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# Imposex and organotin contamination in *Nassarius reticulatus* (L.) along the Portuguese coast

**Ana Sousa[1]*, Sónia Mendo[1] and Carlos Barroso[1,2]**

[1]Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal
[2]CESAM–Centro de Estudos do Ambiente e Mar, Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal

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***Nassarius reticulatus*** **(whelk) imposex levels and organotin body burden (b.b.) were surveyed along the Portuguese coast, from Vila Praia de Âncora (northern limit) to Lagos (southern limit), between May and August 2003. The percentage of females affected with imposex (%I), the relative penis length index (RPLI), the vas deferens sequence index (VDSI) and the degree of female oviduct convolution index (AOS) were used to assess the level of imposex at each site. These imposex indices were determined for 23 sampling stations throughout the coast and were in the range 0.0–100%, 0.0–90%, 0.0–5.0 and 0.0–1.3 respectively. Sterile females (i.e. females carrying aborted egg capsules inside the capsule gland) were found inside the harbours of Viana do Castelo (8.5%) and Aveiro (3.7%). Organotin compounds were assessed at 10 sampling sites spread along the coast. Tributyltin (TBT) b.b. in females varied between 39 and 1679 ng $g^{-1}$ (as tin) dry weight, and dibutyltin (DBT) and monobutyltin (MBT) varied in the ranges 23–1084 ng $g^{-1}$ (as tin) d and 18–939 ng $g^{-1}$ dry wt respectively. Among the butyltins, the major fraction corresponded to TBT (47.4%), followed by DBT (27.6%) and MBT (25.0%), which indicates recent TBT inputs. Triphenyltin (TPT) levels ranged from <5 to 21 ng $g^{-1}$ (as tin), and, when quantifiable, represented on average 10% of that of TBT. TPT was the dominant phenyltin and was detected in 60% of the sampling stations. The imposex was significantly correlated to ln (TBT) (Spearman $r = 0.918$, $p < 0.001$ for RPLI; $r = 0.864$, $p < 0.001$ for VDSI; $r = 0.828$, $p < 0.01$ for AOS). The higher levels of imposex and TBT contamination occurred inside or close to harbours, which we identified as 'hotspots' of pollution along the coast. Comparing the results obtained in the current work with those reported in a similar survey in 2000, imposex and TBT b.b. varied locally but did not reveal any global trend in the variation of TBT pollution along the Portuguese coast over the 3 year period.** 

**KEYWORDS:** organotin; TBT; imposex; sterility; *Nassarius reticulatus*; Portuguese coast

## INTRODUCTION

Organotin compounds, tributyltin (TBT) and to a lesser extent triphenyltin (TPT), have been used as biocides in antifouling paints since the 1960s.[1] Their deleterious effects on non-target organisms were recognized in the early 1980s, mainly as the cause for the decline in oyster production[2] and for imposex[3]—the superimposition of male characters onto prosobranch females.[4] Legislation to ban the use of organotin antifouling paints on boats smaller than 25 m in length was introduced for the first time in France in 1982, mainly motivated by the negative impact of TBT pollution on oyster farming. Latterly, similar legislation was applied throughout Europe; in Portugal, the use of TBT and TPT on small boats (<25 m) was banned in 1993, and 1 year before that the Portuguese Navy imposed a total ban of their use on their ships. Nevertheless, pollution was still high at many sites of coastal and deep-sea waters of countries that adopted this regulation.[5–10] As a consequence, in 2001 the International Maritime Organization (IMO) formulated the 'International Convention on the Control of Harmful Systems on Ships' that bans the application of organotin antifouling paints on any boat after 1 January 2003 and forbids its usage after 2008.

*Correspondence to: Ana Sousa, Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal.
E-mail: anasousa@bio.ua.pt
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The netted whelk *Nassarius* (= *Hinia*) *reticulatus* (L.) is a common European prosobranch gastropod that is distributed from the Black Sea and the Mediterranean Sea, north to Norway and into the western Baltic.[11] Recently, this whelk has been successfully used as a bioindicator of TBT pollution in European coastal waters[12–15] through assessment of imposex level and organotin body burden (b.b.) in the populations. A survey of this type was conducted in 2000 along the Portuguese coast.[15] The present study concerns a resurvey conducted in 2003 that aims to assess the status of TBT pollution on the Portuguese coast in the year that the IMO ban took place; this will provide a baseline to track the future evolution of the TBT pollution in Portuguese waters and to evaluate the effectiveness of this ban in reducing the pollution levels. Furthermore, it aims to evaluate the temporal trend of organotin pollution over the last 3 years.

## METHODS

### Sampling

*N. reticulatus* was collected between May and August 2003 from 23 sampling stations (Stns) along the Portuguese coast, between Vila Praia de Âncora (northern limit) and Lagos (southern limit; Fig. 1; Table 1). Specimens were collected by hand at the intertidal shore and with baited hoop nets at sublittoral sites. The animals were brought to the laboratory and maintained in aquaria with permanent aeration at constant temperature (17 °C ± 1 °C) for a period of about 3 days prior to analysis.

### Biological examinations

About 60 adult specimens (i.e. those presenting white columellar callus and teeth on the outer lip) were analysed per station. They were narcotized using 7% $MgCl_2$ in distilled water for 40 min and the shell heights (distance from shell apex to lip of siphonal canal) were measured with vernier callipers to the nearest 0.1 mm. The shells were then cracked open with a bench vice, and individuals 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 (FPLI) the relative penis length index (RPLI = mean female penis length × 100/mean male penis length), the vas deferens sequence index (VDSI) and the oviduct stage index (AOS) were determined for each station. The percentage of sterile females (%STER), i.e. females carrying aborted egg capsules inside the capsule gland, was also determined. The penis length was measured using 1 mm graduated graph paper under a stereo microscope. The VDSI was classified according to the scoring system developed by Stroben *et al.*,[12] with minor alterations proposed by Barroso *et al.*[15] The degree of oviduct convolution (AOS) was ranked according to the three-stage scale of Barreiro *et al.*[14]

