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Imposex, organotin bioaccumulation and sterility of female *Nassarius reticulatus* in polluted areas of NW Spain

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ABSTRACT: Between July 1998 and January 1999, levels of imposex (superimposition of male characters upon females) and organotin bioaccumulation (tributyltin, TBT; dibutyltin; monobutyltin; triphenyltin) were measured in the gastropod *Nassarius* (*Hinia*) *reticulatus* (L.) at 15 localities in NW Spain. Sampling sites were scattered along 5 rías (rivers), 3 with large commercial harbours, 2 mainly given over to fishing activity. Both imposex and organotin concentrations showed that the rías with large commercial harbours were polluted with TBT to an extent similar to or even higher than contaminated areas in other parts of Europe, even when the data on the latter had been recorded before restrictions on the use of TBT-laden paints. However, rías lacking large vessel traffic also displayed substantial contamination, indicating that TBT pollution was not confined to the vicinity of the large trading ports. The masculinization of the gonadial section of the oviduct was graded in various stages (i.e. degree of convolution resembling the male seminal vesicle). This anomaly affected a substantial proportion of females (26.5%). It was most prevalent in females displaying advanced imposex, suggesting that after exposure to high TBT pollution, imposex is not constrained to the pallial gonoduct. The average degree of convolution (average oviduct stage, AOS) increased with TBT bioaccumulation up to levels above 2000 ng Sn g^{-1} tissue. AOS appears to be a better index for ranking severely polluted sites than customarily applied indices (relative penis length index, RPLI, and vas deferens sequence index, VDSI). A relatively large fraction (4 to 26%) of females suffering from advanced imposex bore masses of aborted egg capsules at all locations within 2 rías highly polluted with TBT. Such a high frequency of functionally sterile females is unparalleled in the scientific literature; *N. reticulatus* has been previously grouped with other gastropod species in which imposex does not causes sterilization. While still lacking an explanation for the mechanism leading to sterilization, logic indicates that imposex might be the cause. Imposex in *N. reticulatus* is a useful bioindicator of TBT in polluted areas, where its indicator ability may be enhanced by assessing the degree of masculinization of the gonadial oviduct. The presence of functionally sterile females at highly polluted sites suggests that TBT-induced imposex may sterilize female *N. reticulatus.*

KEY WORDS: Organotin · Imposex · *Nassarius reticulatus* · Sterile females · NW Spain

INTRODUCTION

The presence of male organs in female gastropods (a syndrome termed 'imposex') was first reported in the early 1970s (Blaber 1970, Poli et al. 1971, Smith 1971). A decade latter, Smith (1981b) showed that the agent causing this syndrome in the American mud snail *Ilyanassa* (*Nassarius*) *obsoleta* was the toxic compound tributyltin (TBT) employed in ship antifouling paints since the 1960s. Since then, the TBT-imposex link has become one of the best documented cases of endocrine disruption by a manufactured xenobiotic (for review see Matthiessen & Gibbs 1998). As early as the 1980s (i.e. before TBT was proved to be the causative agent

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for imposex), research data suggested that we might be facing another case of 'global pollution', as imposex was being identified in gastropods around the world (Smith 1981b). The detrimental effects of TBT on neogastropods and oysters are now well-established and have motivated several countries to regulate TBT-based antifoulants, mainly banning their use on boats shorter than 25 m in length (France in 1982, United Kingdom in 1987, USA in 1988, European Union in 1991).

To date, over 50 neogastropod species have been recorded as exhibiting imposex (Gibbs et al. 1997). Only a few have been studied in any detail and used to bioindicate the presence of TBT in the environment. Among them, the dog-whelk *Nucella lapillus* has been the main species of choice, to the point that imposex in this gastropod is now a 'recommended' biomarker for TBT pollution along the European shoreline (OSPAR-COM 1997). Dog-whelks are excellent indicators and have provided much of the current scientific evidence on the detrimental effects of TBT on neogastropods (reviewed in Gibbs & Bryan 1996). Still, its use as a bioindicator is limited in studies of muddy or sandy shores, low-salinity reaches of estuaries or areas severely contaminated by TBT, conditions under which this species is not usually found. In such cases, the sediment-dwelling netted whelk *Nassarius reticulatus* may be a good alternative. It flourishes in sedimentary areas and tolerates brackish water better than *N. lapillus* (Fretter & Graham 1994). The wider distributional range of *N. reticulatus* further increases its usefulness for studies of geographically extensive regions. *N. lapillus* has its southern distribution limit in Portugal and is largely lacking in Belgium, the Netherlands, Denmark, Baltic Sea and most of Germany. In contrast, *N. reticulatus* ranges south to the Canaries and Azores, and is also present in the Mediterranean and Black Seas (Fretter & Graham 1985). Although *N. reticulatus* has been recommended as a TBT biomonitor alternative to *N. lapillus* at contaminated sites (Stroben et al. 1992b, Bryan et al. 1993), this recommendation has not received much support.

