in United Kingdom the sale of antifouling paints containing TBT was forbidden for small boats and aquaculture structures. In 1989, European Union introduced the directive (89/677/CEE) that forbidden the use of TBT and TPT based antifouling paints in vessels smaller than 25m. Portugal adopted this directive in 1993. Even if these restrictions occur, they were reported as ineffective by some published works (Minchin et al., 1995; Morgan et al., 1998; Barroso and Moreira, 2002; Santos et al., 2002). Therefore in 2001 International Maritime Organization (IMO) formulated the AFS convention that totally bans the application of paints with TBT in all vessels, this convention entered into force in September of 2008. Before 2008, since 1st July 2003, European Union forbade the application of TBT in all vessels and since 1st January of 2008 the presence of this in vessels. These restrictions also were applied for non-European boats that entered in European harbours (Sousa et al., 2009).

1.2. Endocrine disruption

Since the beginning of 90's, increasing concerns about some substances, or the product of their degradation, released in environment that have the ability to cause disruption in the endocrine system has been raised. And so in 1991, Colborn and Clement, formulated the one of the most important hypothesis in environmental sciences that become known as the "Endocrine Disrupting Hypothesis". That hypothesis suggests that several man made chemicals released in the environment by the anthropogenic activity had the potential to disrupt the endocrine system in wildlife and in humans at significant concentrations. These compounds were designated as Endocrine Disrupting Chemicals (EDCs) (Guillette, 2006).

These compounds have the ability to interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. In the environment endocrine disrupters can mimic, enhance (an agonist) or inhibit (an antagonist) the action of hormones.

The compound that causes this effect is known as endocrine disruptor and adopting the definition proposed by a European Conference is defined by:

"An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function. In absence of definitive *in vivo* data, a *potential* endocrine disruptor is defined as a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism." (IEH, 1999)

Several observations of endocrine disruption are well documented, such as estrogenic, androgenic, antiandrogenic and antithyroid actions detected in fishes downstream the sewages or downstream the pulp and paper mills (Jobling and Tyler, 2003); the thinning of the eggshell in birds induced by DDT (Giesy et al., 2003); alterations in steroidogenesis, hormone levels, and alterations in the morphology of endocrine organs (gonad, thyroid) juvenile alligators when exposed to agricultural chemicals (Guillette and Iguchi, 2003). At last, one of the most studied phenomenons in endocrine disruption field is imposex that is considered to be a direct consequence of TBT exposure. TBT act as androgen and it affects molluscs by the induction of male characters in females (Matthiessen, 2003).

1.3. Imposex

Defined by Smith (1971), imposex is the superimposition of male characters onto females of gonochorist gastropods. It was first described by Blaber in females of *Nucella lapillus* (Blaber, 1970) and one year later, Smith reported the same phenomenon in females of *Nassarius obsoleta* and called it imposex (Smith, 1971). In 1981, Smith observed high levels of imposex near the ports and attributed the phenomenon to antifouling paints; the cause effect was then established and TBT was considered the causative agent of this phenomenon (Smith, 1981a, b). Over the last decades, imposex was reported in approximately 200 species of Gastropods (Shi et al., 2005). But not only TBT can cause the induction of imposex. It was already shown that triphenyltin is also an imposex inducer in *Thais clavigera* and *Nassarius reticulatus* (Horiguchi et al., 1997; Barroso and Moreira, 2002). Nevertheless, TPT values in European environments are much lower than the ones for TBT, playing this way a minor role on imposex induction in natural populations (Barroso and Moreira, 2002; Sousa et al., 2009).

TBT pollution monitoring is carried out assessing the levels of imposex as well as environmental and tissues concentrations of TBT. Determination of levels of imposex allows a low cost and effective monitoring of TBT contamination because, in some cases, organisms react to low environmental concentrations (Oehlmann et al., 1996). This determination is based on highly significant correlations founded between imposex and TBT levels in tissues (Stroben et al., 1992b, a; Barroso et al., 2002a; Sousa et al., 2005b). *Nucella lapillus* was first proposed by Gibbs and co-workers as a indicator species of TBT pollution (Gibbs et al., 1987). After that other species were proposed as bioindicator ones. In Europe the most common species used as bioindicators for TBT pollution are *Nassarius reticulatus*, *Nucella lapillus, Hydrobia ulvae*, *Littorina littorea* and *Buccinum undatum* (Gibbs et al., 1987; Stroben et al., 1992c; Ten Hallers-Tjabbes et al., 1994; Matthiessen et al., 1995; SchulteOehlmann et al., 1997; Barroso et al., 2000).

Several indexes are used to evaluate the levels of imposex in gastropod populations. The indexes created for the species used in this work are presented below.

Percentage of females affected by imposex (%I): Percentage of affected females in a station.

Percentage of sterile females (%STER): Percentage of females carrying aborted egg capsules inside the capsule gland.

Vas deferens sequence index (VDSI): Scoring system based on vas deferens development. For *Nassarius reticulatus* this system was developed by Stroben and co-workers (1992b); for *Nucella lapillus* the system was developed by Gibbs and co-workers (1987).

Mean female penis length (FPLI): Mean female penis length in a station.

Relative penis length index (RPLI): Mean female penis length × 100 / mean male penis length. This index is used in assessment of imposex levels in *Nassarius reticulatus*.

Relative penis size index (RPSI): (Mean female penis length)^3 × 100 / (mean male penis length) ^{3}. This index is used in assessment of imposex levels in *Nucella lapillus*.

Average oviduct stage (AOS): Proposed by Barreiro and co-workers (2001) to assess the degree of oviduct convolution in *Nassarius reticulatus*.

1.4. Monitoring of sediment contamination

Chemical contamination in marine sediments is widely spread throughout the world, as a result of human activities. Over the last decades, growing concerns on this problem resulted in an expansion on the scientific work and reports on contamination. The extension of chemical pollution, inputs, accumulation and persistence of chemicals in environment received major attention in early years. Even though, assessing chemical concentrations in sediment is not enough to determine pollution level at one place. Chemical surveys do not give information about the available fraction of contaminants to organisms (bioavailability) or effective biological adverse effects (Chapman and Long, 1983). An integrative study combining both chemical and biological data is recommended for a better knowledge in sediment quality status (Chapman and Long, 1983; Long et al., 1996; Matthiessen et al., 1998; Beiras et al., 2003a).

The effort to assemble all chemical data and biological effects of contaminated sediments lead to the establishment of several quality criteria. These quality criteria are the basis for creation of the Sediment Quality Guidelines (SQGs). These guidelines appeared to help in evaluation of contaminated sediment, to formulate risk management decisions and proved to be useful and predictive of biological effects in many (but not all) marine and freshwater systems (Burton, 2002).

In SQGs, there are empirical approaches that are based on actual field and laboratory data that show adverse effects to benthic organisms when exposed to field-contaminated sediments. It includes the Effects Range Approach, the Effects Level Approach and the Apparent Effects Threshold Approach (Burton, 2002). These approaches set two threshold levels, in which below one no adverse effects are expected to occur, between the two levels adverse effects can occur occasionally, and above the other level adverse effects are frequently expected. For example the Effect Range Approach establishes two guideline values as reference for several contaminants, ER-L (Effects Range Low) and ER-M (Effects Range Median). These values were obtained after a compilation of sediment chemistry and biological effects data and define concentration ranges in which the contaminants can cause rarely, occasionally or frequently adverse biological effects for the organisms. As this approach does not take in consideration the role of bioavailability, measuring the total contaminant concentration in the sediment, the confounding factors and cause effects mechanisms should be used as screening tools in the assessment of pollution (Long et al., 1995).

In later years scientists are trying to develop a "consensus based" guideline that assembles values from empirical approaches described before creating mean values for the threshold levels. As an example of that, the Consensus-Based Sediment Quality Guideline provides two guideline values, the Probable Effect Concentration (PEC) and the Threshold Effect Concentration (TEC). For values below TEC adverse affects are not expected to occur. For values above PEC adverse affects are expected to occur. Although, a very good correlation is achieved between the PEC and the incidence of toxicity, this approach has also some limitations (MacDonald et al., 2000).

One of the biggest problems pointed out to the previously described SQGs is that they don't take in consideration the bioavailability of contaminants, considering only the total concentration in sediment. The Equilibrium Partitioning approach takes that in consideration. It considers the ability of the chemicals to move from one partition to another and reach equilibrium between them. So, this approach is based on the equilibrium between the solid phase, interstitial water and the water organisms. The main advantage of this approach is that it considers the variables that affect the bioavailability of the contaminants in sediment as, for instance, total organic carbon (TOC) for nonpolar organic contaminants and acid volatile sulfides (AVS) for some metals. In spite of some limitations this approach was selected by EPA as the primary source for the formulation of the SQG (EPA, 2005).

In mid 80's a distinct approach was proposed, the concept of sediment quality triad combined toxicity tests, chemical data and assemblage of benthic fauna. While Sediment Quality Guidelines recommend some values for contamination acceptability, this triad has the advantage of acquiring from the environment biological information and compares it with the chemical and toxicity tests (Long and Chapman, 1985b). It was used for the assessment of historically contaminated areas like the Puget Sound, San Francisco Bay, and Galveston Bay (Long and Chapman, 1985a; Chapman et al., 1987; Carr et al., 1996).

Due to sorption properties of some contaminants to sediments, they act as sink of contamination, having concentrations of contaminants three to five times higher than the overlying water column. As a consequence, benthic animals that are in contact to sediments or ingest sediments can uptake large amounts of contaminants and accumulate them in tissues (Bryan and Langston, 1992). The deepest layers of sediment can retain great amounts of contaminants released in environment in the past, being contaminants reservoir for a long time. However these contaminants are neither accessible nor available to sediment and water-dwelling organisms unless severe remobilization processes occur (Gustavson et al., 2008). For example, dredging activities may cause remobilization of contaminants from sediments and that is one of the most critical issues in environmental risk assessment in estuarine areas (Torres et al., 2009). This is especially important in harbours where adequate depth must be maintained for vessels traffic .On the other hand, eventual dispersal of contaminants around the disposal site can affect the local biota. With the remobilization, bioavailability of contaminants is modified and they could become available for organisms (Gustavson et al., 2008).

1.5. Laboratory bioassays – endocrine disruption

Current strategies to evaluate marine pollution integrate both chemical and biological parameters. As part of biological parameters, laboratory bioassays have become effective tools in ecotoxicological studies. Sediment toxicity tests, a non-expensive and effective tool, are normally used for monitoring contaminated places and predict their toxicity. They consist of exposing test organisms to contaminated sediment and compare the results to a reference (uncontaminated) site. Based on the obtained results toxicity of the studied site can be predicted. Those assays can be performed with different fractions of the sediment:

- **The solid phase assay** consists on exposing the test organisms to the whole sediment.
- **The elutriate assay** is carried out after the elutriation process. In this procedure the mixture of standard contaminated sediment and non contaminated water is stirred and consequently the soluble fraction of contaminants in the sediment is released to the water. After the stirring and a certain period of decantation the liquid phase of the mixture is used for the toxicity test. Water column organisms are exposed to this fraction. This approach is frequently used to assess the impact of remobilisation, like dredging situations, because simulates these conditions.
- **The pore water assay** consists of the exposure of water column organisms to the interstitial water of sediment.

These different approaches results in different effects on the organisms The solid phase gives a better realistic approach to the natural environment, although the pore water and the elutriate assay are suitable for some organisms such as bacteria, algae, embryos (Ankley et al., 1991).

