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Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal)

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ABSTRACT: The prosobranchs *Nucella lapillus*, *Nassarius reticulatus*, *Hydrobia ulvae* and *Littorina littorea* were sampled from May to July 1998 at 45 stations in the Ria de Aveiro (NW Portugal) and the adjacent coastal zone. The distribution patterns of these populations were related to the salinity gradients. The tributyltin (TBT) concentration in the water was determined for 17 stations spread over the study area during a neap tide in July 1998 and varied from 9 to 42 ng Sn l^{-1}. The TBT contamination of unsieved sediments taken from 20 sites inside the Ria de Aveiro in July 1998 ranged from <6 to 88 ng Sn g^{-1} dry weight. All populations of the 4 species showed imposex (superimposition of male characters on females) or the related phenomenon intersex, except for *N. reticulatus* at some inshore stations outside the ria. The vas deferens sequence index (VDSI) of *N. reticulatus*, *H. ulvae* and *N. lapillus* varied from 0.0–4.8, 0.2–1.1 and 4.0–4.4, respectively. The intersex index (ISI) for *L. littorea* ranged from 0.3 to 0.5. Sterilization of females was found in *N. lapillus* at 1 site (29%). This muricid was the most sensitive species to TBT, followed, in decreasing order, by *N. reticulatus*, *H. ulvae* and *L. littorea*. The nassariid *N. reticulatus* provided the best discrimination between stations with different TBT environmental contamination. It is proposed that *N. reticulatus* and *H. ulvae* may jointly be used for monitoring sediments of moderately to highly contaminated estuarine systems due to their complementary distributions with regard to salinity, their common type of substrate and their ubiquity on the European coast.

KEY WORDS: *Nassarius reticulatus* · *Nucella lapillus* · *Hydrobia ulvae* · *Littorina littorea* · TBT · Imposex · Intersex · Estuaries · Portugal

INTRODUCTION

Ships' antifouling paints containing tributyltin (TBT) were introduced in the 1960s (Bennett 1996). This compound is known to be harmful to a large taxa range of aquatic organisms, particularly the molluscs (Bryan & Gibbs 1991), and its widespread use in antifouling paints makes it a matter of world concern. Legislation to ban the use of TBT paints on small boats (<25 m) was introduced in France in 1982; similar restrictions were adopted by the United Kingdom in 1987, and later applied in most European (EC directive 89/677/EEC) and other countries (Mora 1996, Stewart 1996). Consequently TBT contamination has declined in many coastal areas in water, sediments and tissues of molluscs, accompanied by a lessening of the intensity of imposex in gastropods and also of shell deformation in oysters (Gibbs & Bryan 1994). Nevertheless, the continued use of TBT paints on large vessels makes the problem still acute in commercial and fishing ports.

In 1993, Portugal adopted the EC directive (89/677/EEC) concerning the ban on the use of TBT paints for ships smaller than 25 m. The Portuguese Navy applied a total ban to their ships in 1992. However, since the

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EC ban only refers to small pleasure craft, it has not influenced the continuing input of TBT into the Ria de Aveiro, where the main traffic is composed of large commercial and fishing vessels and about 85% of these vessels have TBT-based antifoulings. There are also dockyards devoted to the construction and repair of ships longer than 25 m, where TBT antifouling paints are estimated to represent 70% of the total volume of paints applied.

Prosobranchs' imposex, the superimposition of male characters onto the female (Smith 1971), is a worldwide phenomenon. In Europe several species have been proposed and validated for biomonitoring TBT contamination through the imposex response, notably: the muricids *Nucella lapillus* (L.) (Bryan et al. 1986, Gibbs et al. 1987, Gibbs & Bryan 1996), *Ocenebra erinacea* (L.) (Gibbs et al. 1990, Gibbs 1996) and *Ocinebrina aciculata* (Lam.) (Oehlmann et al. 1996); the nassariids *Nassarius* (*Hinia*) *reticulatus* (L.) (Stroben et al. 1992a, Bryan et al. 1993) and *Nassarius* (*Hinia*) *incrassatus* (Ström) (Oehlmann et al. 1998); the buccinid *Buccinum undatum* (L.) (Ten Hallers-Tjabbes et al. 1994, Mensink et al. 1996); the hydrobiid *Hydrobia ulvae* (Pennant) (Schulte-Oehlmann et al. 1997, 1998); and the littorinid *Littorina littorea* (L.) (Bauer et al. 1995, 1997). Biomonitoring may overcome some difficulties related to the chemical measurement of TBT in the environment, mainly the large temporal variations in the concentrations of this compound at fixed locations and the fact that it can be biologically effective at concentrations below the limit of detection. Besides, it is a low cost technique and also a biological meaningful measure revealing the effects of a contaminant on a given ecosystem, at the individual, population and community levels. For biomonitoring a whole estuary, several species need to be examined because different tolerances to salinity restrict the distribution of each species. The objectives of the present work are: (1) to evaluate the ecotoxicological properties of 4 prosobranch species for monitoring TBT pollution over a southern European estuarine system; (2) to assess the impact of TBT pollution on the populations of those species at the Ria de Aveiro; and (3) to propose a multispecific approach for monitoring TBT pollution in this and other similar estuarine systems.

Study area

The Ria de Aveiro is a shallow estuarine system on the north-west coast of Portugal (Fig. 1) which can be classified as a bar-built estuary, according to the definition of Pritchard (1967). 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 (Fig. 1): 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 mouth and dominates the hydrological circulation. The volume of water in the Ria de Aveiro at a mean low tide is approximately 78×10^6 m^3, while the mean tidal prism is about 83×10^6 m^3 (Silva 1994). The River Vouga is the most important river discharging into the ria, accounting for $^2/_3$ of the total mean river input, which is only about 73 m^3 s^{-1} (Silva 1994). The salinity regime in the Ria de Aveiro is characterised later in this paper. The tidal time delay, relative to the mouth, may reach 6 h or more in the far reaches of the channels (Barrosa 1985, Vicente 1985). With regard to substrate type, 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 (see Fig. 2B). For the most part of the ria the bottom is formed by sediments ranging from medium sands to muds (Moreira et al. 1993, 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 surficial sediments are dominated by sand with a low content of gravel and mud (Cunha et al. 1997).