Most of the coast of NW Spain has been surveyed for TBT using imposex in *Nucella lapillus* as a biomarker (Ruiz et al. 1998). In Ruiz et al.'s survey, the absence of this species (either because of the lack of appropriate environment or due to an excessive level of TBT pollution) precluded the study of some areas suspected as being highly polluted by TBT (e.g. those with intense maritime traffic such as the harbour area in the Ria de A Coruña or the entire Ría de Ferrol). TBT pollution at specific locations within those areas was later confirmed by mid-term (5 mo) *in situ* exposure experiments with *N. lapillus* (Quintela et al. 2000). However, a more comprehensive assessment of TBT pollution at allegedly polluted areas would require a more suitable bioindicator such as *Nassarius reticulatus*. In this paper, we present data on TBT bioaccumulation and imposex prevalence in *N. reticulatus*. The same species was sampled at other rías (rivers) not directly affected by large-vessel traffic. Unexpectedly, during this study we found a substantial proportion of *N. reticulatus* females bearing masses of aborted egg capsules at some TBT polluted locations. Such a high frequency of functionally sterile females has not been previously reported and we consider this observation interesting enough to deserve comprehensive examination.

MATERIAL AND METHODS

Sampling. *Nassarius* (*Hinia*) *reticulatus* (L.) were collected by hand from shallow water during spring low tides between July 1998 and January 1999. Only large individuals were selected to assure that they were sexually mature. Sampling sites were located within 5 rías affected by differing levels of maritime traffic (Fig. 1). Both the Ría de Betanzos and the Ría de Muros (sampling Sites 3 to 6 and 12, respectively) have coastal fishing ports and a few small marinas scattered along their shorelines, whereas the Ría de Ferrol (sampling Sites 1 and 2), Ría de A Coruña (7 to 11), and Ría de Vigo (13 to 15), each have a large trading port (e.g. Ferrol harbour received 985 merchant vessels totalling 7.5 million gross register tons [GRT] in 1997, while A Coruña harbour was visited by 1059 ships amounting 10 million GRT during 1998; vessels larger than 10 000 GRT accounted for 75% of the tonnage in both cases). In addition, the Ría de Ferrol accommodates 2 large dry-dock and shipyard facilities. Both A Coruña and Vigo harbours are home ports to important deep-sea fishing fleets (fishing boats ≥30 to 35 m in length) and have shipyard facilities for their maintenance (antifouling painting included). Coastal fishing ports and small marinas are also present within the Ría de A Coruña and Ría de Vigo. Yachts are moored year-round at Site 4 in the Ría de Betanzos and at Site 10 within A Coruña harbour.

Evaluation of imposex. Individuals were narcotised (7% $MgCl_2$) before measurement. After measuring and removing the shell, females were identified by the presence of a dark sperm-ingesting gland. Penis lengths were measured to the nearest 0.01 mm with a digital calliper. The vas deferens sequence (VDS) stage was determined for each female by examining its genitalia under a stereomicroscope. Females were ranked (0 to 4+) by a slightly modified version of the method of Stroben et al. (1992a). Females with only a tiny penis or a very short distal vas deferens (VD) behind the right ocular tentacle were assigned to VDS Stage 1. Those

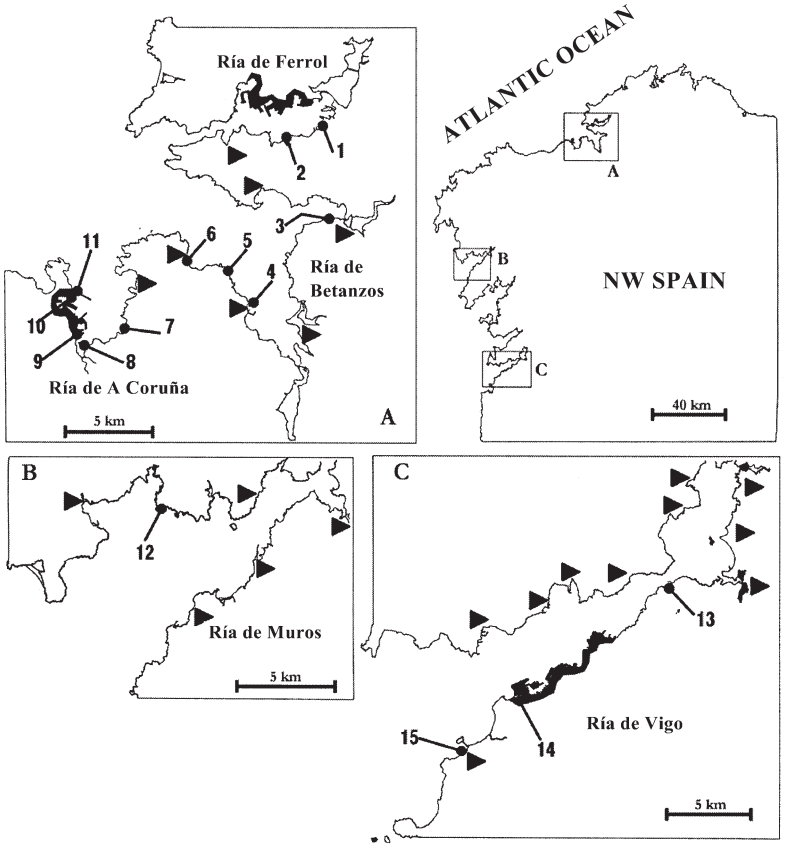

Fig. 1. *Nassarius reticulatus* in Galicia (NW Spain). Sampling sites numbered as in Table Shaded areas indicate major commercial harbours; triangles represent small fishing ports

bearing a penis together with a distal VD were ranked as Stage 2 if VD length was less than one half the distance between the base of the penis and the vulva, and as Stage 3 if it was more than one half that distance. Stage 4 corresponded to females whose VD extended from the penis to the vulva (in *Nassarius reticulatus*, the VD does not contact the oviduct at the genital papilla). Whenever the snail's VD extended further than the genital papilla, the individual was ranked as Stage 4+. At this stage, the VD usually runs into the ventral channel of the capsule gland as described by Stroben et al. (1992a). However, in some specimens the VD followed a course parallel to the oviduct beyond the vulva, ending in the posterior section of the pallial cavity instead of contacting the oviduct. Imposex intensity at each locality was evaluated estimating commonly used indices: the relative penis length index (RPLI, average female length/average male length as percentage) and the vas deferens sequence index (VDSI, average VDS value of a sample). Stage 4+ was given a numerical value of 4.5 for VDSI estimates.