The bioassays can be divided in two types, the acute and the chronic. The acute bioassay measures immediate or short term effects like survival, abnormal growing, and avoidance behaviour. These tests are relevant as screening tools in highly contaminated areas. Amphipods, as for example *Corophium spp.* (Bat and Raffaelli, 1998; Castro et al., 2006) or *Hyalella azteca* (Ankley et al., 1991; Borgmann and Norwood, 2002), polychaetes such as *Nereis diversicolor* (Perez et al., 2004) or *Arenicola marina (Matthiessen et al., 1998)* , and more sensitive life stages such as echinoderm's and bivalve's embryos (His et al., 1999; Beiras et al., 2003b), phytoplankton and bacteria (Microtox) are commonly used in acute bioassays (Matthiessen et al., 1998). In the other hand, chronic bioassays measures long term effects like endocrine disruption, growing and feeding rates, reproduction, fertilization success, among others. They are very important to assess if contaminants are present at a concentration that produce some deleterious effect at a long term. Amphipods, molluscs, polychaetes and fishes are mainly used in this type of assay (Kusk and Petersen, 1997; Castro et al., 2006; Scarlett et al., 2007).

Several ways of assessing endocrine disruptors in wildlife are described extensively for various groups of animals by Ankley and co-workers (1998). In the specific case of gastropods few bioassays are described for endocrine disruption assessment. However some gastropod species had demonstrated to be good test organisms for the assessment of endocrine active chemicals. For further information see (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Tillmann et al., 2001; Duft et al., 2003a; Duft et al., 2003b; Gust et al., 2009). *Potamopyrgus antipodarum*, has been used as test organism in this assessment due to his capacity to react both to estrogens or androgens compounds (Duft et al., 2003a; Duft et al., 2003b). Other prosobranch snails were also used in assessment of endocrine disruption caused by sewage effluents (Santos et al., 2008; Clarke et al., 2009). For specific case of TBT pollution, tests with collected sediment from polluted sites were already published for *Nassarius reticulatus* and *Marisa cornuarietis* with imposex induction as the endpoint (Duft et al., 2007b).

1.6. Species used in this work

In the present work several species were used to illustrate different methods that can be implemented for the assessment of TBT pollution in Ria de Aveiro. The gastropods *Nassarius reticulatus and Nucella lapillus* were used in field monitoring surveys as indicators of TBT pollution levels, by assessing imposex levels in natural populations. *Potamopyrgus antipodarum*, *Hydrobia ulvae* and *N. reticulatus* were tested in chronic sediment solid phase assays. For the evaluation of the endocrine disruption potential of sediments collected from Ria de Aveiro, the sea urchin, *Paracentrotus lividus* was used in acute elutriates assays in order to evaluate general toxicity of sediments.

1.6.1. *Nassarius reticulatus* Linnaeus, 1758

Taxonomical classification
Phylum: Mollusca
Class: Gastropoda
Subclass: Prosobranchia
Order: Neogastropoda
Family: Nassariidae
Genus: *Nassarius*
Species: *Nassarius reticulatus*

Figure 1-1 - Shell of *Nassarius reticulatus*

The netted whelk *Nassarius reticulatus* (Figure 1-1) is a marine prosobranch gastropod. This specie has a wide range of distribution along Europe, from Norway to Azores and Canarias, also founded in the Mediterranean and Black Sea (Graham, 1988).

The shell is a sharply pointed cone with a reticulate surface, an oval aperture with internal teeth. They can reach more than 30 mm height and 14 mm of width. Last whorl occupies 60-70% of shell height and the aperture 40-50%.The head has two ocular tentacles and a siphon, which is long and narrow. The foot is long and narrow (Graham, 1988).

Nassarius reticulatus is common in estuaries and also along the offshore coast. They live in rocky shores or in sediment are scavengers feeding on death matter (Graham, 1988).

The netted whelk has an indirect development. The larvae came from a capsule that can have 50-350 eggs. That capsule is normally attached to shells, rocks or algae. After hatching, veligers start a planktonic stage that lasts 1-2 months. Breeding in Ria de Aveiro occurs in spring and lasts until the end of summer (Sousa et al., 2005a).

1.6.2. *Nucella lapillus* Linnaeus, 1758

Taxonomical classification

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

Figure 1-2 - Shell of *Nucella lapillus*

Commonly named dog whelk, *Nucella lapillus* (Figure 1-2) is a prosobranch snail that is distributed in littoral areas on the North Atlantic. This snail has a solid shell with a large aperture that can grow up to 35 mm and 25 mm. Last whorl occupies 85% of shell height and the aperture occupies about 70%. The outer lip is thick and toothed internally. The colours of shells are very diverse. The animal is white cream and has two ocular tentacles (Graham, 1988).

It occurs on rocky shores where they live gregarious next to mussels and barnacles on which they feed. Breeding occur mainly between spring and autumn. Juveniles emerge from egg capsules with 1mm high and live along with their parents. Each capsule contains in median 600 eggs but only 25-30 grow to emerge as juveniles. Maturation is achieved at 2.5 years (Graham, 1988).

1.6.3. Potamopyrgus antipodarum Gray, 1843

Taxonomical classification

Phylum: Mollusca

Class: Gastropoda

Subclass: Prosobranchia

Order: Caenogastropoda

Family: Hydrobiidae

Genus: *Potamopyrgus*

Species:*Potamopyrgus antipodarum*

Figure 1-3 - Shell of *Potamopyrgus antipodarum*

The gastropod *Potamopyrgus antipodarum* (Figure 1-3) is considered an invasive species. Native from New Zealand is widely dispersed throughout the world due to a high reproduction rate or high capacity for active and passive dispersal (Alonso and Castro-Díez, 2008).

This prosobranch snail has a small, but extended, shell that can reach up to 6-7 mm of height and 3-4 of broad. Last whorl occupies 65-70% and the aperture that occupies 40% of its height. It lives preferably in freshwater but can also be found in waters with low salinity (0-16‰), preferring running water to stagnant. Lives under stones, on plants, on which it feeds, and on substratum, clay, fine sand and mud are the preferable ones. In native ambient reproduction is both sexual and asexual but non native populations are parthenogenetic being composed mainly by females. Females carry their offspring in a brood pouch. Sexual maturity can be reached at 3-3.5mm and one adult can produce 1-6 generations per year with an average of 230 juveniles (Graham, 1988; Alonso and Castro-Díez, 2008).

1.6.4. Hydrobia ulvae Pennant, 1777

Taxonomical classification

Phylum: Mollusca
Class: Gastropoda
Subclass: Prosobranchia
Order: Caenogastropoda
Family: Hydrobiidae
Genus: *Hydrobia*
Species: *Hydrobia ulvae*

This gastropod, commonly named mud snail, occurs almost all over the North Athlantic, from Norway to Senegal and Mediterranean. It has a small shell, up to 6 mm and 2.5-3 mm broad. Last whorl occupies 60-70% and the aperture 40% of shell height. Although it prefers estuarine conditions and occurs in wet banks of sand and mud it also occurs on open coasts. It can live and breed in salinities between 5-40‰. This breeding occurs in spring and summer and normally 3-7 eggs are laid in a jelly mass over sand grains. After 2-3 weeks a veliger larva emerges, adults may live up to 5 years. With feeding habitats very diverse, preferably, eats diatoms collected from the surface, bacteria, silt and algae (Graham, 1988).

1.6.5. Paracentrotus lividus *Lamarck, 1816*

Taxonomical classification

Phylum: Echinodermata
Class: Echinoidea
Order: Echinoida
Family: Echinidae
Genus: *Paracentrotus*
Species: *Paracentrotus lividus*

Paracentrotus lividus, commonly named sea urchin, is distributed from northern Atlantic to southern Morocco including Mediterranean Sea and ranging temperatures from 4ºC to 28ºC. It lives in subtidal areas and in intertidal rock pools. It is sensitive to salinity preferring salinities in the order of 15‰ to 20‰. The shell, of diverse colours, can reach up to 7.5 cm of diameter. It feeds on invertebrates and algae (F Boudouresque et al., 2007).

Breeding occurs in spring and early summer. It as an indirect development and the plutei larvae formed develops in one month until the settling. It feeds on unicellular algae (F Boudouresque et al., 2007).

1.7. Ria de Aveiro

The Ria de Aveiro, located in NW of Portugal, is a shallow estuarine system which can be classified as a bar-built estuary. It covers an area of 47 and 43 km^2 at high and low tide, respectively. The topography of the Ria de Aveiro consists of 3 main channels which radiate from the mouth with several branches, islands and mudflats (Figure 1-4): Mira and Ílhavo channels run to the south and are narrow and shallow; S. Jacinto-Ovar Channel runs to the north and is wide and deep in its southern part but changes northwards, forming secondary narrow and shallow channels and bays. Exchange of water with the sea occurs only through the artificial mouth. Inside the Ria de Aveiro the rocky shores are restricted to artificial banks constructed mainly in the outer part of the channels and along the navigation channel. For the most part of the ria the bottom is formed by sediments ranging from medium sands to mud (Cunha et al., 1999). The adjacent coastal zone consists of a very extensive sandy shore interrupted by a few man-made rocky groynes. Sublittorally, on the continental shelf and upper slope, the superficial sediments are dominated by sand with a low content of gravel and mud.

The potential TBT sources of contamination in the Ria de Aveiro are the ports, dockyards and marinas. The ports and dockyards are located along the main navigation channel that extends 9 km eastwards from the mouth to the city of Aveiro. Also a large number of small local fishing boats and pleasure boats are spread along the banks of the estuary, mainly in the channels inside the city of Aveiro, in the outer part of Mira Channel and, to the north, at Torreira and Murtosa (Barroso et al., 2000).

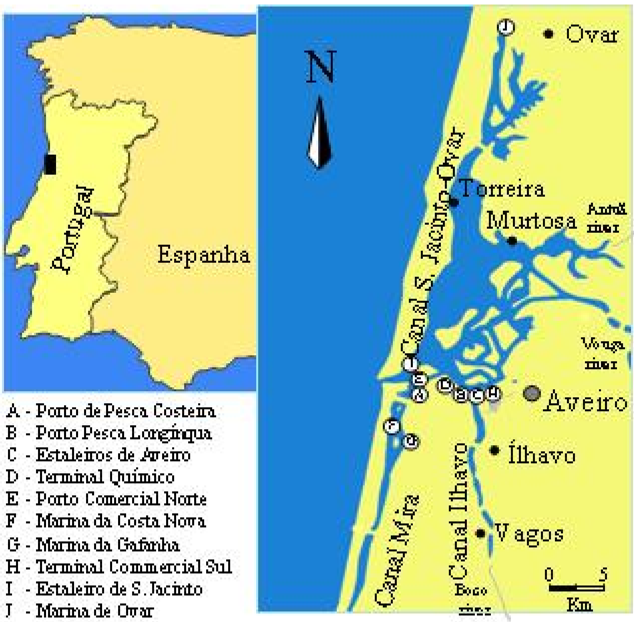

Figure 1-4 Map of Ria de Aveiro, containing information on the main hotspots for TBT contamination.

1.8. Objectives

The objective of the current work is to develop an innovative methodology for the assessment of TBT pollution in estuarine areas, combining data derived from both field monitoring and laboratory bioassays. A special relevance will be given to sediment quality assessment as sediments are known to constitute a long-term reservoir of TBT in estuarine areas. The field monitoring includes the assessment of imposex levels in populations of gastropods and chemical determination of TBT concentration in the sediments. The bioassays consist on exposing gastropods to sediments collected from the study area and measure the females penis growth as an indication of the presence of TBT in the sediments. In a complementary approach, we will also study the global toxicity of sediments using two additional bioassays. One attempts to characterise the endocrine disruption potential of sediments by assessing the effects of different sediment samples on *Potamopyrgus antipodarum* embryo production. The other is more generalist and will evaluate the effect of sediment elutriates on the development of *Paracentrotus lividus* larvae.

1.9. Thesis overview

The thesis is organised in four chapters as described below.

Chapter 1 presents a general introduction to the subjects discussed in this thesis providing a review on relevant literature.

Chapter 2 describes the integrative assessment of TBT pollution in Ria de Aveiro. First subchapter describes TBT field monitoring, presenting results of imposex survey with two bioindicators – *Nassarius reticulatus* and *Nucella lapillus* - and TBT concentration on sediments quantified trough chemical analyses. The second subchapter describes laboratory bioassays to evaluate levels of TBT contamination in sediments collected from Ria de Aveiro, using the gastropods *Nassarius reticulatus* and *Hydrobia ulvae* as test species and the development of imposex as the endpoint.