### Organotin analysis

For 10 selected stations TBT, dibutyltin (DBT), monobutyltin (MBT), TPT and diphenyltin (DPT) were measured in the whole tissues of 10–15 pooled females (Table 1). The analyses were performed by the Servicios Xerais de Apoio á Investigación (Universidade da Coruña). The procedures used are described by Quintela *et al.*[16] and are largely based on the methods of Szpunar *et al.*[17] Briefly, two replicate samples of 0.1 g lyophilized tissue were digested with tetramethylammonium hydroxide by application of microwave power. After adjustment to pH 5, sodium tetraethylborate and isooctane containing tetrabutyltin as an internal standard were successively added. After microwave radiation treatment, the organic phase was recovered and analysed by gas chromatography–mass spectrometry. The methods gave a tin quantification limit of around 20 ng $g^{-1}$ dry weight (dry wt) for the butyltins and 5 ng $g^{-1}$ dry wt for the phenyltins. The procedure was validated with a certified reference material, i.e. the Japanese NIES11 fish tissue (National Institute for Environmental Studies, Japan Environment Agency). Recoveries in routine samples were assessed by a standard addition method and results were corrected accordingly. The analysis was performed for two separate replicates of each sample, and the results are given as mean values with the respective standard deviations. The extraction efficiencies for TBT, DBT and MBT varied in the ranges 68–72%, 97–123% and 37–118% respectively, whereas for TPT and DPT the extraction efficiencies were in the ranges 97–118% and 28–57% respectively.

### Statistical analysis

All statistical analysis was performed using the software Statistica 6.0. The correlation analysis refers to the non-parametric Spearman rank order correlation. Comparison between VDSI values was made through the non-parametric Mann–Whitney *U* test.

## RESULTS

### Imposex and organotin body burden in *N. reticulatus*

Levels of *N. reticulatus* imposex and female organotin b.b. obtained in the current survey are shown in Fig. 1 and Table 1. The %I varied from 0.0 to 100%, the RPLI varied between 0.0 and 90% and the VDSI ranged from 0.0 to 5.0. The degree of female oviduct convolution (AOS) varied from 0.0 to 1.3 and was significantly correlated with VDSI ($r = 0.914$, $p < 0.001$). The oviduct convolution occurred only on females with VDSI > 2 (Fig. 2). Sterile females were found inside the harbours of Viana do Castelo (Stn 2) and Aveiro (Stn 12), with incidences of 8.5% and 3.7% in the population respectively. These sterile females presented advanced imposex stages (VDSI = 5 and AOS > 1) and did not exhibit any vulva occlusion or capsule gland deformation. The TBT female

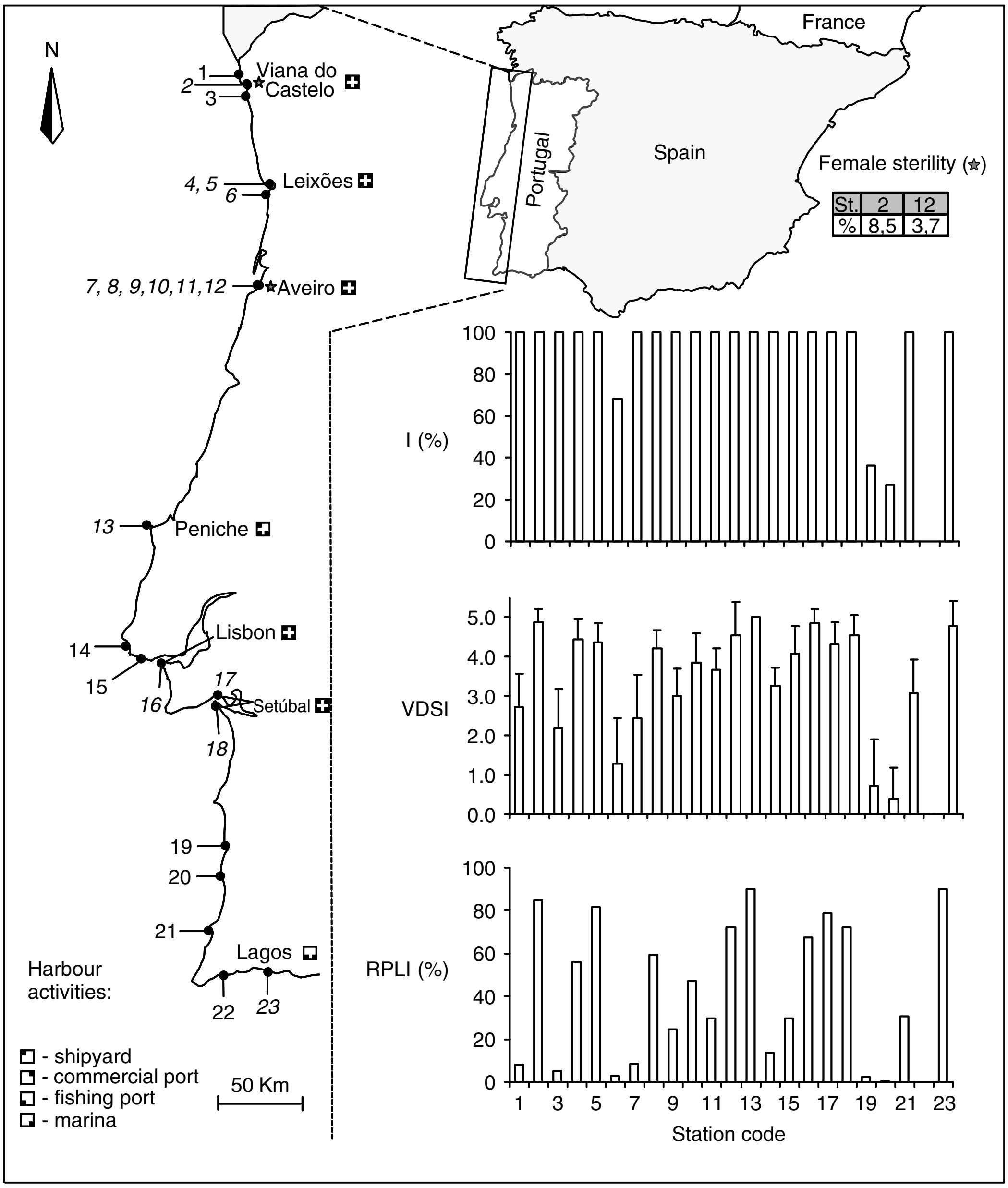


**Figure 1.** *N. reticulatus*. Map of the Portuguese coast indicating the sites (1 to 23) where specimens were collected and the location of the main harbours. Italic code numbers represent sampling stations located inside harbours. The histograms show the values of relative penis length index (RPLI), vas deferens sequence index (VDSI) and imposex incidence. * Occurrence of female sterility.