In 10 of the 15 locations (Sites 1 to 3, 5, 8, and 11 to 15), 260 females were examined for the condition of their gonadial oviduct. After inspecting the first samples, it became apparent that not only the pallial gonoduct but also its gonadial section displayed some degree of masculinization. Similar observations had already been made for *Ilyanassa obsoleta* (Smith 1980), in which the convolution of a normally straight gonadial oviduct was considered a sign of masculinization since it resembled the male seminal vesicle. The seminal vesicle in male *Nassarius reticulatus* is also convoluted, while the gonadial oviduct in females is a thin straight duct. In females expressing imposex, we found changes in the appearance of the gonadial oviduct, which departed from the typically thin and straight condition to become a winding, sometimes thicker, duct. Consequently we tentatively ranked the degree of masculinization of the gonadial oviduct by a simple 3-stage ordinal scale: Oviduct Stage (OS) 0 was used for females with a normal straight oviduct, OS 1 for females whose oviduct was slightly sinuous, and OS 2 for those exhibiting a clearly convoluted gonadial oviduct. At each location, the average condition of the gonadial oviduct was estimated as the average OS value (AOS) and employed as an supplementary index of the degree of imposex.

Organotin analysis. The concentration of organotin (OT) species (monobutyltin, MBT; dibutyltin, DBT; tributyltin, TBT; and triphenyltin, TPhT) was measured in whole tissues of pooled females. Analyses were performed in a blind manner, i.e. analysts did not know which sites (polluted or clean) individuals had come from and therefore had no inital bios as to results, by Laboratoire de Chimie Analytique Bio-inorganique at Environment (LCABIE, Unité Mixte de Recherche [UMR] 5034, Centre National de la Recherche Scientifique [CNRS], Pau, France) following a procedure detailed by Szpunar et al. (1996). Briefly, aliquots of lyophilized tissue were digested with 5 ml of 25% tetramethylammonium hydroxide by low microwave power. Digested samples were diluted with 15 ml of water, their pH adjusted to 5 with concentrated acetic acid, and buffered with 5 ml of 1 M sodium acetate buffer (pH 5.0). OTs were ethylated by adding 1 ml of 1% (w/v) sodium tetraethylborate (made daily) and extracted to 1 ml of isooctane. The organic phase was analyzed by GC atomic emission detection. Tripropyltin was used as internal standard. The procedure was validated using certified reference material (CRM) BCR 477 (mussel tissue); recovery values were 96% for TBT and 92% TPhT. The quantification limit was 10 ng g^{-1} dry weight (DW) as Sn, and results are reported as ng Sn g^{-1} DW.

Table 1. *Nassarius reticulatus*. Biometrical, imposex and organotin data for sites shown in Fig.1. No. with capsules: number of females found with aborted egg capsules. See 'Materials ansd methods' for details of vas deferens sequence (VDS) stages. RPLI: relative penis length index; VDSI: vas deferens sequence index; TBT: tributyltin; DBT: dibatyltin; MBT: monobutyltin; TPhT: triphenyltin; nq: not quantifiable

Site / Ría	TPhT	Males Shell height (n)	Males Shell height mm	Males Shell height SE	Males Penis length mm	Males Penis length SE	Females Shell height (n)	Females Shell height mm	Females Shell height SE	Females Penis length mm	Females Penis length SE	RPLI	VDSI	VDS Stage (n) 0	1	2	3	4	4+	No. with capsules	Tissue (ng Sn g^{-1} DW) TBT	DBT	MBT
Ferrol																							
1 Maniños	68	(36)	24.1	0.37	11.9	0.31	(66)	24.0	0.27	10.3	0.23	86.7	4.5						66	11	1576	897	122
2 Seixo	44	(40)	23.3	0.35	10.1	0.30	(57)	23.2	0.29	9.5	0.25	93.7	4.5						57	7	1502	831	95
Betanzos																							
3 Centroña		(6)	21.3	0.91	10.7	0.77	(14)	22.6	0.59	4.5	0.50	42.3	3.1			1	11	1	1				
4 Sada	125	(92)	21.9	0.23	8.8	0.20	(9)	24.0	0.74	7.8	0.63	88.5	4.2					5	4		866	275	60
5 Arnela	nq	(33)	26.7	0.40	12.7	0.33	(47)	27.7	0.34	2.2	0.28	17.4	2.3	1	5	25	13	2	1		72	114	nq
6 Cirro	nq	(47)	21.4	0.32	9.5	0.27	(50)	22.4	0.31	5.4	0.27	56.6	3.7		2	3	12	16	17		524	214	20
A Coruña																							
7 Santa Cruz	47	(97)	23.7	0.23	9.8	0.19	(50)	25.3	0.31	8.6	0.27	87.3	4.3					18	32	2	708	650	42
8 Sta Cristina	35	(177)	25.6	0.54	9.2	0.46	(31)	25.4	0.52	8.0	0.44	86.9	4.5						31	8	478	275	23
9 Oza	27	(65)	25.7	0.28	11.4	0.23	(42)	26.8	0.34	9.4	0.29	82.9	4.3					14	28	3	1107	471	49