Chapter 3 describes the complementary laboratory bioassays for the assessment of general sediment toxicity. First subchapter describes bioassays exposing the gastropod *Potamopyrgus antipodarum* to sediments collected from Ria de Aveiro and using embryo production as endpoint. The second subchapter describes the *Paracentrotus lividus* larval bioassay with sediment elutriates.

Chapter 4 discusses the presented work as an integrative strategy that allows a comprehensive understanding of the status of TBT pollution in study areas. The obtained results are discussed, giving overall conclusions from the previous chapters, and also for a comprehensive knowledge of this approach hypothetical results, which were not obtained in this work, are given for a better understanding of its scope.

2. Integrative assessment of TBT pollution using Ria de Aveiro as a case of study

2.1. Introduction

According to Goldberg (1986), tributyltin is the most toxic xenobiotic ever produced and deliberately released into marine environment. One of the well known deleterious effects caused by TBT is imposex, which is the best documented example of endocrine disruption in wildlife (Matthiessen and Gibbs, 1998). Imposex is the superimposition of male characters, such as penis and vas deferens, in many species of female's gastropod. It was first described by Blaber (1970) in females of *Nucella lapillus*. One decade later, Smith concluded that TBT was the responsible agent of this phenomenon, conclusions also confirmed by Bryan and co-workers (Smith, 1981b, a; Bryan et al., 1987; Gibbs et al., 1987). Since then, imposex was described in more than 200 species of gastropods (Shi et al., 2005) and has been used as biomarker of TBT pollution in field surveys. Following observed deleterious effects of TBT in ecosystems, such as gastropods populations declines (Bryan et al., 1986; Oehlmann et al., 1996) and reduction in production of oysters (Alzieu, 2000), some restrictions were made on the use of TBT since the decade of 80s, culminating in a global ban of organotin AF-paints in September 2008 imposed by IMO (for further information chapter 1.1.2.1)

Several imposex monitoring studies have been published since the implementation of legislation restricting the use of TBT antifouling paints (Stroben et al., 1992a; Barreiro et al., 2001; Barroso et al., 2002a; Sousa et al., 2005b) in order to verify the legislation effectiveness. Several studies in the late 90's and in the beginning of the new century pointed out the inefficacy of Directive 89/677/EEC that banned the use of TBT and TPT on boats smaller than 25m in length (Minchin et al., 1995; Morgan et al., 1998; Barroso and Moreira, 2002; Santos et al., 2002). However, more recent imposex surveys revealed that levels are clearly decreasing as consequence of the implementation of latest legislation - the EC Regulation 782/2003 - that banned the application of OTs AF paints in all EU ships after 1st of July of 2003 (Galante-Oliveira et al., 2009; Sousa et al., 2009).

In estuarine systems TBT tend to accumulate in sediments and presents a long persistence in this compartment. In anoxic sediments TBT can be stable for at least 2 decades (Dowson et al., 1993). Although it was banned worldwide in 2008, persistence in sediments will cause only a slow decline of this pollutant over time in this compartment and, due to possible remobilisation from sediments, TBT pollution may persist for many years to come. For this reason, monitoring the levels of TBT in sediments is of paramount importance in estuarine areas. Chemical monitoring of sediments may include not only TBT and its degradation compounds but also other organotin species. Another important organotin is triphenyltin (TPT) that is known to cause similar adverse effects on biota – e.g., imposex.

When studying the status of TBT pollution in a given estuary, like Ria de Aveiro, several monitoring methods can be applied, each presenting advantages and disadvantages. For instance, imposex biomonitoring, in comparison with chemical monitoring, is a low cost pollution assessment approach being in some cases even more efficient because some organisms react to TBT concentrations below detection limits of analytic techniques (Oehlmann et al., 1998). It also has the advantage of reflecting average levels of pollution at the site where the gastropod sample was taken. However, as stated by Stroben and co-workers (1992a), imposex is an irreversible phenomenon, which means that imposex surveys only reflect historical contamination at a given site. Chemical monitoring in water, sediments and biota should be performed along with biomonitoring to provide better knowledge about correlations between environmental contamination and imposex, as well as to preview all range of possible adverse effects to biota and to the ecosystem as a whole. Quantification of butyltins concentrations in different compartments also provide information on recent inputs of TBT that cannot be assessed by imposex monitoring (Sousa et al., 2007b). The bio- and chemical monitoring also allows to evaluate if actual levels comply with environmental quality standards determined by legislation.

On the other hand, concentrations of TBT in water and sediments determined by chemical analyses do not give information about the amount of TBT available to organisms. This information could be supplied by using laboratory bioassays where animals are exposed to environmental samples – e.g., sediments – taken form the study area and potential toxic effects investigated.

Hence, an integrative assessment of TBT pollution, combining chemical analyses, imposex surveys and sediment laboratory bioassays provides a better understanding of the ecological quality of estuarine areas. This is the context of the current chapter, which main objective is to develop new methods for assessing the status of TBT pollution in estuarine systems combining imposex surveys with *Nucella lapillus* and *Nassarius reticulatus*, organotin quantification in sediments and laboratory bioassays with *Nassarius reticulatus* and *Hydrobia ulvae*.

2.2. Methods

2.2.1. Field biomonitoring of TBT pollution levels

About 60 to 80 adult specimens of *Nassarius reticulatus* were collected in August of 2009 during the low tide from 4 stations in Ria de Aveiro (Figure 2-1) with a baited hoop net or by hand. Animals were brought to the laboratory and maintained in aquaria with constant aeration. Prior to the imposex analysis, animals were narcotized with a solution of $MgCl_2$ (7%) and shells heights measured with callipers to the nearest 0,1 mm. The shells were cracked open with a bench vice and the animals were sexed and dissected under a stereo microscope. Parasited specimens were discarded from the analysis.

The percentage of females affected by imposex (%I), mean females penis length (FPLI), mean males penis length (MPLI), the relative penis length index (RPLI, mean female penis length x 100/ mean male penis length), the vas deferens sequence index (VDSI), the percentage of sterile female (%STER) and the average oviduct stage (AOS) was determined for each station. The VDSI were classified according to the scoring system developed by Stroben (Stroben et al., 1992b) with minor alterations proposed by (Barroso et al., 2002a). VDS scoring system proposed by (Stroben et al., 1992b)) the stages vary from 0 to 4+, giving the same numerical value of 4 for two different stages of the VDS, 4 and 4+. Barroso stated that when the value of 4+ is computed with the numerical value of 5, a better correlation between VDSI and RPLI is given and therefore allows a better discrimination between stations. The AOS were classified according to Barreiro and co-workers. This index provides a better discrimination between severe polluted sites since gonadial oviduct convolution only occurs in VDSI >2 (Barreiro et al., 2001).

About 60 adult specimens of *Nucella lapillus* were collected by hand in 2 sampling stations in Ria de Aveiro (Figure 2-1) during low tide. Shell height was measured with callipers to the nearest 0,1mm. Shells were removed from the animals with the help of a bench vice and imposex were assessed under a stereomicroscope. The animals weren't narcotized. The relative penis size index [RPSI = mean female penis length $(FPL)^3$ x 100/mean male penis length $(MPL)^3$], the vas deferens sequence index (VDSI) and the percentage of females affected with imposex (%I) were determined for each station, according to Gibbs and co-workers (Gibbs et al., 1987).

Figure 2-1 Map showing sampling stations where gastropods were collected: 1 – Barra; 2 – Magalhães Mira; 3 – Forte da Barra; 4 – Porto Comercial Norte. *N. reticulatus* was collected at all stations and *N. lapillus* was collected at stations 1 and 3.

2.2.2. Chemical monitoring of TBT pollution

Surface sediments (≈2cm depth) were collected at Ria de Aveiro (Figure 2-2) in Muranzel, Ermida, Magalhães-Mira and Porto de Pesca Longínquo in March of 2009. Sediments were collected during the low tide by hand with a spatula. Three replicates of sediment were collected per sampling site, each replicate consisting of about 1 l of surface sediment taken from a square area of 0.04 m^2; the position of the three replicates corresponded to the vertices of an isosceles triangle with 1 m side. The sediments were brought to the laboratory and the three replicates were homogenised. Sediment was then stored in dark at 4ºC.

Organic matter content was obtained by drying sediment samples until constant weight at 60 ºC and then incinerating them at 450 ºC for 24 h. The difference of weight before and after the incineration gives the organic matter. Percentage of fine fraction was obtained after wet sieving sediment samples in 64 µm mesh.

Butyltins (MBT, DBT,TBT), phenyltins (MPT, DPT, TPT) and octyltins (MOT, DOT) quantification in sediment samples was performed by gas chromatography coupled to mass spectrometry (GC–MS) following the method described by Sousa and co-workers (2007b). The analyses were performed by Ana Sousa at Center for Marine Environmental Studies (CMES), Ehime University in Japan in May of 2009. Recoveries values for all the analysed organotin compounds were situated between 58.7 ± 25.6 (for MBT) and 233.0 ± 26.5 (for TPT).

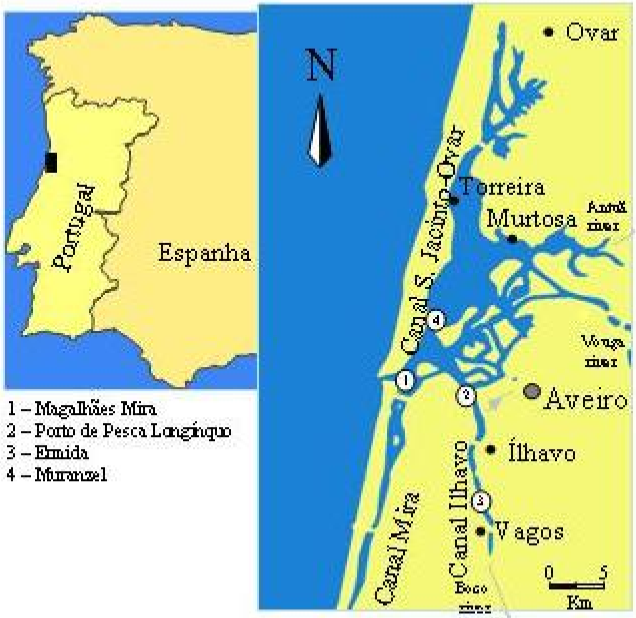

Figure 2-2 Map showing the sediment sampling stations.

2.2.3. Laboratory bioassays

2.2.3.1. *Nassarius reticulatus* sediment bioassay

Some individuals of *Nassarius reticulatus* were collected in one station in Ria de Aveiro close to the mouth of the Ria de Aveiro (Praia do Meio Laranjo), known to present low levels of TBT pollution. They were brought to the laboratory and were narcotized with a solution of $MgCl_2$ 7% in distilled water. The body of the gastropod was pushed to some extent from inside the shell for penis measurement. Selected females with imposex, but with a penis smaller than 1 mm, were left to recover from narcotizing in artificial seawater for one week and then placed into the glass flasks of the experiment.

Organisms were exposed to sediments collected in June 2009 from the following stations in Ria de Aveiro: Magalhães Mira, PPL, Ermida (Figure 2-2) and to a control of artificial seawater. For these experiments sediments were collected as previously described in section 2.2.2. Animals were exposed in 1L glass flask, 10 animals in each flask and 3 flasks for each treatment. Bioassays were performed with 2-3 cm layer of total sediment and a volume of artificial seawater that filled the flask Figure 2-3. Before the bioassay began, with the addition of organisms, the sediment was let to settle for one day. Bioassay was performed at 18±1ºC under constant aeration and the water was renewed weekly as well as the food supply. The animals were exposed for 56 days.

In the end of the experiment *vas deferens* sequence index (VDSI) and female penis length index (FPLI) were assessed. VDSI was classified according to methods described in section 2.2.1. The penis and *vas deferens* were measured with a graduated eyepiece under a stereo microscope. Statistical analyses were performed with SPSS 17.0 software. Differences between treatments were evaluated through one-way ANOVA, followed by Dunnet's post hoc test. Critical significance level was 0.05.