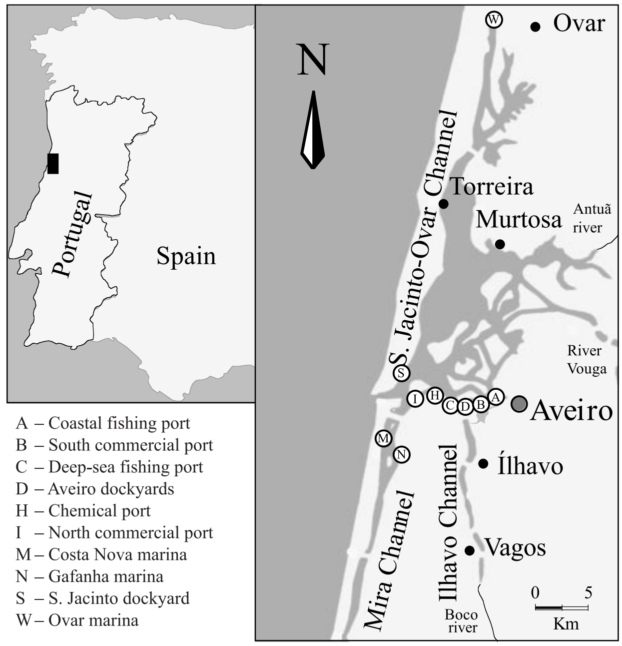


Fig. 1. Ria de Aveiro, showing the main TBT contamination sources. See text for explanation

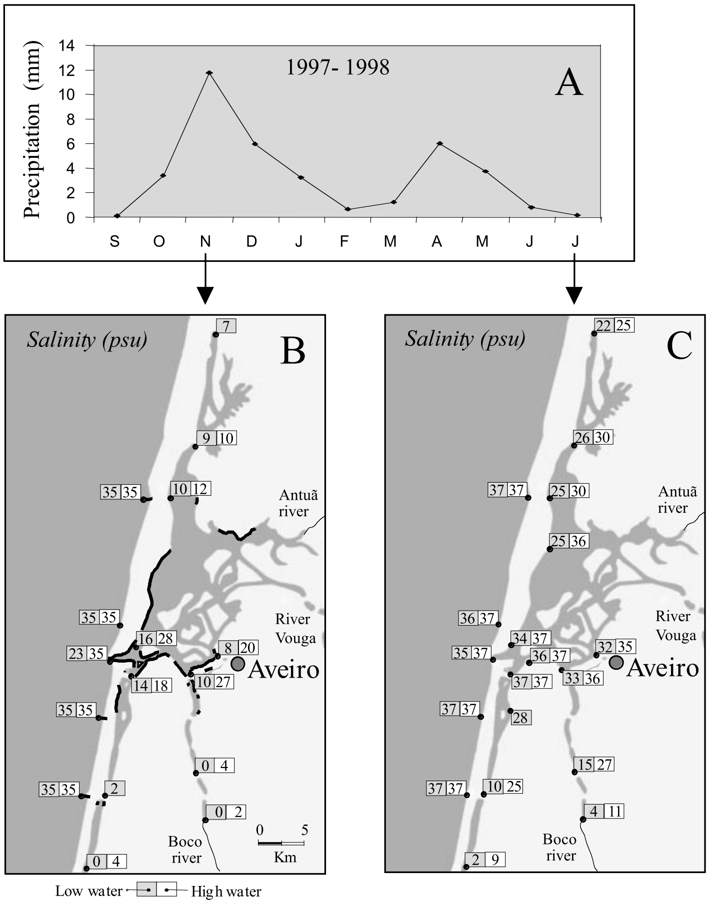


Fig. 2. (A) Seasonal variation of the monthly mean precipitation recorded at Aveiro. (B) Sub-surface water salinity recorded on 3 November 1997 at the low and high water neap tides. Black lines on the banks represent intertidal rocky shore, while those on the sea coast represent rocky groynes. (C) Sub-surface water salinity recorded on 5 July 1998 at the low and high water neap tides

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 (Fig. 1). On the western bank of the initial part of the S. Jacinto-Ovar Channel is a naval construction shipyard (S) for ships of up to 2000 t. On the southern bank of the navigation channel there is an enclosed commercial terminal port (I) with a mean daily frequency (mdf) of 2.2 ships with 88 m mean length (ml) and also a chemical terminal port (H) with a mdf of 0.5 ships (92 m ml). Of major relevance is the deep-sea fishing port (C) with 2 km of wharf, where more than 30 ships ranging from 25 to 80 m are usually docked. The main dockyards of Aveiro are located next to this port (D). Between this point (C) and the city of Aveiro there is a commercial terminal port (B) with a mdf of 0.8 ships (79 m ml) and a coastal fishing port (A) with a mdf of 13 ships (25 m ml). There are also 3 small marinas for up to 100 boats, located at the end of the S. Jacinto-Ovar Channel (W) and on the western (M) and eastern (N) banks of the initial part of the Mira Channel. Additionally, a large number of small local fishing boats and pleasure boats are spread along the banks of the ria, mainly in the channels inside the city of Aveiro, in the outer part of Mira Channel and, to the north, at Torreira and Murtosa.

In spite of the high naval traffic and dockyard activity in the Ria de Aveiro, previous published information regarding TBT contamination is extremely scarce. In a study of the Iberian coast, Peña et al. (1988) give the relative penis size (RPS) of *Nucella lapillus* at 1 site in the Ria de Aveiro in the summer of 1987. Barroso & Moreira (1998) report on the relative penis length (RPL) variation of *Nassarius reticulatus* throughout the reproductive cycle, observed at 1 site during 1992/93.