b.b. (as tin) ranged from 39 to 1679 ng $g^{-1}$ dry wt, and DBT (as tin) and MBT (as tin) varied in the ranges 23–1084 ng $g^{-1}$ dry wt and 18–939 ng $g^{-1}$ dry wt respectively. Among the butyltins, the major fraction corresponded to TBT (47.4%), followed by DBT (27.6%) and MBT (25.0%) (Table 1). TPT was the dominant phenyltin and was quantifiable in 60% of the stations, whereas DPT (as tin) was only detected at one sampling station (Stn 13: 21 ng $g^{-1}$ dry wt). TPT (as tin) levels ranged from <5 to 21 ng $g^{-1}$ and, when quantifiable, represented on average 10% of the TBT residue. No significant correlation ($r = 0.75$, $p = 0.08$) was found between TBT and TPT, although the observed significance was close to 0.05.

All imposex indices were significantly correlated to ln (TBT) ($r = 0.918$, $p < 0.001$ for RPLI; $r = 0.864$, $p < 0.001$ for VDSI; $r = 0.845$, $p < 0.001$ for FPLI; $r = 0.828$, $p < 0.01$ for AOS). Figure 3 illustrates the relationships between the TBT tissue concentration and the RPLI and VDSI indices obtained in the current survey (black circles). The same figure also shows that these relationships are similar to that obtained in 2000[15] (white circles). For VDSI and RPLI there was a rapid increase and then both indices tended to a plateau of

 

**Table 1.** *N. reticulatus*. Data relative to each sampling site with the indication of numbers of males (♂ *N*) and females (♀ *N*) with respective mean shell heights; female oviduct convolution (AOS); mean TBT, DBT, MBT, TPT and DPT whole female b.b. and respective plus/minus one standard deviation. Standard deviations relative to mean shell heights are given as a percentage of the mean: (a) 0 to 5%; (b) 5 to 10%; (c) 10 to 15%; dry wt; na: not analysed; nq: not quantifiable. For additional data see Figure 1

| | | | | | | | Tissue b.b. ± SD (ng $g^{-1}$ dry wt (as tin)) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Station code and name | Coordinates (EUR 50) | ♂ *N* | ♂ shell height | ♀ *N* | ♀ shell height | AOS | TBT | DBT | MBT | TPT | DPT |
| 1Praia Norte | 41°41.85N–8°51.13W | 23 | 21.6[(b)] | 36 | 23.9[(b)] | 0.0 | na | na | na | na | na |
| 2V. Castelo—Estaleiro | 41°41.34N–8°50.26W | 20 | 23.9[(b)] | 32 | 23.9[(b)] | 1.1 | 832 ± 64 | 584 ± 10 | 939 ± 55 | 19 ± 3 | nq |
| 3Praia da Amorosa | 41°38.72N–8°49.31W | 20 | 23.1[(b)] | 41 | 21.1[(b)] | 0.0 | na | na | na | na | na |
| 4Porto Leixoes—Plat. 2 | 41°11.42N–8°41.43W | 20 | 25.9[(a)] | 40 | 26.8[(a)] | 0.9 | 330 ± 13 | 169 ± 18 | 115 ± 29 | nq | nq |
| 5Porto Leixoes—Marina | 41°11.30N–8°42.24W | 20 | 24.2[(b)] | 36 | 25.9[(b)] | 1.2 | na | na | na | na | na |
| 6Praia da Foz | 41°09.78N–8°41.10W | 15 | 22.6[(b)] | 44 | 23.2[(b)] | 0.0 | na | na | na | na | na |
| 7Aveiro—S. Jacinto | 41°39.84N–8°43.56W | 20 | 24.3[(b)] | 31 | 25.0[(b)] | 0.1 | 63 ± 1 | 32 ± 3 | 45 ± 13 | nq | nq |
| 8Aveiro—PCN | 41°39.06N–8°43.76W | 20 | 22.4[(b)] | 34 | 24.0[(b)] | 0.7 | na | na | na | na | na |
| 9Aveiro—Barra | 41°38.71N–8°44.82W | 30 | 23.4[(c)] | 25 | 25.1[(b)] | 0.2 | na | na | na | na | na |
| 10Aveiro—MM | 41°38.65N–8°44.06W | 25 | 24.9[(b)] | 30 | 25.6[(b)] | 0.5 | 218 ± 6 | 61 ± 1 | 69 ± 8 | nq | nq |
| 11Aveiro—FB | 40°38.56N–8°43.59W | 20 | 23.2[(b)] | 34 | 24.8[(b)] | 0.2 | na | na | na | na | na |
| 12Aveiro—PPL | 40°38.24N–8°43.59W | 28 | 25.8[(b)] | 27 | 26.3[(c)] | 1.3 | na | na | na | na | na |
| 13Peniche—Porto Pesca | 39°21.15N–9°22.52W | 24 | 21.8[(b)] | 26 | 21.1[(b)] | 1.1 | 1679 ± 58 | 1084 ± 24 | 637 ± 10 | 21 ± 2 | 21 ± 1 |
| 14Praia do Guincho | 38°43.74N–9°28.46W | 27 | 20.7[(c)] | 15 | 22.5[(c)] | 0.0 | 39 ± 3 | 23 ± 4 | 24 ± 1 | 8 ± 4 | nq |
| 15Praia das Avencas | 38°41.21N–9°21.27W | 15 | 20.8[(b)] | 25 | 21.8[(b)] | 0.2 | 48 ± 3 | 35 ± 2 | 29 ± 8 | 12 ± 1 | nq |
| 16Lisboa—Trafaria | 38°40.55N–9°14.09W | 15 | 19.7[(c)] | 34 | 20.5[(c)] | 0.9 | 400 ± 19 | 103 ± 2 | 69 ± 4 | 8 ± 1 | nq |
| 17Setúbal—Porto Pesca | 38°31.17N–8°52.58W | 34 | 21.2[(b)] | 25 | 21.0[(b)] | 0.7 | na | na | na | na | na |
| 18Setúbal—Tróia | 38°26.25N–9°06.76W | 26 | 20.7[(b)] | 25 | 20.9[(b)] | 1.3 | 185 ± 3 | 143 ± 16 | 103 ± 10 | 16 ± 5 | nq |
| 19Vila Nova Mil Fontes | 37°43.30N–8°47.25W | 16 | 21.7[(c)] | 44 | 22.0[(b)] | 0.0 | na | na | na | na | na |
| 20Zambujeira do Mar | 37°33.20N–8°47.44W | 20 | 21.5[(b)] | 33 | 23.4[(b)] | 0.0 | na | na | na | na | na |
| 21Praia da Arrifana | 37°18.82N–8°52.11W | 22 | 22.0[(b)] | 11 | 22.9[(c)] | 0.1 | 86 ± 1 | 30 ± 2 | 18 ± 1 | nq | nq |
| 22Praia da Luz | 37°05.21N–8°43.64W | 23 | 17.7[(c)] | 36 | 19.3[(b)] | 0.0 | na | na | na | na | na |
| 23Lagos—Marina | 37°06.28N–8°40.19W | 34 | 18[(b)] | 18 | 20.8[(c)] | 0.9 | na | na | na | na | na |

