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# Combination of Field Monitoring and Laboratory Bioassays for the Assessment of TBT Pollution in Ria de Aveiro

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**Abstract**—Despite the use of tributyltin (TBT) antifouling paints had been under regulatory restrictions in Portugal since the 90's, the persistence of this compound in sediments may cause a slow decline in pollution levels over time. In this work, an historical perspective of TBT pollution in Ria de Aveiro is given by both imposex surveys as well as chemical monitoring of TBT in sediments. Additionally, a laboratory bioassay was performed for the assessment of the bioavailability of TBT in sediments: the gastropod *Nassarius reticulatus* was exposed to several sediments collected in Ria de Aveiro and imposex development was used as endpoint. Results of imposex levels and chemical analysis show that TBT pollution decreased in Ria de Aveiro over the last years due to the European Union (EU) ban of TBT antifouling paints in 2003 but TBT levels in the environment are above the quality standards, being still a matter of concern. The laboratory bioassay performed in the current work proved to be an effective tool for the assessment of TBT pollution and should be regarded as a valid option for monitoring programmes in near future.

Keywords: TBT, imposex, sediment, *Nassarius reticulatus*, *Nucella lapillus*

## INTRODUCTION

Tributyltin (TBT) is an organotin (OT) compound that was widely used as biocide in antifouling (AF) paints. Although very effective against biofouling it is also highly toxic for non target species. One of its most well known deleterious effects is imposex, which is considered as the best documented example of endocrine disruption in wildlife (Matthiessen and Gibbs, 1998) and is used worldwide as biomarker of TBT pollution. Imposex is defined as the superimposition of male characters, such as penis and vas deferens, in female gastropods (Smith, 1971) and has already been described in more than 200 species (Shi *et al*., 2005). As a consequence of the deleterious effects in wildlife reported throughout the world (Fent, 1996) several restrictions on its usage started to be implemented, and in Europe, the use of TBT AF paints on boats smaller than 25m in length was banned

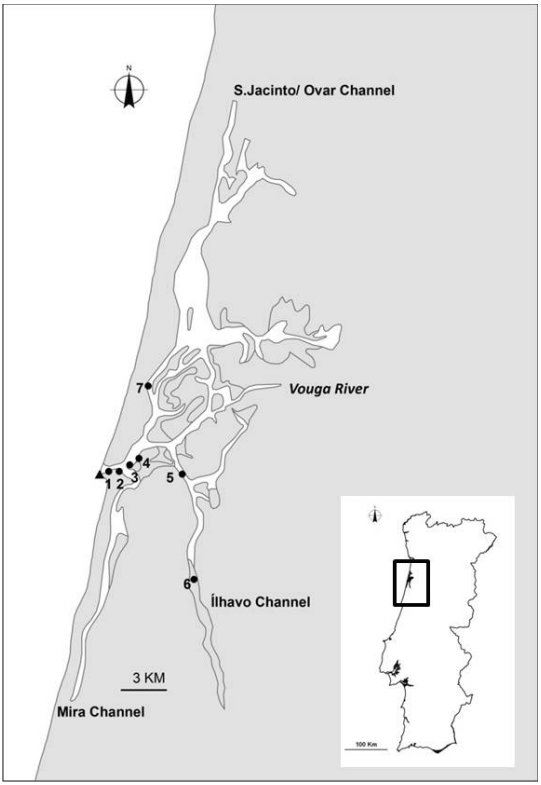


Fig. 1. Ria de Aveiro. Map showing the sampling locations: 1 - Barra; 2 - Forte da Barra, 3 - Magalhães Mira; 4 - Porto Comercial Norte; 5 - Porto de Pesca Longínqua (PPL); 6 - Ermida; 7 - Muranzel. *N. reticulatus* specimens were collected at St. 1-4, *N. lapillus* at St. 1-2. The sediments for the chemical analysis were collected at St. 3, St. 5, St. 6 and St. 7 while for the bioassays were collected at St. 3, St. 5 and St. 6. The animals used in the bioassays were collected at Praia do Meio Laranjo marked with a triangle in the map.

trough the Directive 89/677/EEC. However, this partial ban was not effective in reducing TBT environmental levels (Minchin *et al*., 1995; Morgan *et al*., 1998; Barroso and Moreira, 2002; Santos *et al*., 2002). Therefore, in 2001, the International Maritime Organization (IMO) formulated the AFS convention that totally bans the application of OTs AF paints in all vessels worldwide. This convention entered into force in September 17th 2008. Earlier, since July 1st 2003, the European Union (EU) banned the application of OTs AF paints in all EU ships and since January 1st 2008 its presence in all vessels (EC Regulation 782/2003). As a result, recent imposex surveys in the Portuguese coast revealed that levels are clearly decreasing (Galante-Oliveira *et al*., 2009; Rato *et al*., 2009; Sousa *et al*., 2009a).