After the results of the exposure with sediments from Ria de Aveiro were known, an additional exposure was performed with sediments collected from Nazaré fishing port, one of the most TBT polluted harbours in Portugal. The procedure used was similar to the one described before but the time of exposure was shorter: 45 days.

Figure 2-3 – *Nassarius reticulatus* biossay

2.2.3.2. *Hydrobia ulvae* sediment bioassay

Hydrobia ulvae were collected inside Ria de Aveiro at Ermida in October 2008, during low tide. They were brought to the laboratory and separated by different size classes. Animals belonging to the size class of 2.0-2.5mm were selected for the experiments. These are small animals that are expected to exhibit low imposex levels as they did not reach sexual maturity yet. This pre-requisite is needed in order to guarantee that before the experiment imposex is low enough, as it is impossible to push the body from inside the shell for penis measurement, like it happened with

Nassarius reticulatus. The exposure was performed in a 20 L aquarium filled with tap water where round containers were overlapped to create replicates. Approximately 1 cm sediment layer were placed in a round container (Figure 2-4). After one day of sediment settling, 20 individuals were added to each container. The length of the shell was measured with graduated eye-piece under a stereo microscope at different times of exposure. The imposex assessment would be performed only in case these gastropods could achieve good growth rates and body sizes would be large enough for examination.

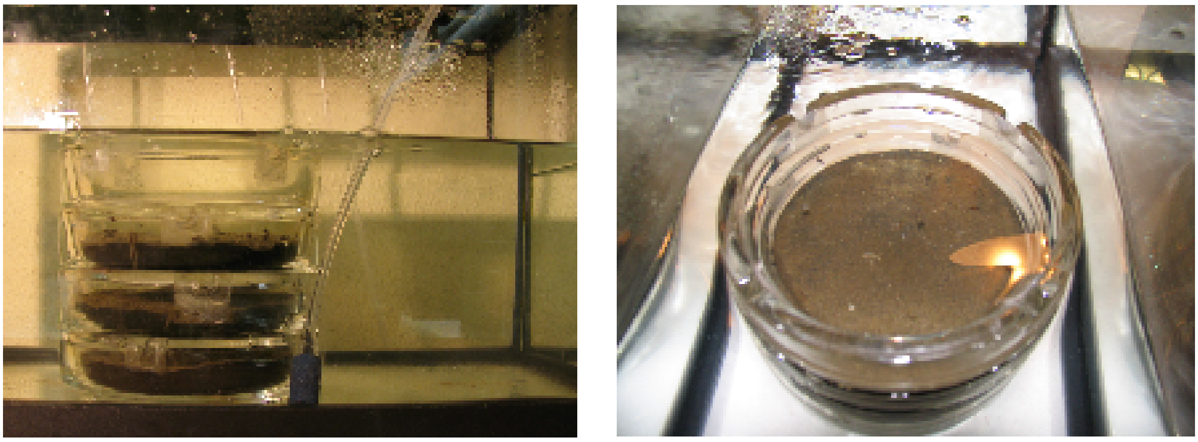

Figure 2-4 - Experimental apparatus for *Hydrobia ulvae* sediment bioassay exposure.

2.3. Results

2.3.1. Field biomonitoring of TBT pollution levels

Imposex levels in Ria de Aveiro are shown in Table 2-1 and Table 2-2 . For *Nassarius reticulatus* (Table 2-1) the percentage of affected females with imposex varied from 33.3% to 83.3% across stations. FPLI and RPLI varied from 0.1 to 0.9 mm and 0.6 to 9.2%, respectively. VDSI ranged from 0.4 to 2.2 and VDS varied from 0 to 4 in all stations except Porto Comercial Norte where VDS stages varied from 0 to 3. AOS levels where 0 in all stations. Magalhães Mira was the station where imposex levels were highest while the lowest levels of imposex were found in Porto Comercial Norte.

Imposex in *Nucella lapillus* was assessed only in Forte da Barra and Marégrafo and the results are summarised in Table 2-2. FPLI ranged from 0.1 to 0.2, RPSI values varied between 0.02-0.1 and VDSI ranged from 1 to 1.2. The percentage of females affected by imposex varied from 79% to 96% in the respective above sampling stations.

Table 2-1 – Imposex levels of *Nassarius reticulatus* in Ria de Aveiro in August 2009: shell height (SH), males penis length in mm (MPLI), mean females penis length in mm (FPLI), relative penis length index (RPLI), the vas deferens sequence index (VDSI), the average oviduct stage (AOS) and percentage of females affected by imposex (%I)

Station code	N♂	SH	MPLI	N♀	SH	FPLI	RPLI	VDSI	AOS	%I
Magalhães Mira	21	24.9 ± 2.2	9.3 ± 1.7	24	25.2 ± 1.9	0.9 ± 0.8	9.2	2.2 ± 1.2	0	83.3
Forte da Barra	29	23.1 ± 2.2	10.9 ± 2.5	30	24.9 ± 2.8	0.2 ± 0.3	1.9	1.2 ± 1.3	0	56.7
Marégrafo	26	23.7 ± 2.4	11.9 ± 1.1	30	25.4 ± 2.00	0.4 ± 0.8	3.4	1.2 ± 1.4	0	50
Porto Comercial Norte	24	22.3 ± 1.6	10.3 ± 1.3	21	24.1 ± 1.8	0.1± 0.2	0.6	0.4 ± 0.7	0	33.3

Table 2-2 – Imposex levels of *Nucella lapillus* in Ria de Aveiro in August 2009 shell height (SH), mean males penis length in mm (MPLI), mean females penis length in mm (FPLI), relative penis size index (RPSI), the vas deferens sequence index (VDSI), females affected by imposex (%I)

Station code	N♂	SH	MPLI	N♀	SH	FPLI	RPSI	VDSI	%I
Forte da Barra	25	25.6 ± 2.2	3.1 ± 0.6	25	27.8 + 4.1	0.2 ± 0.4	0.11	1.0 ± 0.9	79
Marégrafo	22	31.3 ± 3.1	3.6 ± 0.5	23	31.9 ± 4.2	0.1 ± 0.3	0.02	1.2 ± 0.8	96

2.3.2. Chemical monitoring of TBT pollution

The organic matter content and the fraction of fine particles of sediments collected in the studied area are presented in Table 2-3. Granulometric analysis revealed that all sites sampled presented sediments in the class of sands (fraction <63 μm is below 25%). The organic content of sediments at each station varied between 1.3 and 6.1 (% of dw). The concentrations of organotins in sediments are shown in Table 2-4 and expressed in ng Sn/g dw. Concentration values for TBT and DBT were increasingly higher for Muranzel, Ermida, Magalhães Mira and PPL. TBT concentration values ranged from 0.4 in Muranzel to 66.8 ng Sn/g dw in PPL while DBT values ranged from 0.6 to 16.8 ng Sn/g dw for the respective sites. Values of MBT were below the detection limit in all stations except in PPL where it attained 12.8 ng Sn/g dw. Phenyltin compounds were below the detection limit and octyltin compounds (DOT) were only detected in sediments from PPL.

Table 2-3 Values of percentage of organic matter (%OM) and percentage of sediment particle size lower than 63 µm (%<63µm) in sampled stations

	Ermida	Muranzel	Magalhães Mira	PPL
%OM	6.0	5.4	1.3	6.1
%<63µm	10.0	7.6	1.5	14.0

Table 2-4 Organotin concentrations in studied stations.

Concentration (ng Sn /g dw)	Muranzel	Ermida	Magalhães Mira	PPL
MBT	<6.5	<6.5	<6.5	12.8
DBT	0.6	2.1	3.1	16.8
TBT	0.4	1.6	10.9	66.2
MPT	<0.1	<0.1	<0.1	<0.1
DPT	<0.4	<0.4	<0.4	<0.4
TPT	<0.0	<0.0	<0.0	<0.0
MOT	<8.6	<8.6	<8.6	<8.6
DOT	<3.0	<3.0	<3.0	0.3

2.3.3. Laboratory bioassays

2.3.3.1. *Nassarius reticulatus* sediment bioassay

Results from the bioassay with sediments collected in Ria de Aveiro are shown in Figure 2-5 and Figure 2-6. Mortality reached a maximum of 30% only in one replicate of PPL station. In the end of the experiment, statistical differences (p<0.05) were achieved for penis growth. Post-hoc revealed that sediments from PPL showed a statistically significant growth in relation to control. There were no significant differences of VDSI between treatments (Figure 2-6).

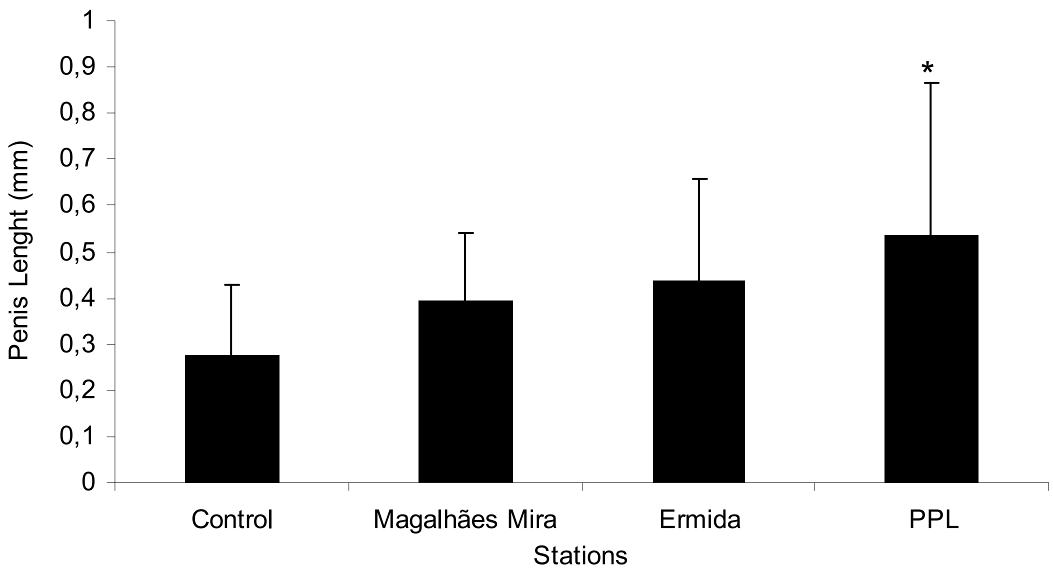

Figure 2-5 - Penis growth in females of *Nassarius reticulatus* exposed to different sediments collected in Ria de Aveiro for 56 days. Statistically significant differences are marked with * (p<0.05).

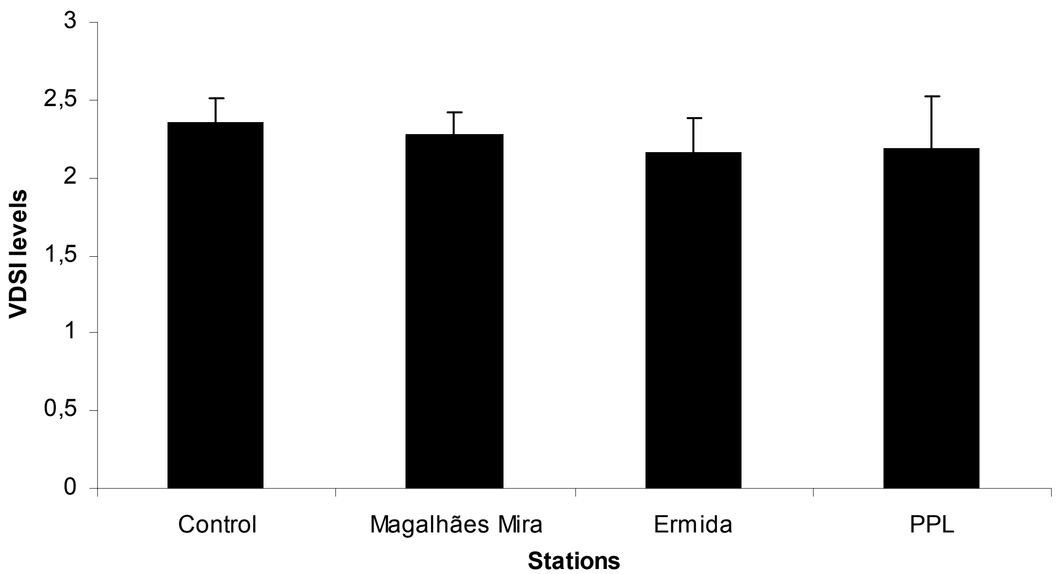

Figure 2-6 – VDSI in females of *Nassarius reticulatus* exposed to different sediments collected in Ria de Aveiro for 56 days

Results from the bioassay with sediments from Nazaré fishing port are shown Figure 2-7. Mortality varied from 0 to 10% among replicates. Differences were highly statistically significant.