RESULTS

Imposex

Biometrical and imposex data for *Nassarius reticulatus* are summarized in Table 1. Average shell length was slightly but significantly larger in females than in males (0.85 mm average difference between genders, $p < 0.0001$ 2-way ANOVA with site and gender as main factors), although differences between sexes varied across samples ($p = 0.034$ for the interaction term). The incidence of imposex was widespread among the studied populations. All collected females showed signs of imposex in 13 of the 15 sampling sites while at sites 5 and 12 more than 95% of females were affected. The degree of imposex, estimated as RPLI and VDSI, exhibited a wider range of values. RPLI estimates went from a low 17% at Site 5 to a high 94% at Sites 2 and 13, while VDSI ranged from 2.3 at Site 5 to 4.5 at Sites 1, 2, 8, and 14 (VDSI 4.5 is the maximum possible value assigned to VDS Stage 4+ in this study, and the value used to record VDS Stage 4+). Both indices were highly correlated across samples ($r = 0.93$); only Site 15 deviated from the general relationship, with a VDSI slightly lower than would be expected from its RPLI value. These imposex indices increased with TBT bioaccumulation only over a narrow range up to 500 ng Sn g^{-1}, levelling out around 85 to 95% for RPLI and 4.3 to 4.5 for VDSI at higher TBT body burdens (Fig. 2a).

The highest degree of imposex was clearly linked to rías with a large commercial harbour. Thus all sites within Ferrol, A Coruña and Vigo, showed the highest values for both indices. Within these estuaries, RPLI regularly exceeded 80%, while VDSI commonly surpassed 4.3, often reaching the maximum of 4.5. An exception was Site 15. Imposex indices (RPLI, VDSI) in the Ría de A Coruña did not differ greatly between sites within the harbour (Sites 9 to 11) and those located on the opposite shoreline (Sites 7 and 8).

Samples from rías with little or no commercial shipping (Betanzos and

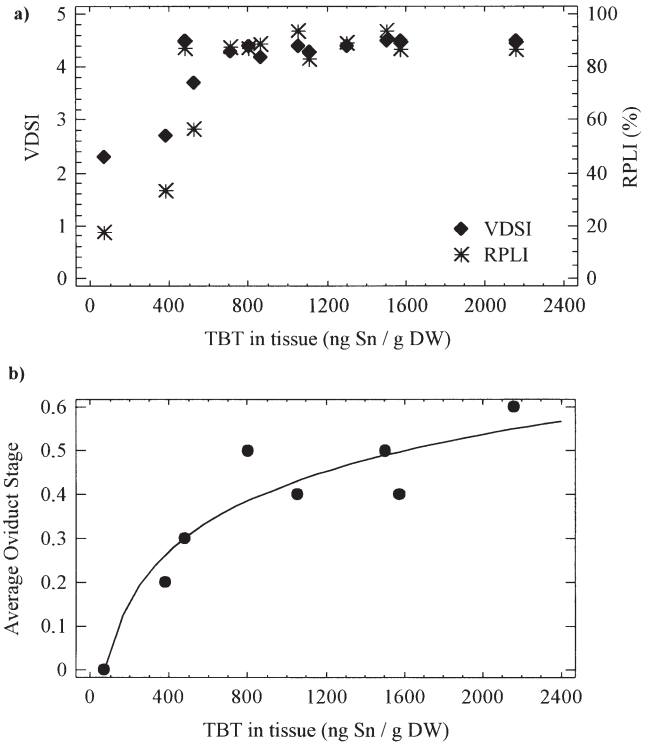

Fig. 2. *Nassarius reticulatus* in Galicia. Relationships between (a) degree of imposex quantified as RPLI or VDSI and body burdens of TBT, and (b) average alteration of the gonadial oviduct and TBT tissue bioaccumulation (line fitted by minimum-squares method, r = 0.94)

Muros) manifested lower degrees of imposex: RPLI mostly below 60% and VDSI below 4.0 (Table 1). Another distinctive feature of these samples was the wide range of VDS stages among sampled females (the entire range of possible VDS stages was found at Sites 5 and 12), in sharp contrast to samples from rías with intense commercial shipping where most females were ranked either as Stage 4 or 4+. Among those sites within rías lacking a large commercial port, Site 4 was the exception. Here the degree of imposex reached levels similar to those at Ferrol, A Coruña or Vigo. These specimens were collected right inside a small marina sheltering yachts and small boats year-round.

Gonadial oviduct abnormalities

Gonadial oviduct anatomy was examined in 260 females from the following sites: Site 1 (39 females), 2 (38), 3 (12), 5 (12), 8 (15), 11 (6), 12 (55), 13 (26), 14 (16), and 15 (41). The purpose was to obtain representatives from the larger data set covering the full range of imposex expression according to VDS stage. Of those examined, 191 (73.5%) presented a normal gonadial oviduct (OS 0), 51 (19.6%) a slightly sinuous gonadial oviduct (OS 1), and 18 (6.9%) a convoluted oviduct (OS 2). Oviduct malformations were observed in every sample examined, except for those collected at Sites 3 and 5. The probability of developing this type of malformation varied significantly as a function of the VDS condition of the female (*G*-test for independence, contingency table OS × VDS, $p < 0.0001$): deformed gonadial oviducts were mostly restricted to females displaying advanced imposex (Fig. 3). The average condition of the gonadial section of the oviduct (AOS) was significantly correlated with TBT body burden in females (correlation coefficient = 0.94), following an asymptotic model (Fig. 2b).