Ria de Aveiro is a shallow estuarine system located in the NW of Portugal

characterized by the presence of numerous industrial activities and naval facilities. The main potential sources of TBT are ports, marinas and dockyards that are located along the three main navigation channels (Fig. 1). In this area, high levels of TBT pollution were found until 2003 and since then a gradual decreasing trend has been observed (Barroso *et al*., 2000; Galante-Oliveira *et al*., 2009; Sousa *et al*., 2009a).

In this work, TBT levels in 2009 were assessed in sediments and imposex levels surveyed using two different species. The obtained results are compared to the ones previously published in order to track the temporal trends of TBT pollution in this estuarine system since 1998. Furthermore, laboratory bioassays using sediments collected in 2009 were performed in order to provide information on the bioavailability of TBT at the present time.

## MATERIALS AND METHODS

### *Imposex field survey*

Imposex levels were assessed in two different species: the netted whelk *Nassarius reticulatus* and the dogwhelk *Nucella lapillus* both collected at Ria de Aveiro, during low tides in August 2009. *N. reticulatus* specimens (about 60 to 80 adults) were collected by hand or with baited hoop nets at four locations in Ria de Aveiro: Barra (St. 1), Forte da Barra (St. 2), Magalhães Mira (St. 3) and Porto Comercial Norte (St. 4) (Fig. 1). Prior to imposex analysis, the animals were narcotized with magnesium chloride 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. Parasitized specimens were discarded from the analysis. The percentage of females affected by imposex (%I), mean female penis length (FPL), mean male penis length (MPL), the relative penis length index (RPLI = FPL × 100/MPL), the vas deferens sequence index (VDSI) and the average oviduct stage (AOS) values were determined for each location. The VDS was classified according to the scoring system developed by Stroben *et al*. (1992) with minor alterations proposed by Barroso *et al*. (2002a). The AOS was classified according to the scheme proposed by Barreiro *et al*. (2001).

*N. lapillus* specimens (about 60 adults) were collected by hand at two sampling stations in Ria de Aveiro (Fig. 1): Barra (St. 1) and Forte da Barra (St. 2) (Fig. 1). The imposex analysis followed the same protocol described for *N. reticulatus* except that the animals were not narcotized and a different index was assessed: the relative penis size index ($RPSI = FPL^3 \times 100/MPL^3$) instead of RPLI. The VDS was classified according to the scoring system developed by Gibbs and co-workers (1987).

### *Chemical analysis*

Surface sediments (≈2 cm depth) were collected at Ria de Aveiro (Fig. 1) in Magalhães Mira (St. 3), Porto de Pesca Longínqua (St. 5), Ermida (St. 6) and

Muranzel (St. 7) in March 2009. Sediments were collected during low tide by hand with a spatula. Three replicates of surface sediments were collected per sampling site, each replicate was taken from a square area of 0.04 $m^2$; the location of the three replicates corresponded to the vertices of an isosceles triangle with 1m side. The sediments were brought refrigerated to the laboratory and the three replicates were homogenised and then stored in dark conditions at 4°C. About 100 g of sediment was freeze dried and preserved at –20°C for chemical analysis. The organic matter content was obtained by measuring the stable loss of weight of the dried sediment sample at 450°C. The percentage of fine fraction was obtained after wet sieving the sediment samples in a 64 um mesh.

Butyltins (monobutyltin (MBT), dibutyltin (DBT), TBT), phenyltins (monophenyltin (MPT), diphenyltin (DPT), triphenyltin (TPT)) and octyltins (monooctyltin (MOT), dioctyltin (DOT)) were measured by GC-MS (gas chromatography coupled to mass spectrometry) and quantified by isotope dilution method following the method described by Sousa *et al.* (2007a). Average recoveries rates were: 57 ± 18.7, 79 ± 13.0, 83 ± 7.5, 80 ± 14.0, 101 ± 14.0, 214 ± 31.8, 83 ± 7.5 and 147 ± 17.3 for MBT, DBT, TBT, MPT, DPT, TPT, MOT and DOT. To assess the QA/QC of the measurements the certified sediment reference material (NIES CRM No. 12) was analysed by the same method. The obtained result from three replicates (0.19 ± 0.001 ug/g TBT) shows a good agreement with the certified one (0.19 ± 0.001 ug/g TBT).