Indicator species

The most suitable prosobranchs for monitoring the Ria de Aveiro are those which have been validated as bioindicator species and in combination give a complete coverage of this coastal system. Based on these criteria 4 species were selected: the dogwhelk *Nucella lapillus*, the nettedwhelk *Nassarius reticulatus* (after revision by Rolán & Luque 1994 and by Sanjuan et al. 1997), the mudsnail *Hydrobia ulvae* and the periwinkle *Littorina littorea*. It has been shown that the degree of development of imposex by the 3 former species and of intersex by *L. littorea* is a graded dependent response to the level of TBT exposure; consequently, the indices for measuring the mean intensity of this response in a specific population—associated with a particular site—provide an estimate of the local levels of TBT environmental contamination. Triphenyltin (TPhT) induces imposex in *Thais clavigera* (Horiguchi et al. 1997), but similar effects have not been reported for the indicator species used in this work. Moreover, the levels of TPhT in the study area are negligible (authors' unpubl. data). Generally, female penis size, vas deferens sequence (VDS), incidence (percentage of affected females = %I) and percentage of sterile females are the main indices that have been applied to evaluate imposex intensity in prosobranchs. In the dogwhelk RPS is mostly used and expresses the mean bulk size (cube of the length) of the female penis as a percentage of that of the male in the same population (Gibbs & Bryan 1994). For the nettedwhelk RPL (= mean female penis length × 100/mean male penis length) index is preferable (Bryan et al. 1993), providing that the populations to be compared are approximately at the same reproductive stage (Barroso & Moreira 1998). For the mudsnail both RPS and RPL indices are useful if moderately or heavily polluted areas are studied; on the other hand, for this species the RPL comparisons are reliable in the sense that male penis size does not vary throughout the reproductive cycle (Schulte-Oehlmann et al. 1997). The more biological meaningful parameter is the vas deferens sequence index (VDSI), which is measured through a scoring system defined for each particular species (see Gibbs et al. 1987, Stroben et al. 1992a, Schulte-Oehlmann et al. 1997). The periwinkle shows a different but related response—intersex—which is assessed by the intersex index (ISI), based on a specific scoring system developed by Bauer et al. (1995, 1997). VDSI and ISI are biological meaningful parameters as they estimate the extent of any reduction in the reproductive capability of the females in a given population.

MATERIAL AND METHODS

Organisms. Sampling of prosobranchs was performed by time searches to obtain estimates of relative abundance. Forty-five stations spread over the whole area of the Ria de Aveiro and the adjacent coastal zone were sampled in the period May to July 1998. Specimens of *Nucella lapillus*, *Hydrobia ulvae* and *Littorina littorea* were collected from the intertidal shore by hand, while for *Nassarius reticulatus* baited hoop nets were used. At the sublittoral stations, *N. reticulatus* was sampled using a small dredge (0.55 m width and net bag of 10 mm mesh size) inside the ria and a larger fishing dredge (1.5 m width and net bag of 25 mm mesh size) at the inshore stations outside the ria. Whenever possible samples of about 60 adult prosobranchs were analysed.

The prosobranchs were maintained in aquaria for about 3 d. *Nucella lapillus* was examined without narcotisation but the other 3 species were narcotised using 4 to 7% $MgCl_2$ in distilled water (according to *in situ* salinity), in order to allow comparisons with data obtained by other authors. Only adult prosobranchs were selected for analysis. The shell height (distance from shell apex to lip of siphonal canal or to base of the last whorl) was measured with vernier callipers to the nearest 0.1 mm. The shells were cracked open with a bench vice and the individuals were sexed and dissected under a stereo microscope. The VDSI or ISI was determined for each species at individual stations according to the classification schemes described by Gibbs et al. (1987) for *N. lapillus*, Stroben et al. (1992a) for *Nassarius reticulatus*, Schulte-Oehlmann et al. (1997) for *Hydrobia ulvae* and Bauer et al. (1995, 1997) for *Littorina littorea*. In the case of *N. reticulatus* the VDSI values of 4^+ were converted to 5 for computation of mean values of a particular site. The RPS was determined for *N. lapillus* and the RPL for *N. reticulatus* and *H. ulvae*. The penis length of the 2 former species was measured to the nearest 0.1 mm using 1 mm graduated graph paper while for *H. ulvae* a graduated eyepiece in a stereo microscope, providing an accuracy of 0.05 mm, was used.

Water. The sub-surface (15 cm below surface) water sampling took place at 17 stations spread over the main channels of the Ria de Aveiro, including the navigation channel, and the adjacent Atlantic coast, during a neap tide on 5 July 1998 (see Fig. 3A,C). Five samples were collected per station covering 1 tidal cycle (12 h) between 2 consecutive high tides. The samples were obtained with a 3 h interval, at corresponding tidal phases at all stations. Volumes of 1 l were collected inside the Ria de Aveiro and 2 l along the adjacent coast. Prior to sample collection, the acid-washed glass bottles used were washed with local water and a sample was taken for salinity determination with a refractometer. After collection the samples were acidified with 5 ml of concentrated hydrochloric acid per litre of water. The methods used for extraction from unfiltered water and TBT analysis are those described by Bryan et al. (1986), providing a detection limit of about 0.2 ng Sn l^{-1}. For each station the TBT concentration is expressed as an arithmetic mean of the individual values observed during the tidal cycle.

Sediments. Sampling occurred between 23 and 25 July 1998 at 20 sites located inside the Ria de Aveiro (see Fig. 3B,C). A portion of the surficial layer (1 cm) of the sediment was removed with a spatula, at about the mid-tide level, placed in a polyethylene bag and deep frozen (–20°C) for storage. Homogenised wet unsieved sub-samples were analysed for TBT following the methods based on Ward et al. (1981) that are fully described by Bryan et al. (1986), rendering a detection limit of about 5 ng Sn g^{-1} dry weight (DW). The moisture content was determined from a separate aliquot. The organotin determination of unsieved, rather than sieved, sediments appears to provide a better indication of the real values to which the prosobranch sediment dwelling species are exposed; moreover their diet is not significantly based on detritus selection. For each station a sediment sub-sample was analysed for granulometric composition and total organic matter. In the former analysis the pretreatment and splitting of silt-clay fraction was performed according to the methods described by Buchanan (1984). As all samples fell within the range of muddy sediments, i.e. with a silt and clay (particles less than 63 µm) content above 5%, they were further classified according to the percentage content of this fraction: muddy sands (<25%), sandy muds (25 to 75%) or muds (>75%). The organic matter content was obtained by measuring the loss of weight on ignition at 450°C of a dried sediment sample.