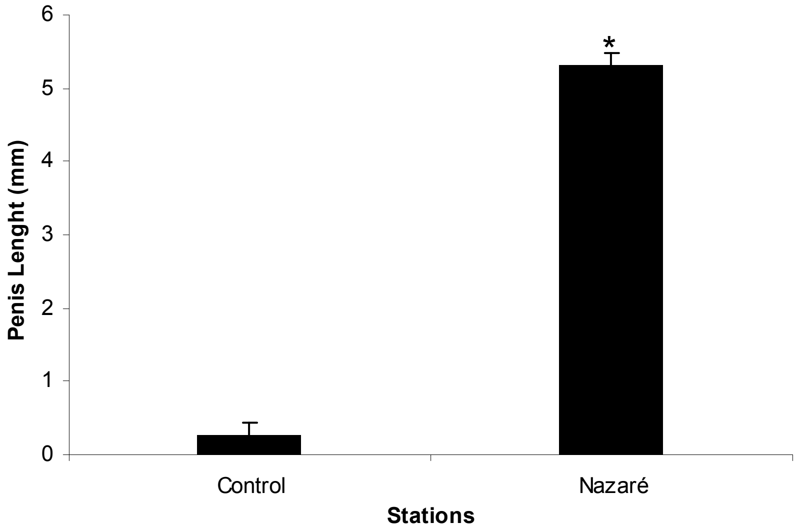

Figure 2-7 - Penis growth in females of *Nassarius reticulatus* exposed to sediment from Nazaré fishing port for 45 days. Statistically significant differences are marked with * (p<0.05).

2.3.3.2. *Hydrobia ulvae* sediment bioassay

This experiment was designed with the same purpose as the *Nassarius reticulatus* bioassay. No results of imposex were obtained due to non-expected low growth rates of the organisms. Therefore only the results of *Hydrobia ulvae* shell growth are shown in Figure 2-8. High mortality was registered at 6 months for sediments from Muranzel.

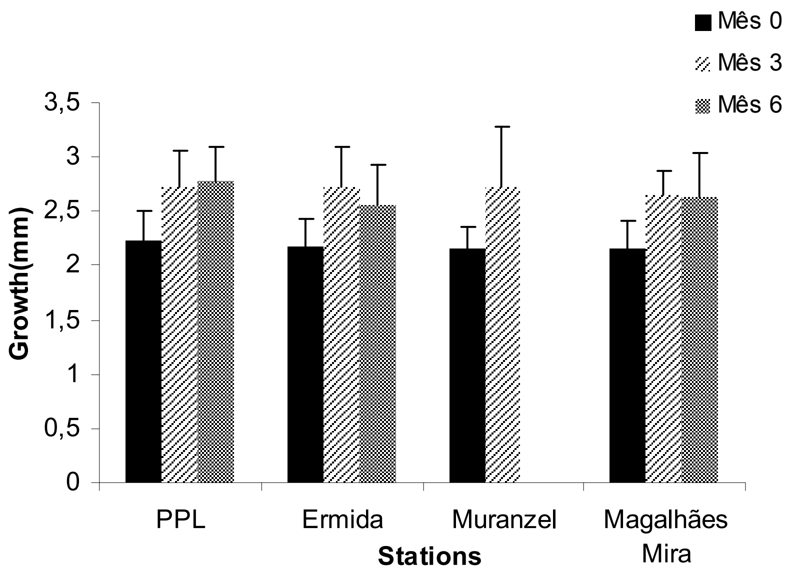

Figure 2-8 – Shell growth of *Hydrobia ulvae* exposed to different sediments collected in Ria de Aveiro,

2.4. *Discussion*

2.4.1. Imposex surveys

The results of the imposex surveys performed in the current study are compared with those reported by other authors for previous years, using the same methodology and common sampling sites (Barroso et al., 2000; Barroso et al., 2002b; Sousa et al., 2005c; Sousa et al., 2007b; Galante-Oliveira et al., 2009; Sousa et al., 2009). Temporal evolution of *N. reticulatus* (Figure 2-9 and Figure 2-10) and *N. lapillus* (**Error! Reference source not found.** and Figure 2-11) imposex levels at Ria de Aveiro over the last decade clearly shows an increase of TBT pollution in the area between 1998 and 2000/2003, followed by a decrease from 2003 to the present time. The progression of TBT pollution in the last decade is most probably a consequence of evolution of naval traffic in Ria de Aveiro and the legislation regarding the use of antifouling paints that was implemented during this period in the European Union. The pollution increase observed from 1998 to 2003 could be caused by the progressive increase in the number of vessels calling the Port of Aveiro during these years(Figure 2-12); in this period TBT based AF-paints were allowed in vessels > 25 m in length (Directive 89/677/EEC) and, consequently, all vessels calling this port could carry these paints. In 2003 was adopted new legislation (EC Regulation 782/2003) prohibiting new applications of TBT AF paints on vessels and, despite the number of ships calling the Aveiro Port continued to increase (Figure 2-12), those carrying TBT AF-paints probably reduced sharply, causing a decrease of TBT pollution. It is interesting to note that the imposex of *Nassarius reticulatus* remained almost identical between 2008 and 2009 contrariwise to what happened to *Nucella lapillus*, which may suggest that sediments may present a lower reduction rate of TBT contamination in comparison to the water column.

TBT compounds are on the Oslo and Paris (OSPAR) Commission 'List of chemicals for priority action' (OSPAR, 2007) and also on the Water Framework Directive 2000/60/EC (2000). Besides the chemical monitoring of TBT environmental concentrations, imposex assessment is also a mandatory element of OSPAR Co-ordinated Environmental Monitoring Programme (CEMP; OSPAR, 2008a). OSPAR Commission adopted specific guidelines to monitor imposex in *Nucella lapillus* and *Nassarius reticulatus*, among other species. Assessment criteria for imposex in different species were developed and Ecological Quality Objectives (EcoQO) set. For *Nucella lapillus* the EcoQO for imposex corresponds to VDSI values below 2 whereas for *Nassarius reticulatus* the EcoQO corresponds to VDSI values below 0.3. For the muriciid the EcoQO is achieved in both surveyed stations in 2009 as VDSI are below 2. In the case of the nassariid the EcoQO is not achieved yet.

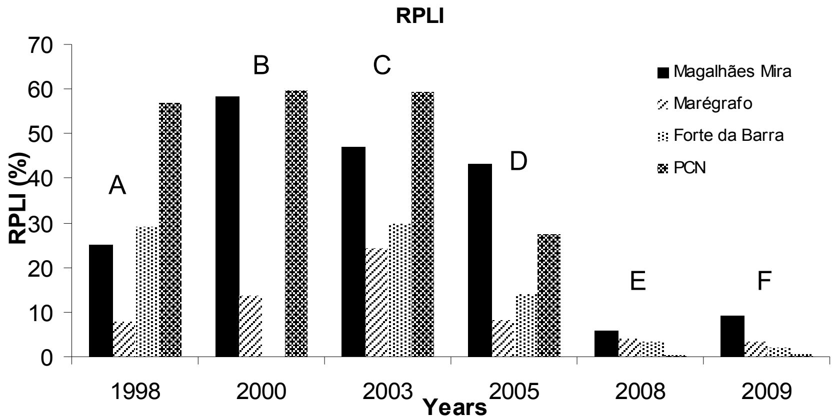

Figure 2-9 RPLI levels in females of *Nassarius reticulatus* over the years. Data on imposex levels was taken from: A - (Barroso et al., 2000); B - (Barroso et al., 2002a); C - (Sousa et al., 2005c); D - (Sousa et al., 2007b); E - (Sousa et al., 2009); F – Present work

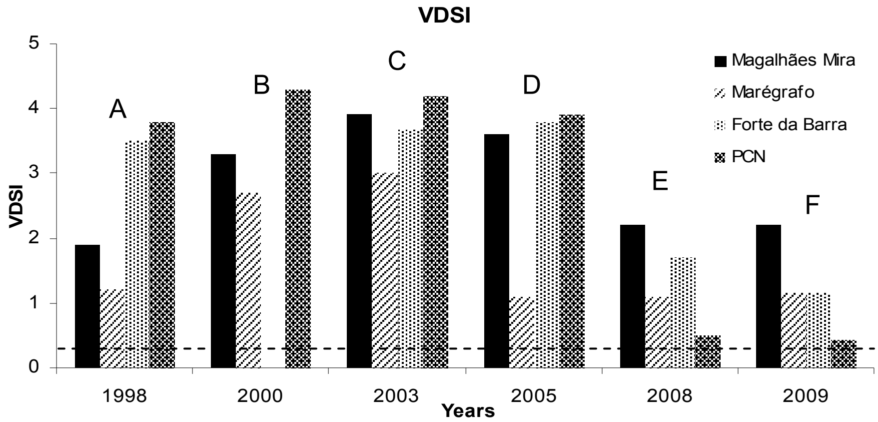

Figure 2-10 VDSI levels in *Nassarius reticulatus* females over the years. Data on imposex levels was taken from: A - (Barroso et al., 2000); B - (Barroso et al., 2002a); C - (Sousa et al., 2005c); D - (Sousa et al., 2007b); E - (Sousa et al., 2009); F – Present work. The line refers to EcoQO proposed by OSPAR.

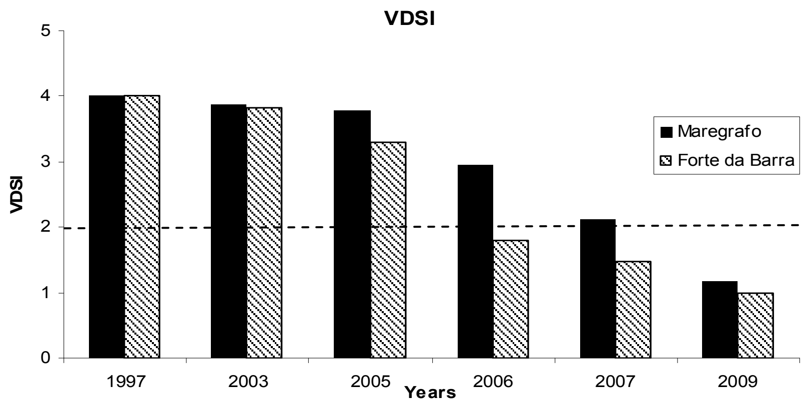

Figure 2-11 VDSI (Vas Deferens Sequence Index) levels in *Nucella lapillus* females over the years. Data were obtained from the work by Galante-Oliveira and co-workers (2009) except for the year of 2009 which is data from this work. The line refers to EcoQO proposed by OSPAR.