*Laboratory bioassays*

*N. reticulatus* specimens were collected with baited hoop nets in Ria de Aveiro at Praia do Meio Laranjo (Fig. 1), a location close to the mouth of the estuarine system with low levels of TBT (Barroso, unpublished data). The animals were brought to the laboratory refrigerated and then narcotized with magnesium chloride ($MgCl_2$ 70 g/L in distilled water) in order to allow sex identification. Only females with penis with less than 1 mm were selected. The females were allowed to recover from narcotization and were maintained in artificial seawater (salinity: 33‰) (Crystal Sea® Bioassay Formulation, Marine Enterprises International) for one week prior to the beginning of the exposures and then placed into the experimental chambers (1 L glass flasks). For each treatment three replicates (i.e., 3 flasks) with 10 animals each were used. The bioassays were performed with 2–3 cm layer of total sediment and a volume of artificial seawater that filled the flask. Before the bioassay began the sediment was left to settle for one day. The bioassay was performed at 18 ± 1°C under constant aeration and weekly water renewal. The animals were fed with mussels collected from a low polluted location (TBT = 3.1 ng TBT-Sn/g dry weight (Sousa *et al.*, 2009b)) once a week. The total exposure time was 54 days. The sediments used in the bioassay were collected in June 2009, following the method described in the previous section, at St. 3, St. 5 and St. 6 (Fig. 1). Artificial seawater with no sediment added was used as a negative control.

At the end of the experiment the vas deferens sequence index (VDSI) and

Table 1. *Nassarius reticulatus* imposex levels in Ria de Aveiro (August 2009): number of males (N♂), shell height (SH), male penis length in mm (MPL), number of females (N♀), mean female penis length in mm (FPL), relative penis length index (RPLI), vas deferens sequence index (VDSI), average oviduct stage (AOS) and percentage of females affected by imposex (%I).

| Station code and name | N♂ | ♂SH | MPL | N♀ | ♀SH | FPL | RPLI | VDSI | AOS | %I |
|---|---|---|---|---|---|---|---|---|---|---|
| St. 1: Barra | 26 | 23.7 ± 2.4 | 11.9 ± 1.1 | 30 | 25.4 ± 2.0 | 0.4 ± 0.8 | 3.4 | 1.2 ± 1.4 | 0 | 50 |
| St. 2: 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 | 57 |
| St. 3: 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 |
| St. 4: Porto Com. 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 |

Table 2. *Nucella lapillus* imposex levels in Ria de Aveiro (August 2009): number of males (N♂), shell height (SH), mean male penis length in mm (MPL), number of females (N♀), mean female penis length in mm (FPL), relative penis size index (RPSI), vas deferens sequence index (VDSI) and percentage of females affected by imposex (%I).

| Station code and name | N♂ | ♂SH | MPL | N♀ | ♀SH | FPL | RPSI | VDSI | %I |
|---|---|---|---|---|---|---|---|---|---|
| St. 1: Barra | 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 |
| St. 2: 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 |

Table 3. Organic matter content (%OM), percentage of sediment particle size lower than 63 μm (%<63 um) and organotin concentrations (ng Sn/g dry weight) in the studied locations.

| Station code and name | St. 3: M. Mira | St. 4: PPL | St. 6: Ermida | St. 7: Muranzel |
|---|---|---|---|---|
| %OM | 1.3 | 6.1 | 5.4 | 6.0 |
| %<63 μm | 1.5 | 14.0 | 7.6 | 10.0 |
| MBT | <6.5 | 13 | <6.5 | <6.5 |
| DBT | 3.1 | 17 | 2.1 | 0.6 |
| TBT | 11 | 66 | 1.6 | 0.4 |
| 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 | 0.3 | <3.0 | <3.0 |

female penis length (FPL) were assessed. 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. The critical significance level adopted was 0.05.