RESULTS

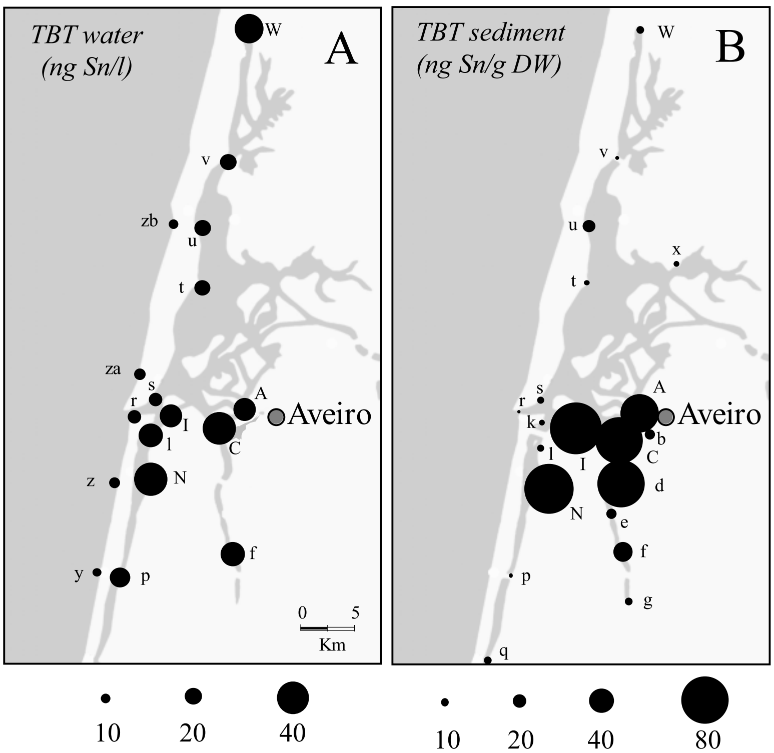
Salinity gradients and substrate types

There are clear salinity longitudinal gradients along the channels of the Ria de Aveiro that are dependent on precipitation and tidal phase (Fig. 2). The Mira and Ílhavo channels presented strong salinity gradients with very low values at the upper reaches, which varied from 0–4 and 0–2 psu in the wet season survey to 2–9 and 4–11 in the dry season survey, respectively. The gradient was less pronounced in S. Jacinto-Ovar Channel, at the top end of which salinity ranged between 7 and 25 psu.

The percentage of fine particles and organic matter in the surficial sediments collected in the Ria de Aveiro is indicated in Fig. 3C. Although muddy sediments were found at all stations, the fine particle content in the Mira Channel is lower than in the Ílhavo and S. Jacinto-Ovar channels, where the sandy muds and muds dominate. The organic matter content varied between 1 and 10% and was significantly correlated to the amount of fine particles in the sediment (r = 0.82, p = 0.000).

Water and sediment TBT contamination of the Ria de Aveiro

There is an evident relationship between the water and sediment TBT concentrations and the proximity of the TBT sources (Figs. 1 & 3). The concentration values in the study area ranged from 9 to 42 ng Sn l^{-1} in the water and from less than 6 to 88 ng Sn g^{-1} (DW) in the total sediment. In the vicinity of ports, dockyards and marinas they varied from 28 to 42 ng Sn l^{-1} and from 65 to 88 ng Sn g^{-1} (DW), respectively, excluding the marina at Stn W. At this station, despite the high value recorded in the water (37 ng Sn l^{-1}), the sediment concentration was only 10 ng Sn g^{-1}. The results reveal a clear increasing gradient of TBT concentration in the water from the sea (9 to 14 ng Sn l^{-1}) into the Ria de Aveiro (15 to 42 ng Sn l^{-1}). In spite of inter-site varia-



St.	Water TBT ng Sn/l	Sediment TBT ng Sn/g	Sediment <63µm %	Sediment OM %
A	28	65	50	5
b		13	31	6
C	41	79	68	7
d		80	87	6
e		14	70	6
f	29	30	93	8
g		9	52	5
I	32	88	73	10
k		6	28	3
l	29	8	51	5
N	42	84	32	3
p	25	nq	10	1
q		11	17	5
r	16	nq	14	2
s	15	8	28	4
t	18	6	59	5
u	19	19	54	5
v	19	nq	81	8
W	37	10	76	10
x		6	80	8
y	9			
z	12			
za	14			
zb	10			

Fig. 3. TBT concentration in (A) the sub-surface water (mean values) recorded during the neap tide of 5 July 1998 and (B) the surface unsieved sediment collected between 23 and 25 July 1998. Capital letters indicate stations located near ports, dockyards or marinas. (C) TBT values recorded in the water and sediment samples, fine particles (<63 µm) and organic matter (OM) content of the sediments. For site codes (St.) compare Table 1 and Fig. 1. Additional site codes: e, Vista Alegre; g, Boco; p, Vagueira; q, Areão

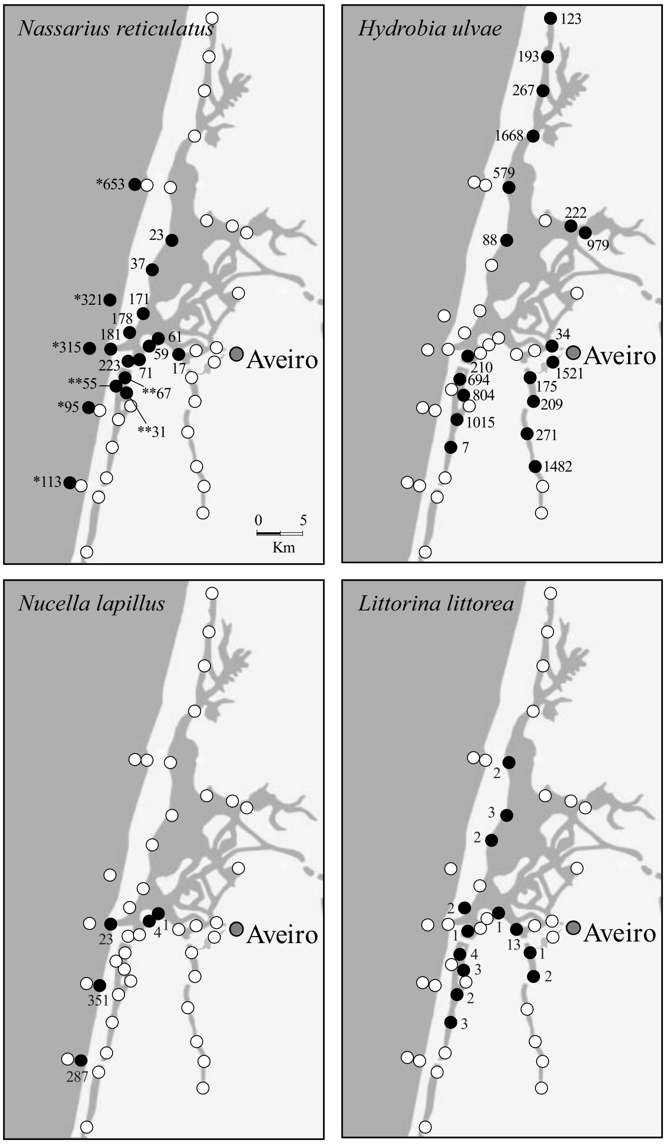


Fig. 4. Station net scheme used for sampling prosobranchs. Each station corresponds to an area of up to about 5000 m^2, depending on the topography. Black and white circles correspond to the presence or absence of a given species, respectively. Numbers represent number of individuals collected after 1 h of sampling by hand (by the same person), for *Hydrobia ulvae*, *Nucella lapillus* and *Littorina littorea*; *Nassarius reticulatus* was collected either with a baited hoop net, with a dredge of 1.5 m width and net bag of 25 mm mesh size (*) or with a dredge of 0.55 m width and net bag of 10 mm mesh size (**)