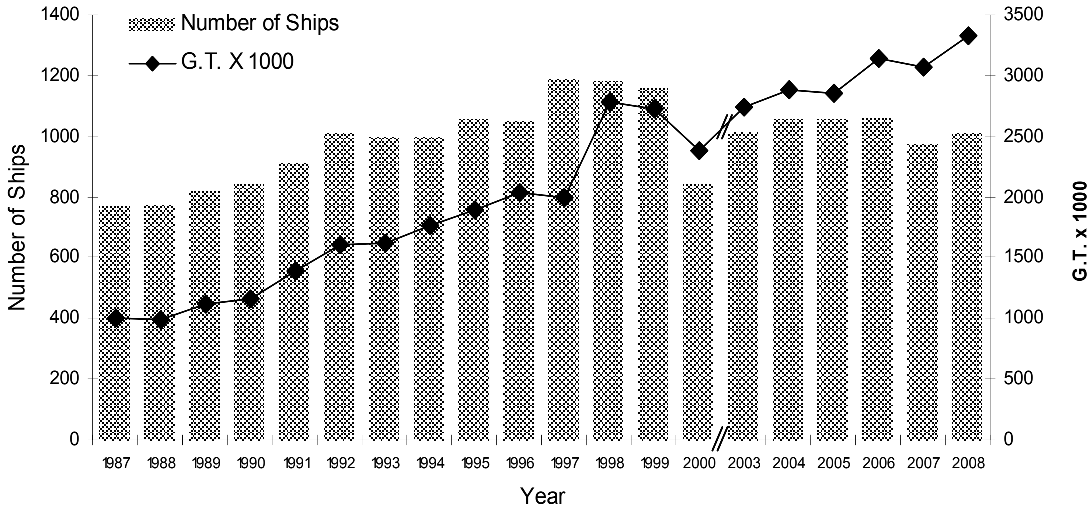

Figure 2-12 Comercial ship traffic activity in Aveiro harbour between 1987 and 2008. G.T. – total gross tonnage; * data not available. (www.portodeaveiro.pt, ; Anonymous, 2000).

RPLI and RPSI seem to be suitable indices to track variations of TBT levels in environment in short periods of time. Analysing the figures presented above, it's possible to see an earliest response in RPLI and RPSI values that the ones from VDSI. However some limitations could be pointed to these indices. In *Nucella lapillus* penis only appears in stage 2; in low polluted areas many females don't exhibit penis and consequently RPSI could be very low or nearly zero (see Table 2-2). For *Nassarius reticulatus*, RPLI also shows limitations because of b-type imposex development and the penis may only appear in VDS stage 4. Described by (Stroben et al., 1992b)), the b-type is an alternative way of VDS development where initial stages of affected females starts to exhibit vas deferens.

The results obtained and comparison with previous works show a clear decline in imposex levels in last years proving legislation effectiveness, however, these levels are still of great concern for Magalhães Mira where *Nassarius reticulatus* VDSI is well above the EcoQO proposed by OSPAR for this species.

Table 2-5 summarizes the advantages and disadvantages of imposex surveys for monitoring TBT pollution.

Table 2-5 Summary of the advantages and disadvantages of field imposex surveys for TBT pollution monitoring, according to species.

	Field Imposex Surveys	
Advantages	• Low cost and fast information. • Periodical imposex surveys allow outline the historical evolution of TBT pollution at a given site and evaluate legislation effectiveness. • Verify legislation compliance	
Disadvantages	• In one survey is impossible to know if contamination is recent or old due to imposex irreversibility and species longevity (up to 10 y).	
	Species	
	Nassarius reticulatus	***Nucella lapillus***
Advantages	• Lives in rocky shores or sediments of offshore or estuarine areas; allows the assessment of TBT contamination in the sediments at diverse depths. • More tolerant to salinity: allows assessment of larger areas of estuaries.	• Lives in rocky intertidal shores: allows the assessment of TBT contamination in the water column. • High TBT sensitivity: allow monitor low polluted areas.
Disadvantages	• Moderate TBT sensitivity: unable to monitor low polluted areas.	• Assessment of TBT pollution only at the shore-line. • Less tolerant to low salinity: unable to monitor higher portion of estuaries.

2.4.2. Chemical monitoring of TBT pollution

One important aspect of the chemical monitoring of TBT in the environment is to relate levels of contamination and the biological effects under study. However, this was not the specific objective of the current work, i.e., it is not pretended to correlate levels of imposex and levels of TBT in the tissues, water or sediments across sites because this relationship is fairly well described in the literature for many study areas, including Ria de Aveiro (Barroso et al., 2000; Oehlmann et al., 2007; Sousa et al., 2007b;

Rodríguez et al., 2009). Most importantly, the data is used here to: (i) evaluate the degree of sediment contamination and verify if they comply with international environmental quality standards (OSPAR and WFD), (ii) analyse if there are fresh TBT inputs to the environment and (iii) establish temporal trends of pollution by comparing recent and past levels of contamination.

Concentration values of TBT ranged from 0.41 to 66.17 (ng Sn/g dw), which are much higher than the upper EAC (Ecotoxicological Assessment Criteria) proposed by OSPAR (0.02 ng Sn/g dw), i. e., concentration for above which there is a concern that negative impacts might be observed in marine organisms (OSPAR, 2004). The obtained values were also higher than the EQS (Environmental Quality Standard), value derived for TBT concentration in sediments proposed by Water Framework Directive (0.008 ng Sn/g dw), i.e. the maximum acceptable concentration of this contaminant in sediment sample (SCTEE, 2005).

TBT recent inputs may be reflected by the higher proportion of TBT over its metabolites or slow degradation rates typical from sediments (Sarradin et al., 1995; Hoch, 2001). Only at PPL it was possible to quantify all butyltin species (TBT, DBT and MBT); in this station TBT represented 69% of total butyltins (ΣBTs) which means that recent contamination might have happened. DBT and MBT represent, respectively, 18 and 13% of ΣBTs

Based on other published works, namely the one published by Sousa and co-workers (2007b), it is possible to establish temporal trends of pollution comparing recent and past levels of contamination. Figure 2-214 shows a clear decrease of TBT concentration in sediments in the sampling stations between 2005 and 2009. PPL and Muranzel show the major values decline while Magalhães Mira shows a minor decline.

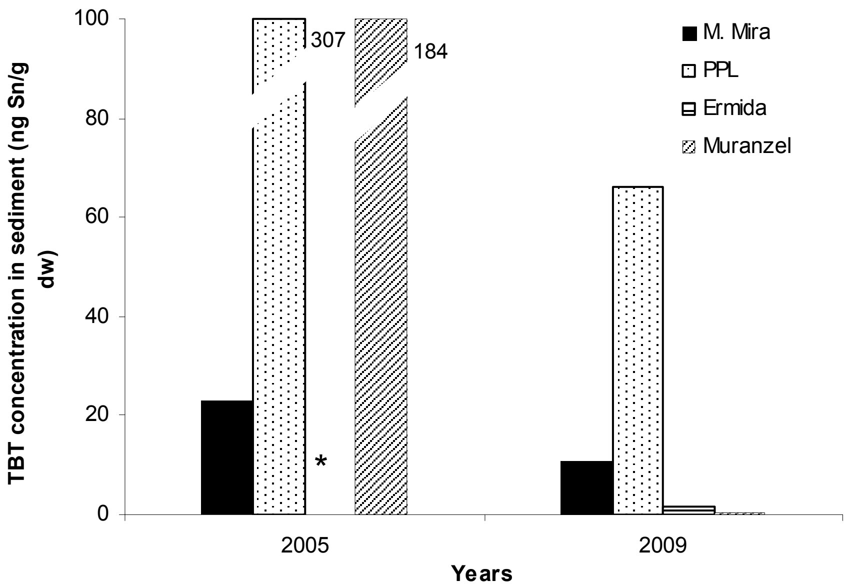

Figure 2-13 TBT concentration (ng Sn/g dw) in sediments collected in Ria de Aveiro in two distinct years: 2005 and 2009. * no data available

These results show that, despite a great decline of TBT levels over the last years, the present concentrations are still much higher than the ones set by OSPAR (EAC) and Water Framework Directive (EQS). Also the results demonstrate possible recent inputs in at least one sampling station. Therefore, these results show that in the present TBT pollution is still a matter of great concern.

Table 2-6 summarizes the advantages and disadvantages of organotin sediment analysis for monitoring TBT pollution.

Table 2-6 Summary of the advantages and disadvantages of chemical monitoring.

	Advantages	**Disadvantages**
Sediment Chemical monitoring	• Provide up to date organotin concentrations in a given place. • Permit to infer on recent TBT inputs based on its degradation process. • Verify legislation compliance	• Don't present information on TBT bioavailability. • Don't provide direct information of effects on biota.

2.4.3. Laboratory bioassays

The bioassays endpoint was the development of imposex in females of gastropods when exposed to sediments. Duft and co workers (2007a) exposed *Nassarius reticulatus* during 30 days to sediments sampled along River Elbe using the VDS as endpoint. Contrary to what happened with sediments of Ria de Aveiro in the current work, they obtained an increase in VDSI in all exposures to sediments from sampled stations, comparing to artificial sediment (control). For Ria de Aveiro, an estuarine area with lower levels of TBT contamination in superficial sediments, the measurement of female penis length (FPL) seems to be a better approach for the assessment of TBT activity. Also FPL seems to be the imposex index to have a faster response for TBT variation levels (Sousa et al., 2007a), as observed with field data (see figure Figure 2-9). Sediments from Nazaré fishing harbour induce a highly penis growth due to high concentration of TBT in this sediment. Sousa reported in 2008 (unpublished data) a TBT concentration of 469.4 ng Sn/g dw. These results show that this bioassay is an effective tool for the assessment of TBT levels and bioavailability in sediments.

The imposex development depends on the timing, duration and level of exposure of the animals to TBT. The imposex level observed in the adult represents a dose-dependent and irreversible response of the total TBT integrated throughout its life, especially during the juvenile stage when the genital tract is in formation (Gibbs and Bryan, 1994). In laboratory bioassays duration of exposure could play an important role in penis growth for low polluted stations where more time would be needed for the animals to accumulate enough TBT into its tissues, whereas for highly polluted sites imposex development would be faster. As an example, for Nazaré sediments, only 45 days were enough for a clear response in terms of penis growth, contrary to what happened with the sediments from Ria de Aveiro that gave a little response in 56 days. Perhaps if duration of exposure would be higher maybe larger penis growth could had been observed in Ria de Aveiro sediment bioassays. A standardization of time of exposure would be very important to set a threshold for the bioassay and to allow the appearance of a rank system for sediment classification in agreement with bioassays results.

One of the major problems concerning chemical monitoring is that it provides information about total concentration of contaminants, however it is impossible to know how much proportion is available for organisms. TBT bioavailability in sediments is assessed by means of bioassays. Sediment from Ermida presents lower concentration of TBT than the one from Magalhães Mira, but the growth of the penis was slightly higher in Ermida than in this last station (although it was not significant); this could be explained by bioavailability of TBT in sediments. Diverse factors can affect this, such as pH and organic matter, which are considered the most important ones. Bioavailability is higher in sediments with neutral or basic pH and with low levels of organic matter (Fent, 1996). The measured organic matter content in sediments shows that Ermida have higher content than Magalhães Mira (Table 2-3), which do not explain the results; however some other parameters not assessed could explain these results.

Also, manipulation of sediment can change some of their properties and their toxicity as well (Anderson et al., 2001).

The *Hydrobia ulvae* bioassay didn't accomplished the objectives for what it was purposed. The purpose of the bioassay was to expose juveniles with no imposex, which corresponds to shell height class of 2-2.5 mm based on Galante-Oliveira and co-workers (Galante-Oliveira et al.), and after 3 months observe the imposex levels in these fast growing animals. However, organisms did not grow enough in order to consent imposex assessment. Results from work on imposex in *Hydrobia ulvae* made in Ria de Aveiro by Galante-Oliveira and co-workers shows that the development of imposex is clearly connected with animal growth. Other results from Mondego estuary, nearby Ria de Aveiro, showed seasonal growth rates, almost ceasing in winter due to reduced temperature and absence of algae as food supply (Cardoso et al., 2002). In laboratory, with controlled conditions the unique explanation for the low growth rates is the absence of food supply in the form of algae, as it seems clear that animals did not graze sediment particles.

Further work has to be done for an improvement of this bioassay because of the ecological importance of *Hydrobia ulvae* in estuarine systems and because it is predominantly a deposit feeder which means that is in direct contact with sediment.