## RESULTS

### *Imposex field survey*

Imposex levels in Ria de Aveiro are shown in Tables 1 and 2. For *N. reticulatus* (Table 1) the percentage of affected females varied from 33% to 83% across stations. FPL 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 locations except St. 4 where VDS stages varied from 0 to 3. AOS levels were 0 in all sites. St. 3 was the location where imposex levels were highest while the lowest levels of imposex were found in St. 4.

Imposex levels in *N. lapillus* are summarised in Table 2. FPL ranged from 0.1 to 0.2, RPSI values varied between 0.02 and 0.1 and VDSI ranged from 1 to 1.2. The percentage of females affected by imposex varied from 79% to 96%.

### *Organotin concentrations in sediments*

The organic matter content, the fraction of fine particles and concentrations of organotins (ng Sn/g dw) in sediments collected in the studied area are presented in Table 3. Granulometric analysis revealed that all sites presented sediments in the class of sands (fraction <63 um below 25%). The organic content of sediments at each station varied between 1.3 and 6.1 (% of dry weight (dw)). TBT and DBT concentrations were increasingly higher for St. 7, St. 6, St. 3 and St. 5. TBT concentrations ranged from 0.4 in St. 7 to 66 ng Sn/g dw in St. 5 while DBT values ranged from 0.6 to 17 ng Sn/g dw, respectively. MBT levels were below the detection limit of the analytical technique (<6.8 ng Sn/g dw) at all stations except

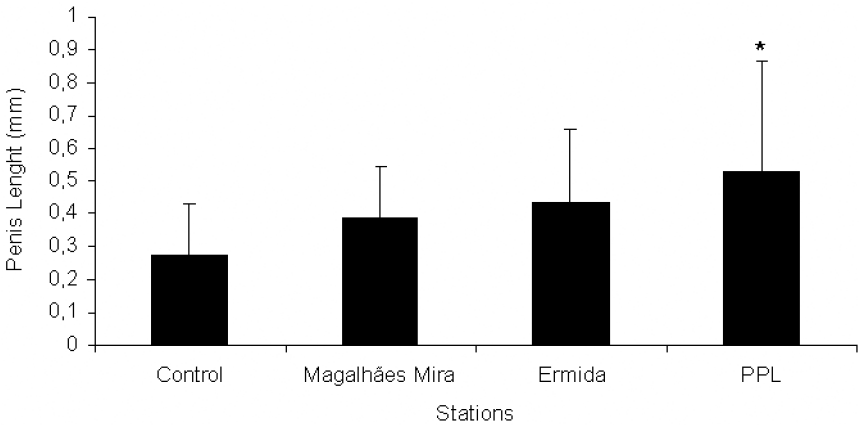


Fig. 2. *Nassarius reticulatus* penis growth in females exposed during 54 days to different sediments collected in Ria de Aveiro. Statistically significant differences are marked * ($p < 0.05$).

in St. 5 where it attained 13 ng Sn/g dw. Phenyltin and octyltin compounds were always below the detection limit (Table 3).

*Laboratory bioassays*

Figure 2 shows the results for the penis growth (FPL) in the bioassays. Mortality reached a maximum of 30% only in one replicate of St. 5. At the end of the experiment, significant penis growth, in relation to the control ($p < 0.05$), was achieved for animals exposed to sediments from St. 5. No statistically significant differences in VDSI values were observed for any treatment.

## DISCUSSION

*Imposex field survey*

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, 2002b; Sousa *et al*., 2005, 2007a, 2009a; Galante-Oliveira *et al*., 2009). Temporal evolution of *N. reticulatus* (Figs. 3 and 4) and *N. lapillus* (Figs. 5 and 6) 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 may had happen because during this period TBT AF-paints were allowed to vessels >25 m in length (Directive 89/677/EEC) and, consequently, all vessels calling

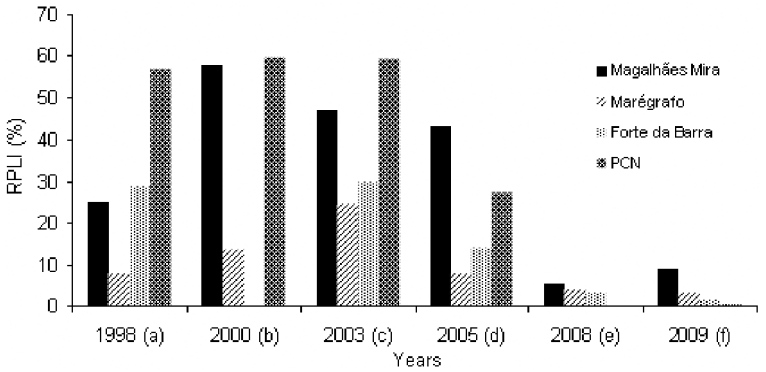


Fig. 3. *Nassarius reticulatus* RPLI (Relative Penis Length Index) levels over the years. Data on imposex levels is cited from: a - Barroso *et al*., 2000; b - Barroso *et al*., 2002a; c - Sousa *et al*., 2005; d - Sousa *et al*., 2007a; e - Sousa *et al*., 2009a; f - Present work.