tion of sediment characteristics (Fig. 3C), a positive significant correlation was established between the TBT concentration in the water and in the sediment ($r = 0.65$, $p = 0.040$). Of the 3 main channels of the Ria de Aveiro, the Ílhavo and Mira channels are the most contaminated ones. This is due, in the former case, to the highly contaminated floodwaters that pass through the deep-sea fishing port (C) and the Aveiro dockyards (D); in the latter, most contamination is probably generated by the marinas (M and N) and small local fishing boats. The lower contamination observed in the S. Jacinto-Ovar Channel is explained by the reduced TBT sources together with a major dilution factor; in the uppermost part of this channel the increasing gradient in the water contamination is related to the marina located at Stn W.

Distribution of the indicator species

The distribution of the selected species in the Ria de Aveiro is shown in Fig. 4. The distribution of the mudsnail *Hydrobia ulvae* extends farthest towards the upper reaches of the channels; it was absent from the oligohaline portion of the Mira and Ílhavo channels, but it was found at the top end of the S. Jacinto-Ovar Channel, where salinity is higher. This species was extremely abundant at the upper levels of intertidal shores in sheltered areas, particularly the muddy flats with high densities of *Ulva* sp. The periwinkle *Littorina littorea* penetrates deeply into the channels, although not so far as the mudsnail. The littorinid was found in the upper levels of the intertidal zone on rocks, stones and muddy flats covered by *Zostera* sp. and *Gracilaria* sp. The nettedwhelk *Nassarius reticulatus* penetrates into the lower estuarine zone of the Ria de Aveiro. It is particularly abundant along the inshore coast of Aveiro, and in the outer part of the ria, where salinity remains high. It was found in various types of sediment, ranging from sand to mud, and occasionally on the rocky shore during egg capsule deposition, between the high water at neap tide and, at least, a depth of 10 m on the shelf. *Nucella lapillus* is a marine species mostly found on the rocky groynes built along Aveiro coast. Its distribution inside the Ria de Aveiro is restricted to the rocky artificial banks in the outer part of the navigation channel, with decreasing abundance from the mouth inwards.

Imposex/intersex levels

The intensity of imposex or intersex exhibited by the indicator species is summarised in Table 1 and the VDSI and ISI levels are also shown in Fig. 5.

Nassarius reticulatus showed a clear increasing gradient of imposex intensity from the sea into the Ria de Aveiro; values for RPLI, VDSI and %I ranged from 0 to 80, 0 to 4.8 and 0 to 100, respectively. At the deep-sea fishing port (Stn C) most nettedwhelks presented the highest possible VDS score of 4^+. VDS stages 1b and 3b, corresponding to the absence of a penis (Stroben et

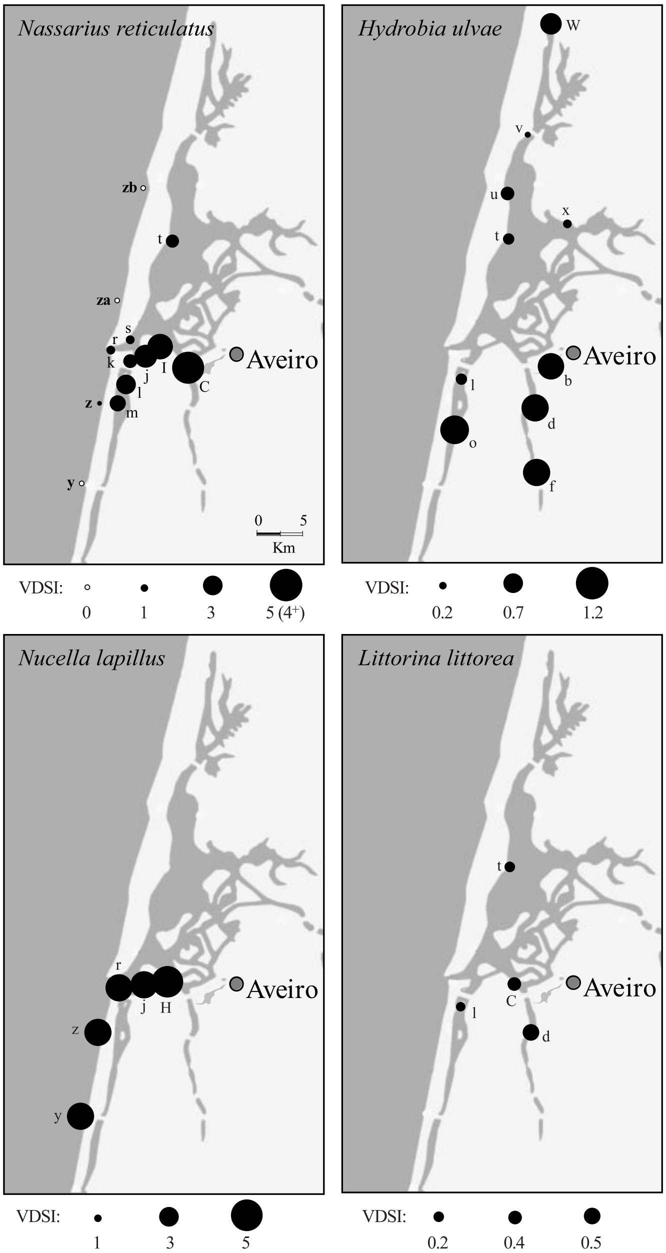


Fig. 5. *Nassarius reticulatus*, *Hydrobia ulvae*, *Nucella lapillus* imposex VDSI levels and *Littorina littorea* intersex ISI levels in the Ria de Aveiro and adjacent coastal area

al. 1992a), were not observed. Significant correlations were established between RPLI and TBT sediment concentration (r = 0.93, p = 0.009) and between VDSI and log-transformed TBT sediment concentration (r = 0.90, p = 0.016).