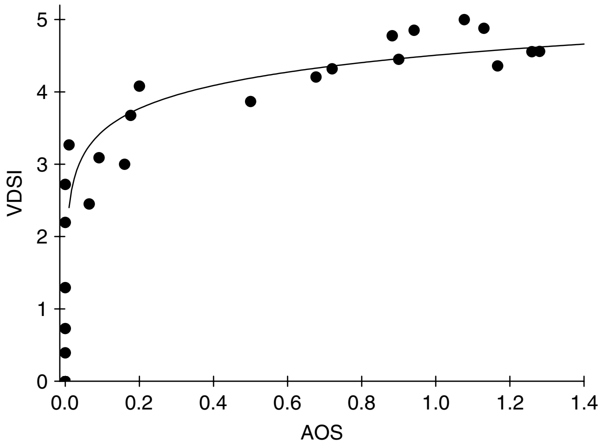


**Figure 2.** Relationship between VDSI and AOS indices in *N. reticulatus*; regression line: VDSI = 4.507 + 0.457 ln (AOS) ($F = 33.6$; $p < 0.001$; $R^2 = 0.671$).

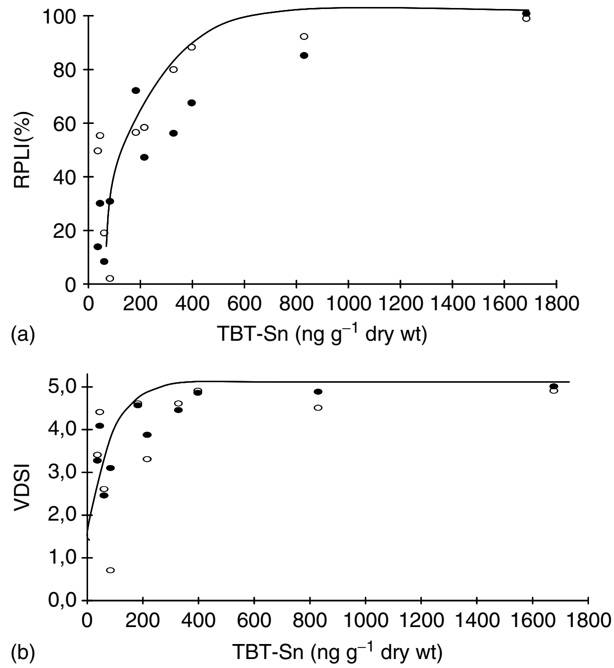


**Figure 3.** *Nassarius reticulatus*. Relationship between TBT body burden and RPL and VDS imposex indices obtained in 2003 survey (black circles) and in 2000 survey (white circles). (a) For TBT > 700 ng Sn g$^{-1}$ dry wt, RPLI = 87%; for TBT < 700 ng Sn g$^{-1}$ dry wt see text; (b) For TBT > 400 ng Sn g$^{-1}$ dry wt, VDSI = 4.6; TBT < 400 ng Sn g$^{-1}$ dry wt, see text.

VDSI = 4.6 and RPLI = 87% at tissue tin concentrations of about 400 ng g$^{-1}$ dry wt and 700 ng g$^{-1}$ dry wt respectively. For the range of values below the plateau, significant regressions were established between ln (TBT) and VDSI ($\mathrm{VDSI} = 1.3\ln(\mathrm{TBT}) - 2.9; F = 50.7, p < 0.001, R^2 = 0.60$) and between ln (TBT) and RPLI ($\mathrm{RPLI} = 27.7\ln(\mathrm{TBT}) - 90.8; F = 141.8, p < 0.001, R^2 = 0.78$).

## Spatial variation of imposex and organotin body burden

An evident relationship was found between the organotin body burden or imposex levels in *N. reticulatus* and the proximity of harbours. The highest TBT values (from 185 to 1679 ng g$^{-1}$ dry wt (as tin) and with an average plus/minus standard deviation of 607 ± 574 ng g$^{-1}$ dry wt (as tin)) were found at stations located inside estuarine systems or embayments enclosing harbours (Stns 2, 4, 10, 13, 16 and 18; see Fig. 1). The lowest values (from 39 to 86 ng g$^{-1}$ dry wt (as tin) and with an average plus/minus standard deviation of 58 ± 25 ng g$^{-1}$ dry wt (as tin)) were found at sites on the open shore distant from harbours (Stns 14, 15, 21), although TBT pollution still occurs due to the presence of a considerable number of small fishing and leisure boats (see Fig. 1). Stn 7 is located at Ria de Aveiro, but nevertheless presents low contamination (63 ng g$^{-1}$ dry wt (as tin)) for being distant from the harbours and for having an intense water renovation from the sea.