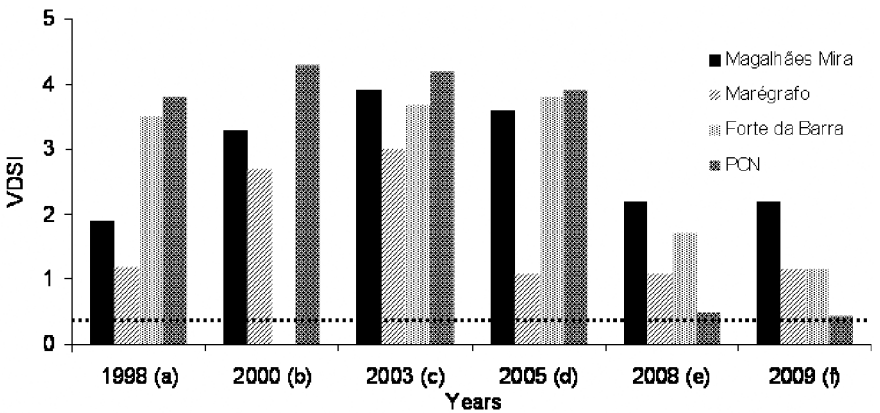


Fig. 4. *Nassarius reticulatus* VDSI (Vas Deferens Sequence Index) levels over the years. Data on imposex levels is cited from: a - Barroso *et al*., 2000; b - Barroso *et al*., 2002a; c - Sousa *et al*., 2005; d - Sousa *et al*., 2007a; e - Sousa *et al*., 2009a; f - Present work. The line refers to EcoQO proposed by OSPAR.

this port could carried these paints and in fact the total GT increased over this period in Ria de Aveiro. In 2003, a new legislation (EC Regulation 782/2003) was adopted prohibiting new applications of TBT AF paints on vessels and, despite the GT of ships calling the Aveiro Port increased (Fig. 7), those carrying TBT AF-paints probably reduced sharply, causing a decrease of TBT pollution. It is interesting to note that the imposex of *N. reticulatus* remained almost identical between 2008 and 2009 contrariwise to what happened to *N. lapillus*, which may suggest that sediments may present a lower reduction rate of TBT contamination

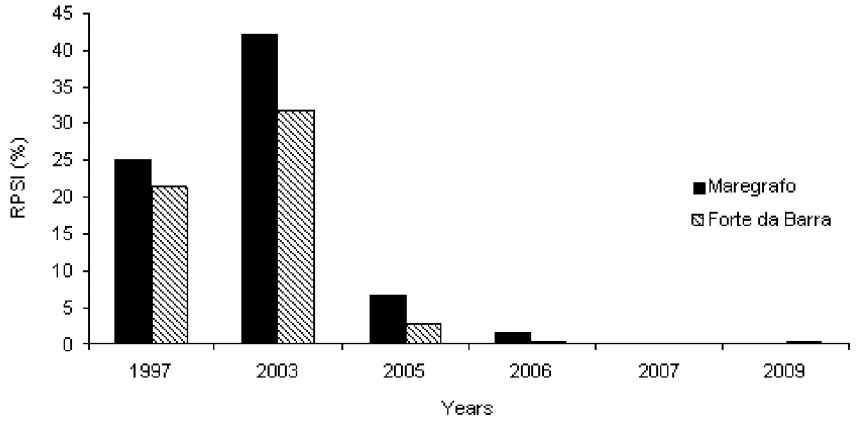


Fig. 5. *Nucella lapillus* RPSI (Relative Penis Size Index) levels over the years. Data was obtained from the work by Galante-Oliveira and co-workers (2009) except for 2009 (present work).