Aborted egg capsules

An irregular brown mass was found inside the capsule gland of some females in 7 out of the 15 localities (Table 1: 'No. with capsules'). These masses were of firm material, with occasionally a rough surface. Some large masses almost filled the capsule gland, measured up to 5 mm in length and 3 mm in width, and were easily distinguishable through the capsule gland walls. Frequently they were slightly flat, resembling somewhat the space between the lobes of the capsule gland. The larger masses appeared to be fragmented. Based on their position within the snails, their appearance, and their similarity with previous observations for *Nucella lapillus* (authors' pers. obs.), these masses were diagnosed as aborted egg capsules; females bearing them are hereafter referred to as 'functionally sterile' or simply 'sterile'. Sterile females always be-

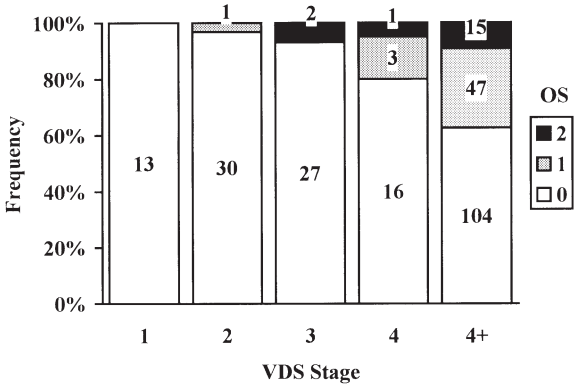

Fig. 3. *Nassarius reticulatus.* Relationship between VDS stage, and condition of the gonadial oviduct in females. Bars represent relative frequency of 3 gonadial oviduct stages (OS) within each VDS stage; numbers are actual number of females (the gonadial oviduct of only 1 female at VDS Stage 0 was examined; it was ranked as Stage 0, and has been excluded from the graph). See 'Materials and methods' for details of oviduct stages and sampling locations

longed to those ranked as VDS Stage 4+, whose VD extended beyond the vulva and ran into the ventral channel of the capsule gland. No obvious external signs were evident to distinguish functionally sterile females from others also ranked as VDS Stage 4+ but lacking aborted capsules.

Sterile females were recorded at every site sampled within the rías of Ferrol and A Coruña, ranging from 4 to 26% of females per site. They were absent from sites sampled at the other 3 rías. The proportion of sterile females (sterility rate) significantly differed between sites (*G*-test for homogeneity of proportions = 74.72 with 14 df, $p < 0.0001$). Groups of sites differing significantly from the others were distinguished by a simultaneous test procedure for unplanned comparisons of the homogeneity of proportions (Sokal & Rohlf 1995). Two overlapping, significantly different groups were identified (Fig. 4). One group comprised the 8 sites with no sterile females plus Site 7 (where sterile females were recorded at their lowest frequency). The other group comprised the 7 sites with sterile females. Although sterility rate did not significantly differ between sites with sterile females, their *G* value was unusally high (*G*-test = 11.92, experimentwise $p = 0.103$; for more detailed explanation of experimenwise error rate, see Sokal & Rohlf 1995). From inspection of the data it seemed apparent that sterility rate might be correlated with the degree of imposex (VDSI) at each site. Thus, a test for a linear trend in the proportion of sterile females was applied to the data from A Coruña and Ferrol using VDSI as the ranking criterion (method by Armitage, cited in: Zar 1996). Testing for trends among proportions is a more powerful procedure than testing for difference among proportions, since the null hypothesis of no trend might be rejected even when the null hypothesis of equality of proportions is not (Zar 1996). Our analysis showed a significant trend of an increasing proportion of sterile females with increasing degrees of imposex (chi-square = 5.6255, 1 df, $p = 0.018$). The lack of significance in the departure from the linear trend ($p = 0.366$) indicates that most of the differences among proportions for the 7 sites with sterile females is due to this linear trend.

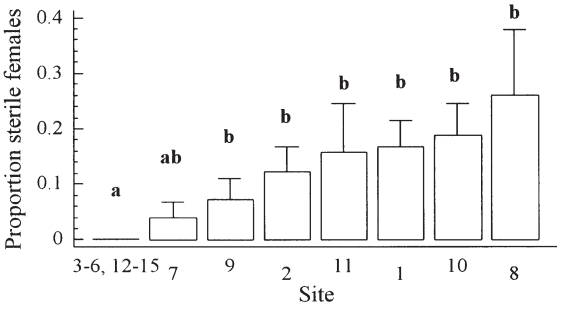

Fig. 4. *Nassarius reticulatus.* Relative frequency (+ SE) of sterile females. Bars sharing a common letter were significantly homogeneous (experimentwise error <0.05) in simultaneous unplanned *G*-tests. Sites with no sterile females have been grouped (3–6, 12–15) for clarity

