USEFULNESS OF *NASSARIUS RETICULATUS* IMPOSEX LEVELS TO MONITOR DECREASING LEVELS IN TBT POLLUTION: FIELD AND LABORATORY STUDIES

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Abstract
Organotin (OTs) compounds were banned from antifouling paints in the European Union in 2003 (2002/62/EC directive) due to the high toxicity to non-target species. The aim of the present work is to assess any obvious decline of the OT environmental levels in Ria de Aveiro (NW Portugal) after the ban. In May of 2005 the organotins - monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), monophenyltin (MPT), diphenyltin (DPT), triphenyltin (TPT), monoctyltin (MOT), dioctyltin (DOT) and trioctyltin (TOT) – were quantified in the gastropod *Nassarius reticulatus*. The imposex (imposition of male characters on females) in this species was additionally used as a biomarker of TBT pollution. The link between imposex and TBT contamination is clearly demonstrated by field correlations and laboratory experiments. Time comparisons show a slight decrease of imposex between 2003 and 2005 probably as a consequence of the EU ban.

Introduction
Organotin compounds (OTs), particularly tributyltin (TBT), have been widely used since de mid 1960s as biocides in antifouling paints. The harmful effects of TBT in marine ecosystems lead governments to adopt restrictions on the use of organotin antifouling paints in the 80's and 90's. Regardless the restrictions, TBT pollution was still high at many sites of coastal and deep-sea waters of countries that adopted TBT regulations [1-6]. As a consequence, in 2001 the International Maritime Organization (IMO) formulated the International Convention on the Control of Harmful Antifouling Systems, which ratification is not completed yet and, consequently, is still not effective. However, the European Union (EU) followed the rational of this convention and introduced the Directive 2002/62/EC that banned the application of organotin antifouling paints on EU boats after 1 January 2003 and forbids its usage by any boats after 2008. The recent concern now is to check the effectiveness of the ban in terms of how fast the OT environmental levels decline in the EU after 2003.

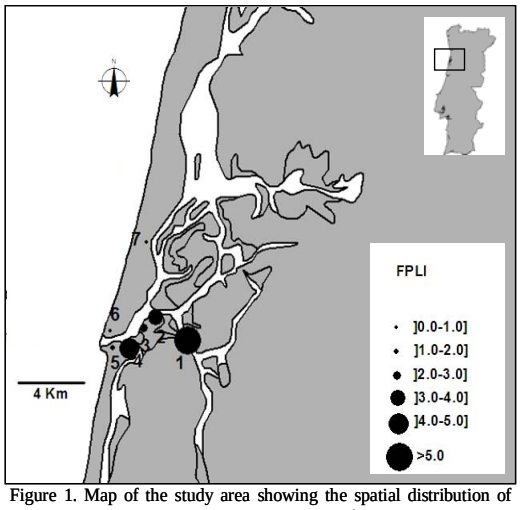

Figure 1. Map of the study area showing the spatial distribution of FPLI across the sampling locations. Deep sea fishing port (1), North Commercial port (2), Forte Barra (3), M. Mira (4), Barra (5), S. Jacinto (6), Muranzel (7)

The main objective of the present study is to evaluate the usefulness of *Nassarius reticulatus* as a bioindicator species to track decreasing levels of TBT, based on the assessment of OTs contamination in the tissues and imposex levels in *N. reticulatus* in 2005, the comparison of the 2005 situation with the one reported for 2003 and the study of the relationship between TBT exposure and imposex in laboratory experiments.

Materials and Methods
Study area: Ria de Aveiro is an important estuarine system located in the NW Portugal [Fig. 1]. The potential sources of organotin contamination in Ria de Aveiro are ports, dockyards and marinas. The main ports and dockyards are located along the main navigation channel. Of major relevance is the deep sea fishing port (St. 1), the Aveiro dockyards (next to St. 1), the commercial port (St. 2), the chemical port and the coastal fishing port [7].

Sampling procedure and imposex analysis: Sampling was performed in May 2005. The whelk *Nassarius reticulatus* was collected at 7 sites inside Ria de Aveiro. Only adult animals were selected for imposex analysis. Prior to observation the selected animals were narcotized in 7% $MgCl_2$ in distilled water. The following imposex indices were assessed: mean female penis length index (FPLI), relative penis length index (RPLI = mean female penis length x 100/mean male penis length), *vas deferens* sequence index (VDSI), incidence (percentage of females affected by imposex = % I) and average oviduct convolution (AOS). The VDSI was classified according to the scoring system proposed by Stroben et al. [8] with minor alterations proposed by Barroso et al. [9]. The degree of oviduct convolution (AOS) was ranked according to the 3-stage scale proposed by Barreiro et al. [10].

Organotin analysis: The organotin analyses were performed at the Center for Marine and Environmental Studies (CMES), Ehime University, Japan following the method described by Iwamura et al. [11] with some modifications [12]. MBT, DBT, TBT, MPT, DPT, TPT, MOT, DOT and TOT average recovery rates (±St Dev) were 38.5±2.77, 84.6±6.03, 86.3±5.62; 6.7±4.33, 54.6±5.30, 126.5±7.98; 51.0±4.26, 118.8±8.19 and 125.8±7.76. Concentrations of MPT were not estimated because recoveries of the internal standard for MPT were less than 10%. Recoveries of internal standards for MBT were around 30 to 50 %, meaning that the concentrations of this compound must be considered as reference values. Our method gave detection limits of: 30 ng/g for MBT, 7.1 ng/g for DBT, 0.42 ng/g for TBT, 0.33 ng/g for MPT, 0.16 ng/g for DPT, 0.08 ng/g for TPT, 3.1 ng/g for MOT, 3.6 ng/g for DOT and 0.43 ng/g for TOT.