Regarding imposex, sites located inside or close to the harbours of Viana do Castelo (Stn 2), Leixões (Stns 4, 5), Aveiro (Stns 8, 10–12), Peniche (Stn 13), Lisboa (Stn 16), Setúbal (Stns 17, 18) and Lagos (Stn 23) presented %I = 100%, RPLI = 30–90% (average plus/minus standard deviation of 69 ± 18%), VDSI = 3.7–5.0 (4.5 ± 0.4) and AOS = 0.2–1.3 (0.9 ± 0.3) (Fig. 1; Table 1). The remaining stations, distant from harbours, but nevertheless subjected to small-boat traffic, presented %I = 0–100% (average plus/minus standard deviation of 76 ± 37%), RPLI = 0–31% (12 ± 12%), VDSI = 0.0–4.1 (2.1 ± 1.3) and AOS = 0–0.2 (0.1 ± 0.1) (Fig. 1; Table 1). On the other hand, increasing gradients of TBT pollution and imposex were observed on approaching the hotspots (Fig. 1). For instance, at Ria de Aveiro, the lowest imposex levels were found in stations near the mouth of the estuary (Stns 7, 9) and the highest levels were registered inside or close to harbours (Stns 8, 11, 12). Similar trends were also observed around the harbours of Viana do Castelo, Leixões and Lisbon. In the latter case, for example, as we approached the harbour there was an increase of TBT b.b. and imposex: Stn 14 (RPLI ≈ 14%, VDSI ≈ 3, TBT b.b. 39 ng g$^{-1}$ (as tin)), Stn 15 (RPLI ≈ 30%, VDSI ≈ 4, TBT b.b. 48 ng g$^{-1}$ (as tin)) and Stn 16 (RPLI ≈ 67%, VDSI ≈ 5, TBT b.b. 400 ng g$^{-1}$ (as tin)).

## Temporal variation of imposex and organotin body burden

Temporal comparisons of imposex levels and organotin b.b. in *N. reticulatus* for common sites sampled in 2000[15] and in 2003 are shown in Table 2. The imposex and organotin analysis were performed on both occasions using identical methods. TPT female b.b. decreased at all but one station analysed, but the TBT levels showed a distinct tendency: they decreased at Stns 2, 4, 7, 14, 15, 16 and increased at Stns 10, 13, 18 and 21 (Table 2). Many factors, such as temporal variations of naval traffic and of shipyard activity, may cause different inputs of TBT to the environment, which will ultimately cause variations in TBT tissue contamination. The same is true for TPT, although inputs from agricultural pesticides must also be considered.[18] Table 3 shows the variation in commercial ship traffic, expressed in terms of tonnage, between the periods of 1998–2000 and 2001–2003, i.e. 3 years before each survey. It is interesting to note the similar decrease among TBT b.b. at Viana do Castelo harbour (Stn 2) and the 9% reduction in commercial traffic between the two periods. Similarly, the TBT b.b. increase at Setúbal harbour (Stn 18) is coincident with a 57% rise in commercial traffic. As mentioned above, change in commercial traffic is not the only cause for TBT b.b. variation. For instance, at Ria de Aveiro, commercial traffic increased only 3%, but the change in TBT (as tin), from 132 to 218 ng $g^{-1}$ dry wt observed at Stn 10 is most likely related to the start of activity in 1999 of a new fishing port close to this site at Canal de Mira. Similarly, we can speculate that the consistent decrease of TBT b.b. at Stns 14–16 in Lisbon is due to the closing of a major national shipyard at this harbour (Lisnave) in 2000, regardless of the slight increase in commercial traffic (0.7%) at Lisbon harbour. Many reasons can be suggested to explain the observed changes and we are infrequently sure of their veracity; hopefully, after the IMO ban there will be a globally consistent decrease in TBT along the whole coast, as the ban will affect all kinds of input sources, which will be easily detected trough imposex and organotin b.b. monitoring.

We also checked whether the imposex evolution between 2000 and 2003 followed the same variation in TBT b.b. in females for a given site. For this we used the regression equations and the plateau line of the relationships in Fig. 3 to estimate theoretical values of RPLI and VDSI for the TBT b.b. registered on both occasions and then we estimated the theoretical variation of these imposex indices from 2000 to 2003. This variation was then compared with the observed difference of the same indices for the same period (Table 4; Fig. 4). Despite some rare exceptions, the theoretical and the observed variations between 2000 and 2003 had a common sign trend, i.e. they generally increase or decrease together, which means that imposex generally accompanied the evolution of TBT concentration in the tissues.

When all stations are compared for imposex variation (Table 2) we conclude that, in the majority of the sites, the VDSI did not differ significantly between 2000 and 2003, and when there was a significant change it was either to increase or to decrease, depending on the site; the RPLI also increased in some sites and decreased in others. Hence, both indices presented some rises or reductions at a local scale, but they did not reveal any global trend in the variation of TBT pollution along the Portuguese coast over the 3 year period.

## DISCUSSION

There is evidence from laboratory experiments that imposex in *N. reticulatus* is induced by TBT, whether administered by injection, by aqueous and sediment exposure or through the diet.[19–22] Further evidence of this relationship is found in the high correlation between imposex and TBT female b.b. in the current field survey; similar correlations were previously reported for the same area[15] and for the coastal waters of France,[12] Britain[13] and Spain.[14] All these studies point out the link of cause and effect between TBT contamination and imposex in the netted whelk.