Organotin bioaccumulation

Total butyltin tissue concentrations (BT) ranged from 186 to 2841 ng Sn g^{-1}. TBT tended to be the dominant species (37 to 72% BT) followed by DBT (22 to 59%), both butyltin species being significantly correlated ($r = 0.78$). MBT constituted only a minor fraction of total BT body residues (2 to 5%). The highest bioaccumulation values (>1500 ng BT-Sn g^{-1}) were measured at places within or near a major trade port (namely the 2 locations within Ferrol, Sites 9 and 10 in A Coruña, and Sites 13 and 14 in Vigo). In contrast specimens sampled at Ría de Betanzos and Ría de Muros accumulated lower BT body burdens (<1000 ng Sn g^{-1}), except at Site 4 where total bioaccumulation (1201 ng BT-Sn g^{-1}) was similar to that measured for rías with intense ocean-going shipping. Finally, TPhT bioaccumulation levels were lower that those for BTs, with a maximum concentration of 125 ng Sn g^{-1}. Its geographical distribution followed a pattern quite different from that for BTs. The highest values were not recorded close to large commercial ports, but at places were yachts are commonly moored (namely 125 ng Sn g^{-1} at Site 4 in Ferrol and 97 ng Sn g^{-1} at Site 10 in A Coruña).

DISCUSSION

TBT pollution in the study area

As in many other parts of Europe, it is virtually impossible to find a pristine, unpolluted place on the coasts of NW Spain. The whole littoral is affected to some extent by shipping activities and, among other compounds, TBT contamination. The approximately 1700 km-long coastline of Galicia supports 5 large commercial harbours and 122 smaller primarily fishing ports, as well as an undetermined number of tiny mooring places. Thus it is not surprising to find TBT spread throughout the region's coast. However, since the partial regulation on TBT, it has been suggested that concern for this toxicant be limited to major commercial ports that are frequented by large vessels, and that the biological hazard posed by ambient levels diminishes with distance from such 'hot-spots'(Evans 1999).

Contrary to this picture, we found TBT at every collection site and at concentrations high enough to induce complete or near complete incidence of imposex in the moderately-sensitive *Nassarius reticulatus*. As

expected, the greatest exposure was within rías with a major commercial port. Here, TBT in *N. reticulatus* was similar to or slightly greater than values reported for polluted sites in other parts of Europe. Thus, *N. reticulatus* collected from Brittany and Normandy between 1988 and 1991 yielded tissue concentrations of 600 to 1400 ng TBT-Sn g^{-1} at places polluted by vessel activity (e.g. Roscoff harbour) (Stroben et al. 1992a), whereas, body burdens from several hundreds up to a maximum of 3000 ng TBT-Sn g^{-1} in pollution-prone sites of SW England were measured in *N. reticulatus* before the 1987 partial ban (Bryan et al. 1993). Moreover, the degree of imposex was even higher in our study: we commonly found RPLI values exceeding 80% while estimates ranged between 60 and 80% in Brittany-Normandy and between 58 and 82% at TBT-polluted places in SW England. Thus, Galician sites linked to major shipping activities are contaminated with TBT to an extent similar to or even greater than other polluted areas in Europe, in cases where the data on the latter were recorded under pre-restriction conditions.

However, TBT pollution is important not only in rías frequented by ocean-going ships. Our study suggests that pollution from large commercial harbours impacts large areas within estuaries. Stroben et al. (1992a) found organotin levels of the order of 15 to 100 ng TBT-Sn g^{-1} at sites some distance away from sources of TBT contamination. According to their estimated relationships, such level of bioaccumulation would correspond to a RPLI lower than 10% and a VDSI below 3. Also, *Nassarius reticulatus* collected some distance from commercial ports in SW England commonly accumulated TBT below 50 ng Sn g^{-1} with a RPLI lower than 59% (usually 10 to 35%) under post-ban conditions (Bryan et al. 1993). In comparison, we found TBT in *N. reticulatus* up to 524 ng Sn g^{-1} and imposex ranging from 17 to 56% for RPLI and 2.3 to 3.7 for VDSI at rías with no commercial shipping (values for Site 4 excluded because of its year-round utilization and resultant atypical data). Hence, while large trade ports appear to be important sources of TBT, rías lacking commercial shipping still display a considerable level of both imposex and TBT tissue concentrations.

Potential sources for this pollution are difficult to identify. On the one hand, TBT pollution from commercial shipping may be affecting areas not immediately adjacent to major ports. Waters exiting highly contaminated rías may feasibly carry high levels of TBT, presumably high enough to induce imposex distant in areas. On the other hand, shipping is not restricted to commercial vessels. Galicia shelters one of the largest fishing fleets in the European Union (over 8800 boats totalling more than 250 000 GRT), mostly devoted to coastal fishing (around 8100 boats). In addition, >1000 boats are employed in aquaculture activities year-round within the main rías. Altogether this represents a substantial level of shipping activity. A large fraction of this fleet is made up of small boats (around 6000 to 7000) and should thus be liable to the restrictions on the use of TBT paints enforced by the European Union in 1991. This still leaves a sizeable number of boats excluded from such restrictions because of their small size. We have observed TBT-laden antifoulants employed at shipyard facilities devoted to the maintenance of fishing boats. Since boats of various lengths are repaired at the same facility, it is feasible that contamination by a large proportion of TBT-containing antifouling paints is not covered by present regulations. In this connection, the Marine Environment Protection Committee of the International Maritime Organization has urged international governments to take appropriate steps to reduce the use of TBT paints on small boats (e.g. extending the current ban for boats <25 m to include those <50 m in length) and those primarily operating in coastal waters (Bosselmann 1996).