Hydrobia ulvae populations contained mostly a-type imposex females, i.e. females which first produce a penis and then gradually develop a vas deferens (Schulte-Oehlmann et al. 1997); only 2 females with b-type imposex (where the vas deferens appears first) were found in a total of about 500 females analysed. The highest observed VDS score was stage 2a and no sterile females were found. Values for RPLI, VDSI and %I ranged from 4 to 20, 0.2 to 1.1 and 36 to 88, respectively. Significant correlations were established between RPLI and TBT sediment concentration (r = 0.77, p = 0.027) and between VDSI and log-transformed TBT sediment concentration (r = 0.73, p = 0.040).

All *Nucella lapillus* females were heavily affected in the study area and none was found with a VDS stage less than 4. RPSI and VDSI varied from 20 to 38 and from 4.0 to 4.4, respectively. Individual VDS stages of 5 and 6 were restricted to 1 single site (Stn H) which corresponds to the inner limit of the species distribution in the Ria de Aveiro. At this station the percentage of female sterility was 29 %. At the rest of the stations all females exhibited a constant VDS stage 4. No correlation analysis was performed between TBT environmental contamination and imposex indices due to the low number of data points.

All *Littorina littorea* populations in the study area were affected by intersex but only females with stages 0 and 1 were found. ISI values ranged from 0.3 to 0.5 and %I varied between 33 and 54 %. (The interpretation of intersex levels found at Stns d, l and o requires caution because sample sizes are small.) No correlation analysis was performed between environmental TBT contamination and ISI levels due to the low number of data points.

DISCUSSION

Distribution of the indicator species

The present study showed that the salinity regime in the Ria de Aveiro is strongly influenced by tidal height and precipitation, as previously observed by other authors (e.g. Moreira et al. 1993, Cunha & Moreira 1995, Cunha et al. 1999). All 3 channels present a longitudinal salinity gradient but this is less pronounced in the S. Jacinto-Ovar Channel. Changes in the benthic community composition related to salinity gradients in the Ria de Aveiro have been described by Moreira et al. (1993) and Cunha et al. (1999).

The sub-surface water salinity surveyed in this work may underestimate the real values to which the soft-bottom species like *Nassarius reticulatus*, *Hydrobia ulvae* and, in some cases, *Littorina littorea* are exposed. However, this effect is probably small since in the Ria de Aveiro the vertical salinity stratification is restricted to periods of rainfall in the lower parts of the channels, not exceeding 5 psu (Queiroga et al. 1994). Many environmental factors may be responsible for

Table 1. Summary of the imposex/intersex intensity levels found in the study area; mean values ($\bar{x}$) and standard deviations (SD) are indicated. In each case the mean and SD of the shell height (mm) for females and males are given. Codes given in italics: stations used for inter-species cross-comparisons of imposex/intersex responses. Capital letters: stations located inside ports, dockyards or marinas. RPLI: relative penis length index; RPSI: relative penis size index; VDSI: vas deferens sequence index; ISI: intersex index; %I: incidence (percentage of affected females)

Station	Code	N ♀	Shell height ($\bar{x}$/SD)♀	N ♂	Shell height ($\bar{x}$)♂	RPLI ($\bar{x}$)	RPSI ($\bar{x}$)	VDSI ($\bar{x}$/SD)	ISI ($\bar{x}$/SD)	%I
Nassarius reticulatus										
Deep-sea fishing port	*C*	27	26.9/2.8	26	26.3/3.1	80		4.8/0.7		100
North commercial port	I	31	25.3/1.9	25	23.6/1.4	57		3.8/0.9		100
Forte da Barra Norte	*j*	25	26.5/1.7	26	26.0/2.1	29		3.5/0.7		100
Av. F. Magalhães, C. Mira	k	26	26.1/2.1	29	25.3/1.8	25		1.9/0.9		100
Ponte Barra, Gramata	*l*	24	27.3/1.8	28	27.3/1.2	20		2.7/1.2		100
Costa Nova	m	24	27.5/1.5	25	27.3/2.0	27		2.3/0.8		100
Barra	*r*	30	26.0/2.3	29	25.4/1.7	8		1.2/0.6		89
S. Jacinto	s	31	26.0/2.5	25	26.0/2.4	10		1.1/0.8		96
Muranzel	*t*	24	25.5/2.2	25	26.7/1.9	10		1.7/1.3		100
Vagueira (Sea)	*y*	25	26.5/1.2	24	26.2/1.3	0		0.0/0.0		0
Costa Nova (Sea)	*z*	27	26.3/1.0	25	27.0/1.3	2		0.2/0.6		30
S. Jacinto (Sea)	za	25	26.3/0.8	26	27.0/1.2	0		0.0/0.0		0
Torreira (Sea)	zb	25	29.0/1.6	25	27.9/1.4	0		0.0/0.0		0
Hydrobia ulvae										
Aveiro, Pêga	b	33	6.0/0.8	31	6.2/0.4	15		0.9/0.7		60
Ponte Ílhavo	*d*	31	5.4/0.4	30	5.5/0.5	20		1.0/0.8		62
Ponte Ermida	f	33	6.5/0.6	31	6.4/0.7	14		1.0/0.4		68
Ponte Barra, Gramata	*l*	29	5.7/0.5	28	5.6/0.5	11		0.4/0.6		36
Juliões	*o*	28	5.8/0.4	28	5.9/0.4	18		1.1/0.7		75
Muranzel	*t*	28	6.6/0.8	27	6.5/0.6	4		0.4/0.5		50
Torreira	u	33	6.1/0.5	33	6.0/0.4	6		0.4/0.6		45
Ponte Varela	v	27	5.9/0.6	26	6.6/0.4	5		0.2/0.3		44
Ovar marina	W	27	5.7/0.4	27	5.8/0.4	10		0.8/0.4		88
Murtosa	x	28	6.2/0.2	26	6.2/0.2	5		0.3/0.4		50
Nucella lapillus										
Chemical port	H	7	27.2/3.6	4	23.5/2.0		38	4.4/0.5		100
Forte da Barra Norte	*j*	15	28.4/2.8	15	27.2/1.6		23	4.0/0.0		100
Barra	*r*	20	25.6/3.1	20	25.4/2.7		22	4.0/0.0		100
Vagueira (Sea)	*y*	25	24.6/1.5	26	23.7/1.3		20	4.0/0.0		100
Costa Nova (Sea)	*z*	22	24.3/1.2	24	22.5/1.2		22	4.0/0.0		100
Littorina littorea										
Deep-sea fishing port	*C*	28	25.9/1.7	15	26.3/1.5				0.4/0.4	54
Ponte Ílhavo	*d*	9	26.1/2.3	5	25.8/2.0				0.5/0.4	50
Ponte Barra, Gramata	*l*	9	26.5/2.5	7	25.3/1.9				0.3/0.5	33
Juliões	*o*	6	25.9/2.1	3	26.0/2.1				0.3/0.4	33
Muranzel	*t*	12	27.3/3.2	10	25.0/2.6				0.3/0.5	33