It is known that TBT acts as an androgenic endocrine disruptor that leads to the masculinization of the females.[23,24] This masculinization varies between different species of prosobranchs, but the common trend is the growth of a penis and a vas deferens over the female genital tract. This has been observed in *N. reticulatus* in previous studies and was also shown in the current survey, since the RPLI and the VDSI were significantly correlated with TBT b.b. Barreiro *et al*.[14] pointed out that females with advanced developed penis and vas deferens collected in northwest Spain also exhibited a convoluted gonadial oviduct, resembling the sinuous seminal vesicle of the males. The current survey and the 2000 survey[15] showed the same pattern along the Portuguese coast, i.e. the convolution of the gonadial oviduct is correlated with VDSI and TBT b.b. and increases rapidly for VDSI $> 2$. Hence, oviduct convolution is most likely to be another masculinization effect of TBT pollution. Neither of the above-mentioned effects seems to cause sterility in *N. reticulatus* females, because they do not lead to vulva blocking or capsule gland malformation, as happens with some other prosobranch species.[25] Nevertheless, sterility was found inside the harbours of Viana do Castelo (Stn 2) and Aveiro (Stn 12) in females with advanced imposex stages (VDSI $= 5$ and AOS $> 1$). Sterile females with advanced stages of imposex were also reported for the Portuguese coast in 2000, although none was found at Aveiro.[15] The consistency of these findings, together with very similar data reported for western France[26] and northwest Spain,[14] strongly suggests that sterilization may be a consequence of imposex development. This may hypothetically result from any deformation that occurs inside the capsule gland that is not easily detected by visual inspection, which needs to be studied further. TBT pollution may thus have a stronger adverse impact on *N. reticulatus* populations than previously thought.

Hopefully, TBT pollution levels will reduce in future years as a consequence of the application of the IMO ban in 2003. Under the scope of assessing the effectiveness of this ban in the future, we have created an updated baseline regarding the status of *N. reticulatus* organotin b.b. and imposex in 2003. This showed the occurrence of increasing TBT b.b. and imposex levels in proximity to harbours, which are identified as 'hotspots' of pollution in Portugal. In these areas, many ships are anchored or being repaired or repainted at dockyards, and high quantities of TBT are leached to the surrounding medium. This is easily detected in the TBT concentrations in the tissues. In fact, we registered a high mean TBT b.b. level of about 600 ng $g^{-1}$ dry wt (as tin) inside or close to

 

**Table 2.** Time comparisons of *N. reticulatus* imposex indices and organotin female b.b, between 2000[15] and 2003 (present survey), at common sites along the Portuguese coast. *U*: Mann–Whitney *U*-test result; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns: not significant; na: not analysed; nq: not quantifiable; (–) just one replicate

| Station code | RPLI | | FPLI | | | | VDSI | | | | *I* (%) | | Tissue b.b. ± SD (ng g$^{-1}$ dry wt (as tin)) TBT | | DBT | | TPT | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2000 | 2003 | 2000 | 2003 | *U* | *p* | 2000 | 2003 | *U* | *p* | 2000 | 2003 | 2000 | 2003 | 2000 | 2003 | 2000 | 2003 |
| 1 | 6.3 | 8.0 | 0.8 | 0.8 | 376.0 | ns | 2.5 | 2.7 | 362.0 | ns | 100 | 100 | 77 ± 3 | na | 63 ± 3 | na | nq | na |
| 2 | 92.1 | 85.1 | 11.5 | 7.5 | 14.0 | *** | 4.5 | 4.9 | 322.0 | * | 100 | 100 | 1368 ± 43 | 832 ± 64 | 654 ± 6 | 584 ± 10 | 112 ± 5 | 19 ± 3 |
| 3 | 2.3 | 5.1 | 0.3 | 0.5 | 551.0 | ns | 1.7 | 2.2 | 454.0 | * | 81 | 100 | 37 ± 7 | na | 37 ± 8 | na | nq | na |
| 4 | 79.8 | 56.0 | 10.4 | 5.9 | 17.0 | *** | 4.6 | 4.5 | 389.5 | ns | 100 | 100 | 481(–) | 330 ± 13 | 205(–) | 169 ± 18 | 18(–) | nq |
| 5 | 88.8 | 81.5 | 10.7 | 8.7 | 165 | *** | 4.4 | 4.4 | 347.5 | ns | 100 | 100 | 602 ± 3 | na | 354 ± 6 | na | 84 ± 7 | na |
| 6 | 10.7 | 3.0 | 1.3 | 0.3 | 76.5 | *** | 2.5 | 1.3 | 219.5 | *** | 100 | 68 | 55 ± 4 | na | 44 ± 3 | na | nq | na |
| 7 | 18.0 | 8.3 | 2.6 | 1.0 | 123.5 | *** | 2.6 | 2.5 | 284.5 | ns | 94 | 100 | 66 ± 10 | 63 ± 1 | 49 ± 8 | 32 ± 3 | nq | nq |
| 8 | 59.8 | 59.4 | 7.2 | 6.1 | 274.0 | ** | 4.3 | 4.2 | 413.5 | ns | 100 | 100 | 262 ± 25 | na | 131 ± 18 | na | nq | na |
| 9 | 13.8 | 24.5 | 1.8 | 2.5 | 221.0 | * | 2.7 | 3.0 | 255.5 | ns | 100 | 100 | 60 ± 1 | na | 62 ± 7 | na | nq | na |
| 10 | 58.2 | 47.1 | 7.0 | 5.5 | 195.5 | ns | 3.3 | 3.9 | 182.0 | ns | 100 | 100 | 132 ± 12 | 218 ± 6 | 81 ± 2 | 61 ± 1 | nq | nq |
| 13 | 88.1 | 90.0 | 8.5 | 6.7 | 72.5 | *** | 4.9 | 5.0 | 221.0 | ns | 100 | 100 | 912 ± 162 | 1679 ± 58 | 671 ± 36 | 1084 ± 24 | 73 ± 8 | 21 ± 2 |
| 14 | 49.5 | 13.7 | 7.3 | 1.6 | 7.5 | *** | 3.4 | 3.3 | 171.5 | ns | 100 | 100 | 90 ± 6 | 39 ± 3 | 103 ± 5 | 23 | nq | 8 ± 4 |
| 15 | 55.2 | 29.9 | 7.6 | 3.5 | 20.0 | *** | 4.4 | 4.1 | 274.5 | ns | 100 | 100 | 128 ± 3 | 48 ± 3 | 121 ± 1 | 35 | 15 ± 3 | 12 ± 1 |
| 16 | 88.1 | 67.4 | 7.7 | 7.0 | 242.0 | ns | 4.9 | 4.9 | 292.5 | ns | 100 | 100 | 488 ± 7 | 400 ± 19 | 275 ± 1 | 103 ± 2 | 23 ± 2 | 8 ± 1 |
| 17 | 85.9 | 78.7 | 7.9 | 9.4 | 158.5 | * | 4.9 | 4.3 | 126.0 | ** | 100 | 100 | 459 ± 26 | na | 413 ± 2 | na | 52 ± 11 | nq |
| 18 | 56.4 | 72.0 | 5.9 | 7.3 | 157.5 | ** | 4.6 | 4.6 | 286.0 | ns | 100 | 100 | 124 ± 3 | 185 ± 3 | 90 ± 4 | 143 ± 16 | 20 ± 1 | 16 ± 5 |
| 19 | 1.2 | 2.2 | 0.1 | 0.3 | 536.5 | ns | 0.5 | 0.7 | 529.0 | ns | 26 | 36 | 23 ± 2 | na | nq | na | nq | nq |
| 20 | 0.1 | 0.6 | 0.01 | 0.1 | 325.5 | ns | 0.6 | 0.4 | 246.0 | ns | 58 | 27 | nq | na | nq | na | nq | nq |
| 21 | 1.9 | 30.7 | 0.2 | 5.0 | 14.5 | *** | 0.7 | 3.1 | 16.0 | *** | 44 | 100 | 21 ± 1 | 86 ± 1 | nq | 30 ± 2 | 13 ± 1 | nq |
| 22 | 0 | 0.0 | 0.0 | 0.0 | 576.0 | ns | 0.0 | 0.0 | 576.0 | ns | 0 | 0 | nq | na | nq | na | nq | nq |