Gonadial oviduct malformation

Largely forgotten in imposex studies is that not only the pallial oviduct but also its gonadial portion is susceptible to masculinization in some gastropods. Most references to a deformed ovarian duct are restricted to the mud snail *Ilyanassa obsoleta* (Smith 1971), for which the degree of convolution has been employed to quantify (Smith 1980), diagnose (Bryan et al. 1989) or categorize the degree of imposex (Curtis and Kinley 1998). However, it is known that gonadial oviduct malformations are not limited to *I. obsoleta* and a coiled oviduct has been observed for *Nassarius reticulatus* as well (Stroben et al. 1992a).

Our results show that an important portion of females (26.5%) exhibited signs of masculinization of their gonadial oviduct. The presence of a malformed gonadial oviduct significantly depends on the VDS stage of the female ($p < 0.0001$, Fig. 3). Thus, masculinization of the gonadial section of the oviduct is a 'late development', mostly limited to females exposed to high toxicant levels and belonging to VDS Stages 4 or 4+. Of those females ranked as VDS Stage 4+ and 4, 37 and 20%, respectively, displayed an abnormal gonadial oviduct. In contrast, this deformity was observed in only 7 and 3% of those assigned to VDS Stages 3 and 2, and never appeared in females ranked as VDS Stages 1 or 0. This agrees with the previous observation that penis presence is more common than a VD and the frequency of the VD is significantly greater than that of the abnormal gonadial oviduct in *Ilyanassa obsoleta* (Smith 1980). Such a 'late-detection' method

is a serious drawback for monitoring purposes, especially when the degree of imposex (penis growth, VD development) is readily quantifiable by sensitive and widely accepted indices (RPLI, VDSI). Likewise, determination of gonadial oviduct condition and even VD development have occasionally been discarded for the evaluation of the degree of imposex in favour of penis development because of the ease of measurement and higher sensitivity of the latter method (Smith 1981a).

However, changes in the gonadial oviduct could be used to discriminate the degree of pollution in highly polluted areas. Our results illustrate that both RPLI and VDSI reach a plateau at moderate TBT bioaccumulation (about 500 ng TBT-Sn g^{-1}: Fig. 2a). Similar results have been obtained for *Nassarius reticulatus* (Stroben et al. 1992b, Bryan et al. 1993) as well as for other gastropods (*Ocenebra erinacea*, *Nucella lapillus*; e.g. Huet et al. 1995) whose imposex indices, after reaching a plateau, could no longer distinguish the degree of pollution in heavily polluted locations. In our study, both indices were unable to differentiate between *N. reticulatus* living under polluted conditions that resulted in body residues of (say) 1000 ng TBT-Sn g^{-1} and others in which a more intense contamination had induced a 2-fold higher bioaccumulation. In contrast, the average condition of the gonadial section of the oviduct (AOS) allows ranking of highly polluted places. According to Fig. 2b, AOS is related to TBT levels in females and increases with TBT bioaccumulation beyond the saturation levels found for RPLI or VDSI. From an AOS of 0 at low-pollution locations (<400 ng TBT-Sn g^{-1}), AOS increases to 0.2–0.3 at moderately polluted sites (~400 to 500 ng TBT-Sn g^{-1}), rises to 0.4–0.5 at clearly contaminated places (800 to 1600 ng TBT-Sn g^{-1}) and is still able to differentiate to 0.6 at severely polluted areas (>2000 ng TBT-Sn g^{-1}).

Even though the evidence presented above is based on a limited number of sampling locations, it does suggest that alterations in the gonadial oviduct in *Nassarius reticulatus* have potential as a supplementary index for ranking areas severely polluted with TBT. *N. reticulatus* is one of the few TBT biomonitors usually available at heavily contaminated sites. The low sensitivity of the present protocol recommends the use of imposex in *N. reticulatus* as a biomarker of TBT pollution along European coasts, particularly for monitoring whether pollution is actually decreasing at highly polluted sites as a result of legislative measures.

Aborted egg capsules

As stated in the 'Introduction', *Nassarius reticulatus* may surpass the dog-whelk *Nucella lapillus* as a bioindicator of TBT pollution in muddy-sandy areas, low-salinity reaches and highly polluted areas. However, dog-whelk populations may decline or even die out as a result of the sterilization caused by blockage of the pallial oviduct (Bryan et al. 1986). No comparable effect has been established for *Nassarius reticulatus*, in spite of the fact that the latter species has been the object of several imposex studies (Stroben et al. 1992a,b, Bryan et al. 1993, Huet et al. 1995, Stroben 1996). Even at places heavily polluted with TBT, female *N. reticulatus* retain a functionally normal ovary, female glands and genital papilla in the presence of imposex (Stroben et al. 1992a). Hence, imposex in *N. reticulatus*, while constituting a useful biomarker, lacks the appeal and relevance of the critical population impact in *N. lapillus*.

In contrast to this widespread perception, we have found a statistically significant portion of female *Nassarius reticulatus* bearing aborted egg capsules within their capsule gland at every location sampled within the 2 most polluted rías (Ría de A Coruña and Ría de Ferrol). Our observations suggest a relationship between female functional sterility and the type and extent of development of the VD due to TBT exposure. Aborted capsules were found only in females displaying a well developed VD (Stage 4+), and only in those individuals whose VD ran into the ventral channel of the capsule gland. Statistical tests also revealed a significant trend for the proportion of sterile females to increase with the increasing degree of imposex among sites at A Coruña and Ferrol. This further supports the possible link between the development of imposex and functional sterilization of females. The range of imposex values (VDSI) employed for this test was necessarily narrow (4.3 to 4.5) as sterile females were found only at sites heavily polluted with TBT. However, it still represented a change in the intensity of imposex at every site. VDSI values below 4.5 indicated that a portion of females was still liable to further imposex advance (those ranked VDS Stage 4), while at sites with a VDSI of 4.5 (100% of females staged 4+) none could develop imposex further.