Table 2-7 presents an overview of the performed bioassays and their role in TBT pollution monitoring with their advantages and disadvantages.

Table 2-7 Summary of the advantages and disadvantages of sediment bioassays using *Nassarius reticulatus* **and** *Hydrobia ulvae* **.**

	Laboratory bioassays	
Advantages	• Low cost information. • Information on levels and bioavailability of TBT existing in sediment. • Allow the use of sediments from stations where there are no available organisms for imposex survey.	
Disadvantages	• Duration of exposure can affect results.	
	Species	
	Nassarius reticulatus	***Hydrobia ulvae***
Advantages	• Larger animals: provide easy imposex assessment	• Animals resistant to manipulation and experimental conditions Ingest sediment particles and may accumulate more TBT.
Disadvantages	• Animals less resistant to manipulation and experimental conditions	• Smaller animals: do not allow easy imposex assessment •

2.5. General Conclusion

The purpose of this work was to validate the combined use of several methodologies as a more accurate approach for the integrative assessment of TBT pollution in estuarine areas. Generally, biomonitoring of TBT pollution only refers to chemical analyses and imposex surveys. The addition of sediments bioassays allows a better knowledge on actual status of the sediment as well as TBT bioavailability. The combination of these three parameters is essential to provide an integrative assessment of TBT contamination giving an historical perspective as well as the actual status of pollution. In this case, the integrative assessment was directed to a particular contaminant, TBT. This compound is expected to occur in estuarine areas with intense harbour activities that are common along the European coast. The specific bioassay and field surveys here presented is complemented with the results regarding physical and chemical characterisation of sediments.

Using Ria de Aveiro as study area, the results presented are only demonstrative of the usefulness of an integrated approach for TBT pollution monitoring. Only in Magalhães Mira a comparison between the three monitoring components was possible. Although one of the advantages of bioassays is to allow that at least some of the components are used for some sampling sites (e.g., there are sites with no available TBT indicator species and where biomonitoring is impossible to implement), more stations with the possibility of integrating imposex & chemical surveys and laboratory bioassays should be included in monitoring programmes.

In Magalhães Mira station it's possible to conclude that despite presenting the highest levels of imposex in the present, the sediments don't show a significant androgenic activity and TBT concentration is only moderate (around 11 ng Sn/g dw.). Past contamination explains levels of imposex that, as shown in Figure 2-9 and Figure 2-10, are decreasing over the last years. In general, TBT pollution in Ria de Aveiro is decreasing since 2003 after the implementation of EC Regulation 782/2003 as shown by the evolution of imposex and TBT concentration in sediments, and, at the present time, sediments from Ria de Aveiro have relatively low androgenic activity exposure, when compared to high polluted sediments from Nazaré fishing port.

3. Complementary assessment of general sediment toxicity

3.1. Introduction

The ship traffic, diverse industries, agriculture run-off, domestic sewage discharges among other threats release into the estuarine areas a variety of contaminants. The integrative assessment of the pollution has been for several years accepted as the most effective way to predict the environment toxicity (Chapman and Long, 1983; Matthiessen et al., 1998; Bellas et al., 2008). As part of biological parameters, laboratory bioassays constitute an important step in the assessment of marine environment quality, providing an integrated measure of toxicity, and becoming widely used in monitoring programmes, dredging studies, and other regulatory activities. Low cost information given by the test organisms on the quality of the environment will complement the information given by chemical analyses (Long et al., 1996; Beiras et al., 2003b).

Potamopyrgus antipodarum has been recently used in bioassays to assess endocrine disruption compounds present in environmental sediments (Duft et al., 2003a; Oehlmann et al., 2007; Mazurová et al., 2008). The endpoint of this bioassay is the embryos production as a response of exposure to possible endocrine disruptor compounds (Duft et al., 2003a; Duft et al., 2003b). An increase in embryo production has been used in laboratory assays as an indication of estrogenic compounds present in sediments, whereas a decrease indicates the presence of androgenic or other toxic compounds (Duft et al., 2007a). When testing field sediments it has to be considered possible effects of mixtures of compounds that may affect fecundity.

The technique of elutriation has made possible to use water-column organisms, such as early developmental stages of marine invertebrates, in sediment toxicity bioassays (Long et al., 1996). Some authors demonstrate that these water-phase bioassays were found to be more sensitive than solid-phase as for example the amphipods sediment bioassay (Long et al., 1990; Carr et al., 1996). One of the marine invertebrates larvae mainly used in sediment elutriates bioassay is sea urchin (Long et al., 1996; Geffard et al., 2001; Beiras et al., 2003a; Bellas et al., 2008). Generally, in these bioassays the endpoint tested is the percentage of normal larvae but Beiras (2002) in his work stated that recording the larval length could improve the sensitivity and discriminating power of the bioassay.

The purpose of this work is to complement the integrative assessment of TBT pollution with general toxicity bioassays to evaluate sediment quality. Chronic exposures to sediment with *Potamopyrgus antipodarum* and acute toxicity bioassays with *Paracentrotus lividus* larvae exposed to sediment elutriate were performed in order to achieve that objective. However, it should be noticed, that the major goal of this chapter is to explore the implementation of these bioassays in the laboratory for the first time. This means that it is pretended to achieved information regarding how species are easily collected from the field, how they adapt to manipulation and laboratory conditions and how do they respond to sediment bioassays. These preliminary results will serve as a basis for the development of protocols to be implemented in future routine monitoring programmes.

3.2. Methods

3.2.1. **Sediment laboratory bioassays with *Potamopyrgus antipodarum***

Potamopyrgus antipodarum were captured in Rio Minho near Valença, Portugal, following the location described by Sousa and co-workers (2007c) in their published work Animals were brought to laboratory and kept in aquaria with tap water and under constant aeration.

Two exposures were performed with some differences in their procedure and separated in time. In the first one, individuals of *Potamopyrgus antipodarum* were collected from the field in October 2008 and kept in laboratory, under controlled conditions (18±1ºC; photoperiod of 16:8h (light:dark), for 4 months in aerated tap water with natural food supply (algae) ad libitum. After 4 months they were randomly selected to the bioassay. The organisms were exposed to sediments from two stations in Ria de Aveiro: Ermida and Porto de Pesca Longíquo (PPL). The exposure was performed in overlapped round containers as described previously in chapter 2.2.3.2. After one day of sediment settling, 10 individuals of *Potamopyrgus antipodarum* larger than 3.5 mm of shell heigh were added to each container. Size selection took in consideration sexual maturity, which is accomplished between 3.0-3.5 mm (Alonso and Castro-Díez, 2008). After 2 months of exposure animals were measured and removed from the shell. Shells were cracked open with a bench vice and shell parts were removed. The brood pouch of the animals was gently opened and embryos were removed. The experiment was performed at a temperature of 18±1ºC and a photoperiod of 16:8h (light:dark). The procedure of the sediment bioassay with parthenogenic females was originally developed by Duft and co-workers (2003b) and some of the proposed guidelines were followed. Statistical analyses were performed with SPSS 17.0 software. Differences between treatments were evaluated with one-way ANOVA.

In the second exposure, the animals used were collected one week before the beginning of the experiment, in the same place as described before and they were brought to laboratory and acclimatized in 20 L aquaria with aerated tap water (18±1ºC; photoperiod of 16:8h (light:dark). To the sediments stations already studied, two more were assessed: Muranzel and Magalhães Mira. The time of exposure was changed to one month. Statistical analysis performed were the same as described before.

3.2.2. *Paracentrotus lividus* larval bioassay with sediment elutriates.

The toxicity of sediment was evaluated by elutriates bioassays with embryos of *Paracentrotus lividus*. Elutriates were obtained by rotatory mixing of 100 g wet weight (ww) of sediment with 500 mL of artificial seawater for 30 minutes in closed flasks, and overnight decantation at 20 ºC. Elutriates were siphoned into a beaker and gentle aerated for 10 minutes to avoid irrelevant toxicity potentially caused by H_2S (Beiras, 2002). After this procedure, salinity, temperature and pH were recorded in each elutriate sample. Elutriates were tested undiluted (200g/L) and then diluted with the same volume of ASW (corresponding to 100 g/L) and with the double volume of ASW (corresponding to 50 g/L).

Paracentrotus lividus gametes were obtained by dissection of adults and observed on a stereomicroscope in order to check their quality (round eggs and motile sperm). In a small beaker, the eggs and the sperm obtained were added to 50 ml of filtered ASW and were gentile stirred to allow the fertilization. The percentage of fertilization (% of eggs showing a membrane of fertilization) and number of eggs per ml were determined. The suspension with fertilized eggs was added to vials containing 4 ml of elutriate solution in order to obtain a density of 40 eggs per ml. Control was performed with artificial seawater An extra series of control were fixed with few drops of 38% formalin after the fertilization. Four replicates were made per treatment. After 48-h of incubation at 20 ºC, the larvae were fixed adding a couple of drops of 38% formalin to the vials. Mean larval length in the different treatments was used as endpoint. The effects of the sediment elutriates on larval length were tested for significance with one way ANOVA ($p<0.05$). When differences among groups were significant each experimental group was compared to the control by using the Dunnett's test.

3.3. Results

3.3.1. Sediment laboratory bioassays with *Potamopyrgus antipodarum*

Results from the first sediment bioassay are presented in Figure 3-1. Mortality in this test ranged from 0% to 30% between replicates; higher levels of mortality were registered in PPL with two replicates reaching 30% of mortality. The average number of embryos produced by females in each sediment was statistically significant (p<0.05).

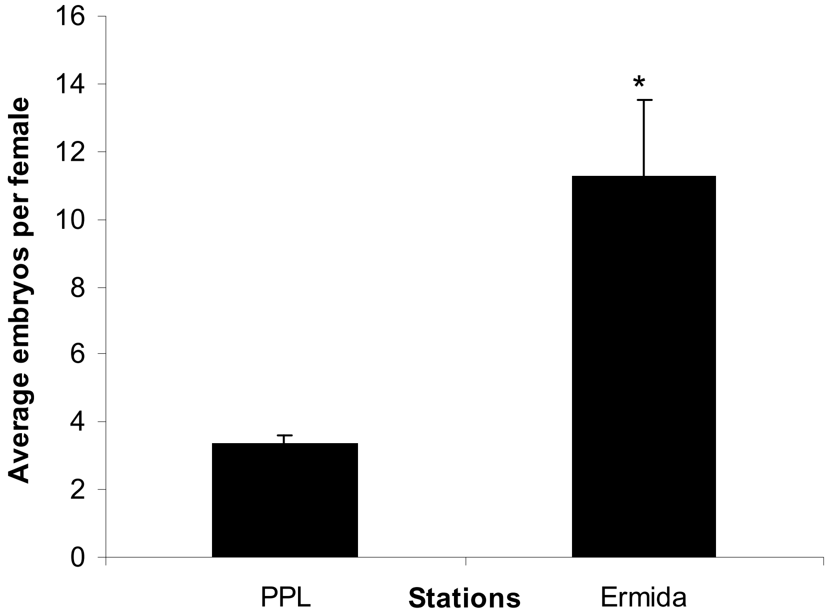

Figure 3-1 Average number of embryos produced by females of Potamopyrgus antipodarum exposed for 56 days to different sediments collected in Ria de Aveiro. Statistical differences are represented by * (p<0,05).

Results from the second bioassay are present in Figure 3-2. Mortality was very low reaching 10 % in some replicates. In this case, there were no differences between the average numbers of embryos produced by females in the different sediment. Surprisingly, PPL sediments induced a higher embryo production, in average 5 embryos more than the other stations, which contrast with the results of the first bioassay.