the observed distribution patterns of the species in the study area. The type of substrate, for instance, is known to be a major limiting factor, particularly to *Nucella lapillus*, which is highly selective for rocky shores. However, the clear relationship between the distribution patterns of the 4 studied species and the spatial trends of salinity in the Ria de Aveiro suggests that this is probably a key limiting factor of their distribution. The hydrobiid appears to be the most tolerant to low salinity as its distribution extends farthest towards the upper reaches of the channels. Despite the very low abundance of *L. littorea*, its distribution reflects a good tolerance to reduced salinity, probably ranking the species as the second most tolerant. The distribution patterns of *N. reticulatus* and *N. lapillus* suggest that these species respectively follow the periwinkle in decreasing order of tolerance to reduced salinity. This is in accordance with the limits of tolerance referred to in the literature: the mudsnail can live and breed between 5 and 40 psu (Graham 1988); the periwinkle can live indefinitely at 15 psu (Gowanloch & Hayes 1926 in Fretter & Graham 1994) and can be 'rather active' at 10 psu (Rosenberg & Rosenberg 1973 in Fretter & Graham 1994); the nettedwhelk can withstand a reduction of salinity to about 16 psu (Fretter & Graham 1984); and the dogwhelk is unable to feed under brackish conditions and does not breed down to 10 psu (Crothers 1985).

The observed complementary distributions of *Hydrobia ulvae* and *Nassarius reticulatus* almost cover the entire area of the Ria de Aveiro and the adjacent coastal zone. Moreover, both species are very abundant and sediment dwellers. In view of that, these 2 prosobranchs are particularly adequate to be used as key species for biomonitoring purposes in this environment. With regards to *Littorina littorea*, despite its tolerance to decreased salinity, its low abundance, probably linked to TBT-induced reproductive failure, reduces the number of available sites for monitoring. *Nucella lapillus* is restricted to the outer part of the Ria de Aveiro and to the groynes on the adjacent coast. This pattern cannot be merely explained by the substrate type since there are long artificial rocky banks in the S. Jacinto-Ovar, Mira and Ílhavo channels as well as in the main navigation channel, with plenty of food, where the species nevertheless is absent. Apart from other factors, its high vulnerability to TBT contamination may also contribute to the observed pattern.

Relative sensitivity to TBT

At a given site the individuals of each species may be contaminated by distinct sources of TBT due to differences of habitat and diet. It is known that sediments trap high quantities of dissolved TBT due to its preferential partitioning onto colloidal and particulate surfaces, which ultimately accumulate in the bottom (Batley 1996). The species *Nassarius reticulatus* and *Hydrobia ulvae* are sediment-dwellers and, consequently, may be directly exposed to higher environmental levels of TBT than, for instance, *Nucella lapillus*. *Littorina littorea* has a wider range of habitats in the Ria de Aveiro that prevents a straightforward sort of prediction. On the other hand, the diet is another main source of TBT contamination. The nassariid is a scavenger, with a preference from detritus among juveniles to carrion among adult snails (Tallmark 1980). The hydrobiid feeds on diatoms, some bacteria, silt and green algae like *Ulva* sp. and *Enteromorpha* sp. (Graham 1988). *N. lapillus* is a predator that feeds preferentially on barnacles and mussels (Crothers 1985). *L. littorea* feeds mainly on green algae, particularly those referred to above, and vegetable detritus (Graham 1988). Under favourable experimental conditions, half or more of the TBT in *N. lapillus* may originate from the diet, although in natural populations it can be considerably less (Bryan et al. 1989). In the case of *N. reticulatus*, food may contribute more than half of the TBT body burden (Stroben et al. 1992b). The latter authors found that, under laboratory conditions, both species present similar bioconcentration factors when exposed to the same level of water contamination and fed with a diet nearly free of TBT, and attributed different TBT accumulation in natural populations to the distinct contamination of each species' food. According to Bauer et al. (1997), the TBT bioconcentration factors for herbivorous prosobranchs, like the periwinkle, are generally lower than for carnivorous species, also mainly due to the different contamination of their food.

Another important factor of variation is related to the time of exposure, which, to a certain extent, is related to longevity: 1 to 5 yr for *Hydrobia ulvae* (Graham 1988, Sola 1996, Schulte-Oehlmann et al. 1997); 5 to more than 10 yr for *Nucella lapillus* (Crothers 1985, Gibbs & Bryan 1994); 10 to 15 yr for *Nassarius reticulatus* (Tallmark 1980); and more than 9 yr for *Littorina littorea* (Heller 1990). Furthermore, the intersex in *L. littorea* can be induced only in juvenile and sexually immature females (Bauer et al. 1997) whereas in the other species this does not strictly happen. Thus, different species may be subjected to different TBT levels of exposure at a given site, according to their life cycles and the local contamination history.

Despite these sources of variation, cross-comparisons of imposex/intersex responses of the different species at common sites provide very useful general information about the relative sensitivity to TBT (Table 2, Fig. 5). Considering the VDSI and ISI *Nucella lapillus* is the most TBT-sensitive species. No specimen exhibited a VDSI less than 4, and this level was found at sites where *Nassarius reticulatus* presented VDSIs from 0.0 to 3.5. The nettedwhelk may be ranked as the second most sensitive species, presenting in the study area the whole range of possible VDSI values; VDSIs of 1.7 and 2.7 in this species corresponded to a VDSI of 0.4 in *Hydrobia ulvae* and an ISI of 0.3 in *Littorina littorea*; the highest score attained at Stn C, a VDSI of 4.8, is related at the same site to an ISI of only 0.4 in the littorinid. The mudsnail and the periwinkle are thus the

Table 2. Cross-comparisons of the imposex or intersex intensity levels shown by the different species at common sites. At Stns y and z *Nucella lapillus* was collected at the intertidal and *Nassarius reticulatus* at 5 to 7 m depth (see text). The values represent RPLI/VDSI/%I for *N. reticulatus* and *Hydrobia ulvae*, RPSI/VDSI/%I for *N. lapillus* and ISI/%I for *Littorina littorea*