The suggested relationship requires further investigation. Other females with equally advanced imposex retained their breeding ability. Nevertheless, it does not preclude the possibility that imposex VD development may be responsible for the functional sterility signalled by the presence of aborted egg capsules. For example, *Nucella lapillus* females are currently exposed to TBT in NW Spain coastal waters to a point where most females (>70% on a regional basis) develop a complete VD whilst retaining their ability to release egg capsules, yet a few (<18%) females do display further development of their VD, rendering them sterile (Barreiro et al. 1999). The mechanism precluding the release of egg capsules in *N. lapillus* is known, and this condition can be determined visually; sterility is indicated by a VD which either

overgrows, constricts or displaces the genital papilla. However, we presently lack comparable evidence supporting a role of the VD in preventing egg capsule release in *Nassarius reticulatus*.

Based on the arrangement of the imposed male organs in imposex-affected females, *Nassarius reticulatus* has been classified among other gastropods in which imposex does not lead to female sterility (Gibbs et al. 1997). External inspection of the female genitalia, employed in this study, has failed to reveal what happens to the VD once it reaches the ventral channel (i.e. whether it stops outside the gland or keeps growing within the ventral channel). In *Ilyanassa obsoleta*, another nasariid, the imposex VD forms a continuous passage with the ventral channel of the pallial oviduct (Smith 1980). Should the VD evolve in a comparable way in *N. reticulatus*, then its growth might interfere with the normal function of the pallial oviduct. Alternatively, Huet et al. (1995) reported finding 2 sterile *N. reticulatus* females (1 with a testis) at a site highly contaminated with TBT. These authors disregarded the VD tissue proliferation blocking the vulva as the reason for sterilization, but noted the presence of excrescences of tissues in the bursa copulatrix narrowing the vaginal channel, the latter being interpreted as vaginal neoplasic tissues. According to this interpretation, sterility in *N. reticulatus* may not be directly linked to VD development. Nonetheless, Huet et al. did ascribe sterilization to TBT, although adding that high concentrations of the toxicant were required to achieve it in *N. reticulatus*.

In this study, female *Nassarius reticulatus* displaying advanced imposex were encountered in several estuaries, but sterile individuals were recorded in only 2 of them. Further, samples from rías other than Ferrol and A Coruña (e.g. Sites 13 and 14 from Vigo) also reached maximum VDSI while lacking sterile females. There is no obvious difference in the level of exposure to TBT between females at highly polluted locations where sterile females are never found (Ría de Vigo), and those collected at locations where sterile females are often found (Ría de A Coruña and Ría de Ferrol). Differences in time of exposure are improbable. In these 3 rías, TBT stems from a similar source, a large port with year-round activity. In contrast to other sources of TBT where pronounced seasonality might be expected (e.g. marinas with a TBT peak in spring-summer when most recreational boats are repainted) there is no reason for a comparable seasonal fluctuation in TBT at commercial harbours. Because of the similar sources of the TBT in these 3 rías, there should be little significant difference in the way their *N. reticulatus* populations are exposed to the toxicant.

Sampling errors can also be ruled out as the reason for the lack of sterile females at Sites 13 and 14. The proportion of sterile females differed significantly between sites with and sites without sterile females, despite the use of conservative experimentwise error rates (Fig. 4). Statistical tests failed only to detect very small differences among proportions; e.g. such as those between Site 7 (sterility rate = 0.04) and sites with a sterility rate of zero. Impracticably large samples would be required to attain the statistical power required to detect differences on this small scale.

On the other hand, the expression of imposex is variabile. This is well-established for *Nucella lapillus*, where variants include the development of the VD but not the penis (Oehlmann et al. 1991) or resistance to the masculinizing effect of TBT (Gibbs 1993). *Nassarius reticulatus* is also liable to comparable morphological variations; a small number of females have been found with a developed a VD but not a penis in populations around Brittany and Normandy (Stroben et al. 1992a) as well as in the present study. Thus, the described differences between populations may reflect variations in the expression of imposex between populations inhabiting the various rías. However, this hypothesis is difficult to test until we have identified the exact malformation mechanism that renders these females sterile.

The lack of a clear understanding of the mechanism of sterilization in *Nassarius reticulatus* should warn against an unfounded hasty linkage to TBT exposure. Clearly, further studies are needed to elucidate it. However, one cannot ignore the evidence presented here that those sterile female *N. reticulatus* that displayed the most advanced degree of imposex were only found in highly polluted areas, and their incidence increased with increasing degree of imposex. Consequently, we would at least like to suggest the possibility that TBT-induced imposex may be the causative factor leading to sterilization in *N. reticulatus* in such areas. In this connection, we intend to carry out a geographically broader survey within the region in search of further clues as to whether sterile female *N. reticulatus* are found in other locations/rías and whether sterility is always linked to advanced imposex stages. We also plan to expend further effort on discovering the mechanism responsible for this sterility.

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