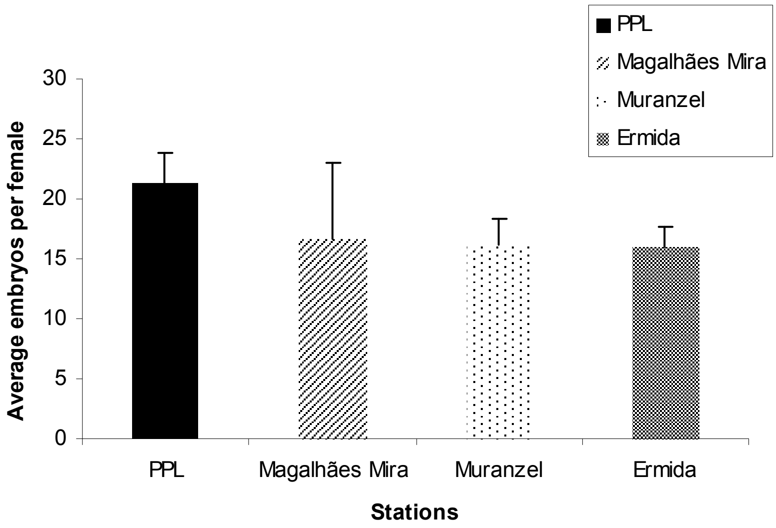

Figure 3-2 – Average number of embryos produced by females Potamopyrgus antipodarum exposed for 28 days to different sediments collected in Ria de Aveiro. No statistical differences were observed (*p*<0.05).

3.3.2. *Paracentrotus lividus* larval bioassay with sediment elutriates

Figure 3-3 shows larval growth results from elutriate bioassays. In general, sediments from Ria de Aveiro showed low toxicity to *Paracentrotus lividus* larvae. In comparison to control, undiluted elutriates from Magalhães-Mira and Muranzel caused a significant decrease in larval growth (*p*<0.05). The two times diluted elutriate from Muranzel also caused a significant similar effect.

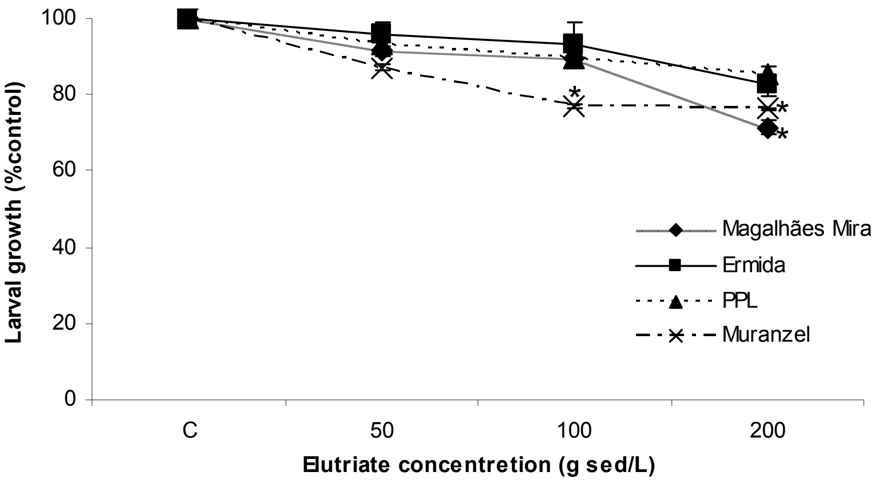

Figure 3-3 *Paracentrotus lividus* larval growth after 48 h exposure of fertilised eggs to different dilutions of elutriates from sediments sampled at Ria de Aveiro. Error bars represent the standard deviations; * Significant differences at *p*<0.05.

3.4. Discussion

3.4.1. Sediment laboratory bioassays with *Potamopyrgus antipodarum*

Production of embryos suffered a great variation for sediments of PPL between the two bioassays. The first bioassay presented low production of embryos for this sediment (around 3 per female) while in the second the highest levels of embryo production were recorded for this station (around 21 per female). Major differences occurred between the two bioassays that can explain the contrasting results. One is the fact that for the first experiment the animals were acclimatized for a long period (4 months) whilst in the second case they were collected only one week before the beginning of the experiment. On the other hand, possible seasonality in the reproductive cycle of the species may have caused strong variations on fecundity. Moreover, sediments were collected at different times (4 months of difference) which mean that they could present different chemical/physical conditions.

Mazurová and co-workers (2008) in their exposures to sediment with *Potamopyrgus antipodarum* reported high variability even within individuals among the same exposure replica or in control. This was also observed in these experiments. Although good results were observed in laboratory experiments with spike of single contaminants (Duft et al., 2003a; Duft et al., 2003b; Gust et al., 2009; Pedersen et al., 2009), when animals are exposed to environmental sediments results seem to be variable and difficult to interpret due to a complex matrix that could comprehend several types of contaminants (Duft et al., 2007a; Mazurová et al., 2008). Duft and co-workers (2007a) recommended that, in exposures to environmental sediments, an increasing in embryo production could be related to the presence of compounds with some estrogenic activity. However a phenomenon of hormesis, an increase in embryo production at low concentration of contaminant, was registered by Gust and co-workers (2009) when exposed these organisms to fluoxetine. This means that also conclusions on the presence of endocrine disruptor compounds could be precipitated. On the other hand, a decreasing in embryo production could be caused by androgens or other compounds with reproductive toxicity such as metals or PAHs (Duft et al., 2007a).

Taking into consideration our preliminary results and the literature revision, *Potamopyrgus antipodarum* should probably be used only as a test species for laboratory assays with spiked contaminants. The use of this species to assess global environmental sediment pollution could lead to erroneous conclusions.

In Table 3-1 is presented a summary of this bioassay, advantages, disadvantages and recommendations.

Table 3-1 Overview on *Potamopyrgus antipodarum* bioassays with collected sediments.

	***Potamopyrgus antipodarum* bioassay**
Advantages	• Low cost information. • Response to endocrine disruptors compounds, yet is advised only to use in assays with single contaminants
Disadvantages	• Difficult to distinguish androgenic effects from general reproductive toxicity when testing field sediments

3.4.2. *Paracentrotus lividus* larval bioassay with sediment elutriates

The larval *Paracentrotus lividus* bioassay with sediment elutriates presented in this work has the main purpose to give a general idea of the acute toxicity caused by sediments from Ria de Aveiro to the development of sea urchin larvae as a proxy to estimate general toxicity to biota. Results reveal low levels of toxicity when compared to results with the same species in other estuarine areas (Beiras et al., 2003a; Beiras et al., 2003b; Bellas et al., 2008) where sediments totally inhibit larval development. In Table 3-2 is shown an overview of the presented bioassay.

Table 3-2 Overview on larval bioassays with sediment elutriates

	***Paracentrotus lividus* bioassay**
Advantages	• Low cost information. • Elutriates allow to estimate possible effects of remobilisation of contaminants from the sediments into the water column. • Elutriates allow testing organisms widely used in water bioassays for sediment monitoring • Elutriates allow testing a life stage sensitive to a wide range of contaminants.
Disadvantages	• Non specific response to chemical contaminants but to the natural sediment composition itself. • High artificial approach

3.5. General conclusions

This work is a complementary approach to the integrative assessment to TBT pollution in Ria de Aveiro. These assays intended to demonstrate global toxic effects of sediments to the organisms under study, using acute and chronic exposures. Acute toxicity bioassay with *Paracentrotus lividus* shows that sediments from Magalhães-Mira and Muranzel are the ones that cause more effects in larval normal growth. On the other hand, sediment from PPL showed little effect on larval growth. A comparison to *Potampoyrgus antipodarum* bioassay should not be made because divergent results were obtained in this particular case.

4. Conclusion

In this work was explored different methodologies in order to perform an integrative assessment of TBT pollution in Ria de Aveiro. Three main components – field biomonitoring, chemical monitoring and laboratory bioassays - are for the first time applied together and their advantages and disadvantages are discussed along this work. Table 4.1 shows several examples of possible results that can occur using this approach and their interpretation. Also results for the sampling station Magalhães Mira are presented and interpreted, as this was the only site in Ria de Aveiro for which all the methodologies were implemented.

Table 4.1 Summary of possible results and their interpretation. Levels of imposex, TBT sediment concentration and toxicity of sediments are described qualitatively by: 0 – none; + – low; ++ – moderate; +++ – high. Production of embryos: (<) - decrease; > - increase.

	Imposex Survey	Sed. TBT concentration	N. reticulatus bioassay	P. antipodarum bioassay	P. lividus bioassay	Interpretation
Example 1	+++	+++	+++	+++ (<)	+++	Sediment with a high androgenic activity, at the present time, showed by high penis growth. Acute toxicity and low embryo production could be caused by other contaminants.
Example 2	+++	++	+	0	++	Sediment with a history of high levels of TBT contamination, but in recovery given by low penis growth. Nevertheless some moderate acute toxicity was registered.
Example 3	0	0	0	+++ (>)	++	Sediment with hypothetic estrogenic activity that causes a high production of embryos by *P. antipodarum*. Acute toxicity could be caused by other contaminants
Magalhães Mira	++	+	0	0	+	Sediment contaminated by TBT in the past, considering moderate levels of imposex, but in recovery due to low penis growth. Low acute toxicity was registered.

Example 1 of Table 4.1. refers to a sampling station where imposex levels are high and, consequently, high levels of TBT pollution occurred or still occur at this site. Accordingly, TBT concentration in the sediments is also high. *Nassarius reticulatus* bioassay indicates that the sediment has a great androgenic potential due to the presence of TBT that is bioavailable, which is corroborated by the decrease of embryo production in the *Potamopyrgus antipodarum*. The high acute toxicity of sediment elutriate to sea urchin larvae obtained in the respective bioassay, indicates that TBT or other toxic contaminants are present. A summary of this interpretation is found in the right column of Table 4.1. The overall conclusion is that this site is presently highly polluted and of high concern, requiring urgent management by competent authorities.

If, in an hypothetical scenario, this station would be subjected to dredging, the disposal site should be very carefully chosen. The examples 2 and 3 provided in Table 4.1 have an adequate corresponding interpretation in the right column of the table.

In the case of Magalhães-Mira, past contamination by TBT is evident considering the moderate levels of imposex, but presently the TBT levels in sediments are low and its androgenic potential is very reduced or null. On the other hand, the low acute toxicity to sea urchin larvae indicates that this is a site that probably presents low concern to local authorities. However, *Nassarius reticulatus* imposex levels (VDSI=2.2) and TBT concentration in sediments (10.9 ng Sn/g dw) are well above environmental quality standards and must be reduced in order to comply with international ecological objectives for the near future.

Globally the results obtained in the current work show that, in terms of TBT pollution, Ria de Aveiro is an estuarine area in fast recovery since 2003, presently showing low levels of imposex and low to moderate TBT contamination of sediments. Nevertheless, all of these parameters are well above the ecological objectives settled by the European Union and OSPAR commission. On the other hand, sediments of the selected sites exhibit low potential to induce the growth of the penis in females of the gastropod *Nassarius reticulatus* and low toxicity to sea urchin larvae.

In conclusion, the integrative assessment of TBT pollution in Ria de Aveiro combining field biomonitoring, chemical monitoring and laboratory bioassays seems to be effective towards a better understanding of the ecological quality of this estuarine system regarding this xenobiotic compound. This works constitutes the first preliminary approach towards this strategy and so there are many methodological aspects that need to be improved. For instance, species used in bioassays should be cultured in the laboratory for at least one generation to provide more consistent and comparable results. This is mostly important in the case of *Potamopyrgus antipodarum* as the results varied considerably perhaps derived from the different conditions this species was used in the bioassays; however, due to its resistance and easy culture in laboratory this gastropod presents good potential as a test species and its usefulness for this kind of studies should be better studied in the future using more reliable and consistent experimental conditions. Also for its ecological relevance, the bioassay with *Hydrobia ulvae* should be improved. Results pointed out that the organisms did not grow without algae food supplement and this should be corrected in forthcoming experimental designs.

However, the association of *Nassarius reticulatus* bioassay with the imposex surveys and chemical monitoring showed satisfactory results. As evidenced in this work TBT concentrations in the environment are still above the quality standards, being a matter of concern. Therefore, implementation of the integrative assessment proposed in this work, combining laboratory bioassays and field monitoring, in near future could be an important improvement for a better knowledge on the quality of sediment regarding TBT pollution.

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