Stns:	C	d	l	j	o	r	t	y	z
N. reticulatus	80/4.8/100		20/2.7/100	29/3.5/100		8/1.2/89	10/1.7/100	0/0.0/0	2/0.2/30
H. ulvae		20/1.0/62	11/0.4/36		18/1.1/75		4/0.4/50		
N. lapillus				23/4.0/100		22/4.0/100		20/4.0/100	22/4.0/100
L. littorea	0.4/54	0.5/50	0.3/33		0.3/33		0.3/33		

least sensitive species, presenting in the Ria de Aveiro VDS and intersex stages not higher than 2a and 1, respectively. In spite of the low general abundance of *L. littorea* in the study area, the results (Table 1) indicate that this species has a lower potential for inter-site discrimination than *H. ulvae*. In general the results obtained in this study agree with the TBT water concentration threshold values for the development of imposex/intersex cited in the literature: less than 0.5 ng Sn l^{-1} in the water for *N. lapillus* (Gibbs et al. 1991); more than 1 ng Sn l^{-1} for *N. reticulatus* (Bryan et al. 1993); in the range of 20 ng Sn l^{-1} for *H. ulvae* (Schulte-Oehlmann et al. 1998); and about 10 ng Sn l^{-1} for *L. littorea* (Bauer et al. 1997). Despite the lower threshold value cited for *L. littorea* compared to that for *H. ulvae*, our results agree with those of Schulte-Oehlmann et al. (1998), who found that the mudsnail is more sensitive than the periwinkle at lower environmental TBT concentrations.

At Stns z and y *Nucella lapillus* was collected from the rocky groynes while *Nassarius reticulatus* was only found on neighbouring clean sandy bottoms (5 to 7 m depth). The low imposex levels of the nassariid (VDSI of 0.0 and 0.2) in comparison to the muricid (VDSI of 4) enhances the importance of distinct TBT exposure related to differences in habitat type and associated food items. Similarly, though to a lesser extent, the data reported by Huet et al. (1995) for the West French coast include some samples where low imposex levels in *N. reticulatus* (VDSIs of about 0.2 to 0.4) are related to high levels in *N. lapillus* (VDSIs of about 3.4 to 3.8).

As expected, the imposex/intersex intensities exhibited by the 4 species present a positive relation to TBT environmental contamination. In this sense all species provide useful additional information that helps to define TBT spatial and temporal trends, highlighting the importance of the availability of indigenous biomonitoring species. Nevertheless, the usefulness of the species is not equal and depends, among other factors, on their TBT sensitivity and the level of contamination of the area under study. Regarding this aspect, *Nassarius reticulatus* is the most appropriate species for monitoring the Ria de Aveiro as it presented a full range of imposex levels in response to the existing environmental gradient of TBT. While *Nucella lapillus* exhibited relatively uniform high values of RPSI, VDSI and %I, *N. reticulatus* showed a wide range of increasing values in the same area. This is in accordance with the findings of Bryan et al. (1993) and Huet et al. (1995), which consider the dogwhelk a useful test species for low concentrations of TBT (below 2 ng Sn l^{-1}) while the nettedwhelk is more appropriate for higher TBT levels. Similarly, but in the opposite direction, *Littorina littorea* and *Hydrobia ulvae* are too insensitive compared to *N. reticulatus*, particularly the former, which shows almost uniform ISI values for sites (e.g. Stns C and t) where the nassariid exhibits very distinct imposex responses.

Reproductive failure

No sterilised females of *Nassarius reticulatus* or *Hydrobia ulvae* were found in the Ria de Aveiro. However, the *Nucella lapillus* population at Stn H showed 29% sterilised females (females with a blocked vulva preventing egg capsule laying). This suggests that the species may have disappeared from the innermost sites as well as from the vicinity of Stn I due to the high levels of contamination rather than natural ecological factors. The present state of the remaining populations that show a VDSI of 4 highlights the risk of mass extinction if contamination increases in the future. At all sites sampled for *Littorina littorea*, 33 to 54% of the females were affected at ISI stage 1, i.e. the female genital opening is enlarged and the bursa copulatrix is split ventrally; consequently a loss of sperm during copulation is possible and the reproductive success may be reduced (Bauer et al. 1997). This could be possibly related to the notably decreasing abundance of this species in the Ria de Aveiro since 1992 (authors' unpubl. data).

Biomonitoring programs in the Ria de Aveiro

Biomonitoring environmental TBT contamination depends on the availability of indigenous indicator species and on a full knowledge of their ecotoxicological properties. In estuarine waters the typical half-life of TBT is 6 to 7 d at 28°C and in oxic surface sediments is of a similar magnitude; however, in deeper anoxic sediments degradation is much slower with half-lives varying from 1.9 to 3.8 yr (Batley 1996). The combination of a long half-life of TBT and the continuing inputs ensures that sediments in many areas will remain as a reservoir and source of TBT for many years (Langston & Pope 1995). Long-term TBT monitoring of sediment and water is thus of considerable importance in estuarine systems. We propose that *Nassarius reticulatus* and *Hydrobia ulvae* may jointly be used for sediment monitoring programs in the Ria de Aveiro and other similar estuarine systems. Their complementary distributions, common substrate and geographical ubiquity in the European coast make these species quite appropriate for monitoring sediments of moderately to highly contaminated estuarine systems. However, the different longevity of the species must be taken into account for temporal trend monitoring. It should also be considered that mudsnail juveniles are highly mobile, dispersing by floating at the water surface through routes where they may aggregate at temporary 'satellite' sites (Armonies & Hartke 1995); this high mobility has been recorded in the Ria de Aveiro (Meireles 1998) and may, at least in part, be responsible for the variability in the observed imposex responses. The distribution of *Nucella lapillus*, limited to the outer part of the Ria de Aveiro and Atlantic shore, restricts its usefulness for monitoring widescale spatial trends of TBT contamination but nevertheless permits a good assessment of general temporal trends since all water passes through the mouth region. Despite the high TBT sensitivity of the species, the levels of RPSI, VDSI and the percentage of sterile females in the populations of the Ria de Aveiro may still increase significantly. *Littorina littorea* appears to be a less useful species for monitoring the Ria de Aveiro because (1) its sensitivity to TBT is low, not providing a good inter-site discrimination, and (2) there are now very few available sites for obtaining good size samples. However, we strongly recommend that the populations of this species continue to be monitored and further studied in relevant aspects of its biology in order to find an explanation for its recent decline in the Ria de Aveiro.

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