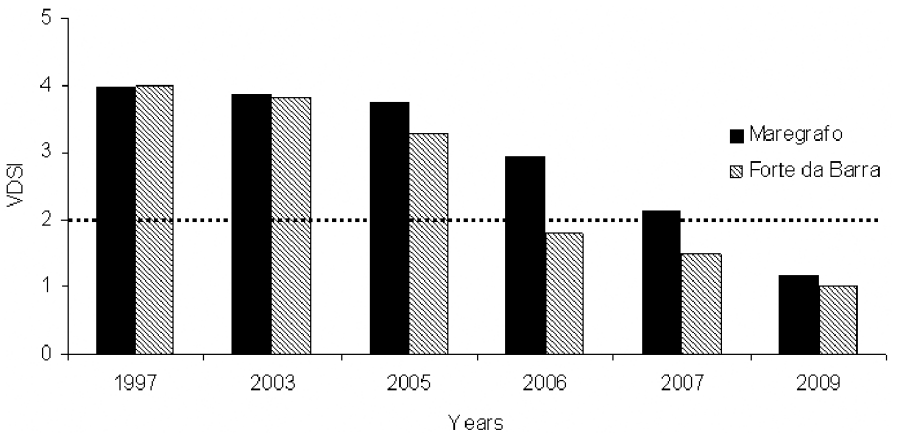


Fig. 6. *Nucella lapillus* VDSI (Vas Deferens Sequence Index) levels over the years. Data was obtained from the work by Galante-Oliveira and co-workers (2009) except for 2009 (present work). The line refers to EcoQO proposed by OSPAR.

in comparison to the water column.

TBT compounds are on the Oslo and Paris (OSPAR) Commission "List of chemicals for priority action" (OSPAR, 2007a) and also on the Water Framework Directive 2000/60/EC. Besides the chemical monitoring of TBT environmental concentrations, imposex assessment is also a mandatory element of OSPAR Co-ordinated Environmental Monitoring Programme (CEMP). OSPAR Commission adopted specific guidelines to monitor imposex in *N. reticulatus* and *N. lapillus*, among other species. Assessment criteria for imposex in different species were

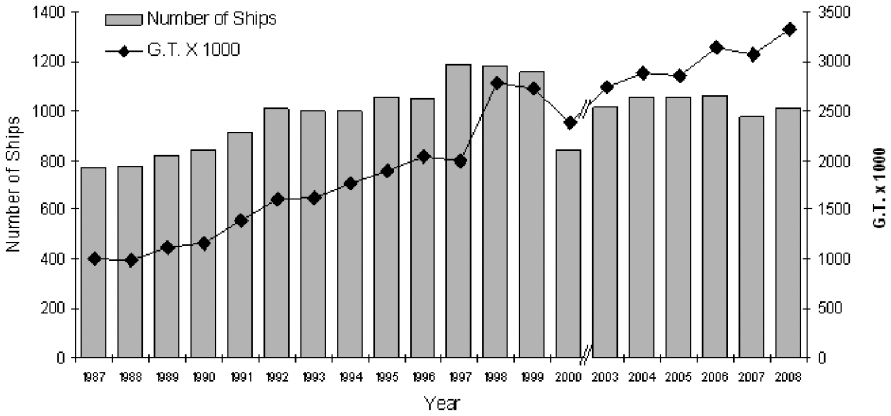


Fig. 7. Commercial ship traffic activity in Aveiro harbour between 1987 and 2008. No data available for 2001 and 2002. G.T. - total gross tonnage (www.portodeaveiro.pt; Anonymous, 2000).

developed and Ecological Quality Objectives (EcoQO) was set (OSPAR, 2007b). For *N. reticulatus* the EcoQO corresponds to VDSI values below 0.3 whereas for *N. lapillus* the EcoQO for imposex corresponds to VDSI values below 2. For the nassariid the EcoQO is not yet achieved in any station. While in the case of the muriciid the EcoQO is achieved in both surveyed stations in 2009 as VDSI levels are below 2.

RPLI and RPSI seem to be suitable indices to track variations of TBT levels in environment over short time periods. Analysing the figures presented above, it's possible to see an earliest response in RPLI and RPSI values than the ones from VDSI. However, some limitations to these indices can be pointed. In *N. 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). For *N. 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.* (1992), the b-type is an alternative way of VDS development where initial stages of affected females start to exhibit vas deferens. Other problem with these indices is that male penis length varies seasonally with reproductive cycle, which may induce variations in RPLI or RPSI, making the use of VDSI more relevant in monitoring programmes.