**Table 3.** Commercial ship traffic activity in main Portuguese ports: total number of commercial ships calling as at each port during 1998–2003 expressed in terms of total gross tonnage stood (GTs)

| Port | GTs ($\times 10^6$t) | | | | | |
|---|---|---|---|---|---|---|
| | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 |
| V. Castelo | 0.92 | 1.13 | 1.19 | 1.15 | 0.93 | 0.87 |
| Leixões | 19.71 | 20.66 | 20.90 | 22.00 | 21.74 | 20.94 |
| Aveiro | 2.70 | 2.67 | 2.34 | 2.54 | 2.70 | 2.73 |
| Lisboa | 36.96 | 37.92 | 37.99 | 35.25 | 37.80 | 40.55 |
| Setúbal | 15.73 | 17.31 | 20.93 | 29.52 | 30.91 | 29.36 |

**Table 4.** Estimated and observed variations in RPLI and VDSI from 2000 to 2003

| Station | RPLI (%) | | VDSI (%) | |
|---|---|---|---|---|
| | Theoretical | Observed | Theoretical | Observed |
| 2 | 0.0 | −7.1 | 0.0 | 0.4 |
| 4 | −10.4 | −23.8 | −0.1 | −0.1 |
| 7 | −1.3 | −9.7 | −0.1 | −0.1 |
| 10 | 13.9 | −11.1 | 0.7 | 0.6 |
| 13 | 0.0 | 1.9 | 0.0 | 0.1 |
| 14 | −23.2 | −35.8 | −1.1 | −0.1 |
| 15 | −27.2 | −25.3 | −1.3 | −0.3 |
| 16 | −5.5 | −20.7 | 0.0 | 0.0 |
| 18 | 11.1 | 15.6 | 0.5 | 0.0 |
| 21 | 39.1 | 28.8 | 1.9 | 2.4 |

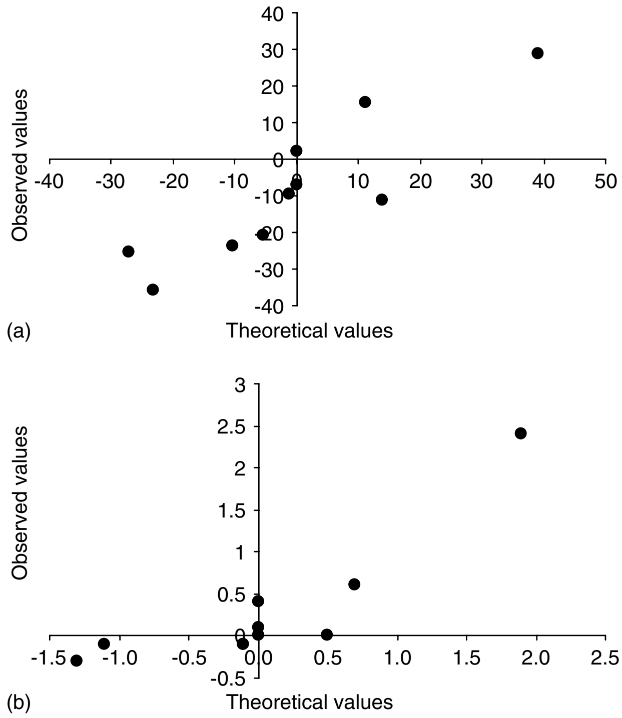


**Figure 4.** Relationship between estimated and observed variations in RPLI (a) and VDSI (b) in *N. reticulatus* from 2000 to 2003.

harbours, and a value as high as 1679 ng $g^{-1}$ was found at one port. These levels are similar to those observed for the same area in the 2000 survey (mean value of ~580 ng $g^{-1}$ dry wt).[15] Comparison of *N. reticulatus* b.b. obtained in this study with results from other areas may be meaningless, because pollution varies drastically with the choice of sampling site and distance to the pollution source. Nevertheless, the values reported in this study are similar to those reported for northwest Spain (mean value of ~850 ng $g^{-1}$ dry wt (as tin)),[14] but they are generally higher than those reported for southwest England (most values below 50 ng $g^{-1}$ dry wt (as tin)).[13] Recent TBT inputs along the Portuguese coast are suggested by the TBT × 100/(TBT + DBT) value, with values varying between 56 and 80%. Such high levels have been reported for the 2000 survey (values varying between 34 and 63%)[5] and for northwest Spain in 1998 near pollution hotspots.[27]

*N. reticulatus* also exhibited high levels of imposex inside harbours, where *I* was 100% and the RPLI and VDSI were, on average, 69% and 4.5 respectively. These values resemble those reported by other workers for the most polluted harbours in Spain (e.g. Coruña, Ferrol and Vigo),[14] France (e.g. Roscoff harbour)[12] and Britain (Dart Estuary and Plymouth Sound).[13] The high imposex and TBT female b.b found in 2003 and the overall TBT content in comparison with its debutylated forms indicate that TBT paints are still largely used along the Portuguese coast. This is corroborated by the analysis of organotin and imposex evolution between 2000 and 2003 that indicates that there was no global change in the levels of TBT pollution. Moreover, when one attempts to compare the relationships between imposex indices and TBT b.b. between the two surveys, no differences as recorded by Bryan *et al.*[13] are observed, reinforcing the idea that no TBT decline has occurred. However, in the present survey the TPT levels were much lower (between 62 and 83% at Stns 2, 4, 13, 16, 21 and 20% at Stns 15, 18) than in 2000, which suggests a strong reduction of the input of this triorganotin. This can be derived either from a lesser usage of TBT as a co-toxicant in antifouling paints or from a diminishing application of TPT-based pesticides in agriculture.

To assess the future impact of the IMO ban using *N. reticulatus* as a bioindicator, it is important to know whether the species is in fact sensitive to track temporal changes in TBT pollution. Since TBT residues are lost from tissues with half-lives of about 2–3 months,[28] this parameter can be used as a reliable tool for monitoring rapid changes in pollution. However, imposex in many prosobranch species has been considered an irreversible phenomenon, so that if TBT pollution decreases then the imposex will only slowly decline as new mature whelks are recruited into the population. Considering that *N. reticulatus* can live for 15–17 years[13,29] and that sexual maturation is achieved in about 4–5 years, imposex monitoring would not detect rapid changes in TBT pollution. However, the current study shows that the variation of RPLI and the VDSI between 2000 and 2003 matches, in general terms, the evolution of the TBT female b.b. in the same period for most of the sites analysed. This suggests that the renovation of generations in the population may be faster than predicted or that imposex is, at least in part, reversible. Bryan *et al*.[13] have already noticed that there is a slow reduction in the female penis length of the netted whelk over time, but further research is needed to ascertain whether the same applies to the VDSI.

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## REFERENCES


1. De Mora SJ. The tributyltin debate: ocean transportation versus seafood harvesting. In *Tributyltin: Case Study of an Environmental Contaminant*, de Mora SJ (ed.). Cambridge Environmental Chemistry Series 8. Cambridge University Press: Cambridge, 1996; 1–13.
2. Alzieu C, Héral M, Thibaud Y, Dardignac MJ, Feuillet M. *Rev. Trav. Péc. Marit.* 1981; **45**: 101.
3. Smith BS. *J. Appl. Toxicol.* 1981; **1**: 15.
4. Smith BS. *Proc. Malaco. Soc.* 1971; **39**: 377.
5. Barroso CM, Moreira MH. *Mar. Pollut. Bull.* 2002; **44**: 480.
6. Santos MM, ten Hallers-Tjabbes CC, Santos AM, Vieira N. *J. Sea Res.* 2002; **48**: 217.
7. Ten Hallers-Tjabbes CC, Wegener J-W, van Hattum B, Kemp JF, ten Hallers E, Reitsema TJ, Boon JP. *Mar. Environ. Res.* 2003; **55**: 203.
8. Michel P, Averty B, Andral B, Chiffoleau J-F, Galgani F. *Mar. Pollut. Bull.* 2001; **42**: 1128.
9. Minchin D, Stroben E, Oehlmann J, Bauer B, Duggan CB, Keatinge M. *Mar. Pollut. Bull.* 1996; **32**: 188.
10. Morgan E, Murphy J, Lyons R. *Mar. Pollut. Bull.* 1998; **36**: 840.
11. Graham AFRS. Molluscs: prosobranch and pyramidellid gastropods. In *Synopses of the British Fauna*, 2nd edn. Kermack DM, Barnes SK (eds). The Linnean Society of London and the Brackish-Water Science Association: London, 1986; 186–189.
12. Stroben E, Oehlmann J, Fioroni P. *Mar. Biol.* 1992; **113**: 625.
13. Bryan GW, Burt GR, Gibbs PE, Pascoe PL. *J. Mar. Biol. Assoc. U. K.* 1993; **73**: 913.
14. Barreiro R, Gonzáles R, Quintela M, Ruiz JM. *Mar. Ecol. Prog. Ser.* 2001; **218**: 203.
15. Barroso CM, Moreira MH, Bebiano MJ. *Mar. Ecol. Prog. Ser.* 2002; **230**: 127.
16. Quintela M, Barreiro R, Ruiz JM. *Sci. Tot. Environ.* 2000; **247**: 227.
17. Szpunar J, Schmitt VO, Lobinski R. *J. Anal. At. Spectrose.* 1996; **11**: 193.
18. Fent K. *Crit. Rev. Toxicol.* 1996; **26**: 1.
19. Stroben E, Oehlmann J, Fioroni P. *Mar. Biol.* 1992; **114**: 289.
20. Bettin C, Oehlmann J, Stroben E. *Helgol. Meeres.* 1996; **50**: 299.
21. Pope ND. PhD thesis, University of Plymouth, 1998.
22. Barroso CM, Reis-Henriques MA, Ferreira MS, Moreira MH. *J. Mar. Biol. Assoc. U. K.* 2002; **89**: 249.
23. Matthiessen P, Gibbs PE. *Environ. Toxicol. Chem.* 1998; **17**: 37.
24. Oberdorster E, Cheek OA. *Environ. Toxicol. Chem.* 2001; **20**: 23.
25. Gibbs PE, Bryan GW. TBT-induced imposex in neogastropod snails: masculinization to mass extinction. In *Tributyltin: Case Study of an Environmental Contaminant*, de Mora SJ (ed.). Cambridge Environmental Chemistry Series 8. Cambridge University Press: Cambridge, 1996; 212–236.
26. Huet M, Fioroni P, Oehlmann J, Stroben E. *Hydrobiologia* 1995; **309**: 29.
27. Ruiz JM, Quintela M, Barreiro R. *Mar. Ecol. Prog. Ser.* 1998; **164**: 237.
28. OSPAR Joint Assessment and Monitoring Program (JAMP)—Guidelines for contaminant specific biological effects monitoring. OSPAR Commission, London, 1998; 38.
29. Tallmark B. *Mar. Ecol. Prog. Ser.* 1980; **3**: 51.