*Chemical monitoring of TBT pollution*

Results obtained from chemical analyses of sediments from Ria de Aveiro allows to withdraw some conclusions on TBT pollution as 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.

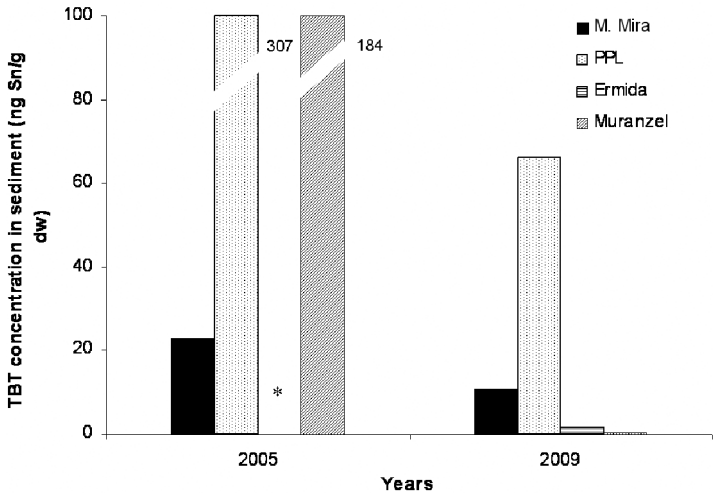


Fig. 8. TBT sediments concentrations (ng Sn/g dw) collected in Ria de Aveiro in 2005 and 2009. * data not available. Data from 2005 is cited from Sousa *et al*. (2007a).

Concentration values of TBT ranged from 0.4 to 66 (ng Sn/g dw), which are much higher than the upper EAC (Ecotoxicological Assessment Criteria) proposed by OSPAR, i.e., concentration 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, i.e., the maximum acceptable concentration of this contaminant in sediment samples (SCTEE, 2005).

TBT recent inputs may be reflected by the higher proportion of TBT over its metabolites, DBT and MBT (Sarradin *et al*., 1995; Hoch, 2001). Only at St. 5 it was possible to quantify all butyltin species (TBT, DBT and MBT); in this station TBT represented 69% of total butyltins which means that recent contamination might have happened. DBT and MBT represent, respectively, 18 and 13% of total BTs.

Based on published work by Sousa and co-workers (2007a), it is possible to establish temporal trends of pollution comparing recent and past levels of contamination. Figure 8 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.

*Laboratory bioassay*

The selected endpoint for this bioassay was the penis growth in females of *N. reticulatus* when exposed to sediments, in order to evaluate current TBT activity and bioavailability. As expected, the penis growth was greater in females

exposed to sediments from PPL station due to its higher TBT concentration. Duft and co workers (2007) exposed *N. reticulatus* during 30 days to sediments sampled along River Elbe using the VDS as endpoint. Contrary to what happened in the current work with sediments of Ria de Aveiro, they obtained an increase in VDSI in all sampled stations, comparing to artificial sediment (control). For Ria de Aveiro, 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.*, 2007b), as observed with our field data (see Fig. 3). These results show that this bioassay is an effective tool for the assessment of TBT levels and bioavailability in sediments. Also this bioassay allows the use of sediments from locations where there are no available organisms for the imposex survey.

## GENERAL CONCLUSION

The aim of this work is to provide an historical perspective of TBT pollution in Ria de Aveiro. Globally the results obtained show that, in terms of TBT pollution, Ria de Aveiro is an estuarine area in fast recovery since 2003, demonstrating the legislation effectiveness. Presently, it shows low levels of imposex and low to moderate TBT contamination of sediments with some TBT activity in this compartment revealed by laboratory bioassays. Nevertheless, all of these parameters are well above the ecological objectives settled by the European Union and OSPAR commission and further recovery is required in the near future.

Generally, biomonitoring of TBT pollution only refers to chemical analyses and imposex surveys. The addition of sediment bioassays, proposed in this work, allows a better knowledge on the actual status of contamination as well as TBT bioavailability in the sediments. The combination of these three parameters is essential to provide an integrative assessment giving an historical perspective, as well as the actual status of pollution. Therefore, the implementation of this integrative assessment could be an important improvement for a better understanding of the quality of sediment regarding TBT pollution as this compound is, still, expected to occur in estuarine areas with intense harbour activities that are common along the European coast.

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