Laboratory experiments: Females of *N. reticulatus* were collected inside Ria de Aveiro at St. 4 in February 2007. The animals were narcotized with 7% $MgCl_2$ in distilled water and only females exhibiting low levels of imposex (FPLI<2mm) were selected. Exposure was performed in 1L glass bottles with 5 females each and 5 replicates per treatment. The temperature was constant during the experiment (18º±0.5ºC) and all solutions were constantly aerated and renewed weekly. Five different TBT treatments were chosen: 50, 100, 150, 200 and 250 ng TBT-Sn/L plus a Seawater control. For TBT solutions ethanol was used as a solvent since it doesn't have any effect on *N. reticulatus* penis length (unpublished data). The lowest concentration used (50 ng TBT-Sn/L) refers to the environmental TBT levels at the collection site [13].

Table 1. *Nassarius reticulatus.* Data relative to each sampling site with the indication of: numbers of males (♂ N) and females (♀N) with respective mean shell heights ± StDev ; male penis length index (MPLI); female penis length index (FPLI); relative penis length index (RPLI), *vas deferens* sequence index (VDSI); female oviduct convolution (AOS) and percentage of affected females (a) For St. 7 *vas deferens* development didn't follow the usual scheme; instead the alternative b-way was observed in 80% of the affected females.

Station	Code	N♂	♂Shell height	♂ PLI	N♀	♀Shell height	FPLI	RPLI (%)	VDSI	AOS	%I
Deep Sea fishing Port	1	7	24.3±4.46	15.3±3,24	12	25.0±2.23	7.8±1.21	50.9	4.8±0.39	1.3±0.75	100
North commercial Port	2	30	21.8±1.26	13.4±3.43	30	22.8±1.53	3.7±1.84	27.3	3.9±0.86	0.1±0.43	100
Forte Barra	3	30	24.2±1.53	15.5±3.03	30	24.4±3.14	2.1±1.18	13.9	3.8±0.57	0.1±0.25	100
M. Mira	4	32	22.9±3.13	10.4±3.93	32	24.6±2.53	4.5±2.32	43.3	3.6±0.70	0.1±0.35	100
Barra	5	28	25.6±2.16	13.8±2.50	35	26.6±2.38	1.1±1.84	8.1	2.2±1.33	0.0±0.00	91.4
S. Jacinto	6	23	24.3±1.68	13.2±4.43	42	25.8±1.93	0.9±1.24	6.8	2.4±1.04	0.0±0.00	90.5
Muranzel	7	26	22.0±1.19	13.0±3.01	26	22.9±1.75	0.1±0.23[(a)]	0.8	1.2±0.83[(a)]	0.0±0.00	84.6

Results

Nassarius reticulatus: imposex levels and OTs tissue concentrations

Levels of imposex in *N. reticulatus* showed a clear decreasing gradient with the distance from ports and dockyards [Table 1, Fig. 1]. This spatial pattern follows a clear ship traffic gradient as already noticed in previous surveys [7, 14-16]. Organotin concentrations in female's *N. reticulatus* are shown in Table 2. Among the organotin compounds the

butyltins represent by far the highest fraction with about 95% in relation to total organic tin (∑OT) quantifiable by our method. Phenyltin compounds represented only 1% of ∑OT. Octyltin compounds are almost negligible in the study area [Table 2]. The TBT tissue concentration in *N. reticulatus* was highly significantly correlated to the imposex indices [Spearman rank order correlation: VDSI (r= 0.84; p<0.001), FPLI (r= 0.87; p<0.001), and AOS (r= 0.84; p<0.001)].

Table 2. Organotin concentrations (Sn ng/g dry wt) in *Nassarius reticulatus* across the sampling stations with indication of the moisture content (% of dw). [1] Reference values

Coordinates	Code	Moisture	MBT[1]	DBT	TBT	MPT[1]	DPT	TPT	MOT	DOT	TOT
Nassarius reticulatus											
40º38'24N-8º43´59W	1	67.6	51	50	40	<9.7	<0.16	0.32	<3.1	<3.6	<0.43
41º39'06N-8º43´76W	2	72.5	71	40	60	<9.7	<0.16	0.4	5.4	8.1	<0.43
41º38'56N-8º43´59W	3	-	-	-	-	-	-	-	-	-	-
41º38'65N-8º44´06W	4	69.8	32	28	73	<9.7	<0.16	1.3	<3.1	7.4	<0.43
41º38'71N-8º44´82W	5	73.7	54	18	25	<9.7	<0.16	0.34	11.8	<3.6	<0.43
41º39'84N-8º43´56W	6	73.5	37	25	38	<9.7	<0.16	0.66	<3.1	<3.6	<0.43
40º43'13N-8º41'55W	7	67.4	<30	11	15	<9.7	<0.16	0.34	<3.1	<3.6	<0.43

Temporal variation of imposex and organotin body burden

Temporal comparisons of imposex levels for the same locations between 2003 [16] and the present survey are shown in Fig. 2 [Mann Whitney *U* test]. We found no significant differences in the VDSI for all stations between 2003 and 2005. In what regards to FPLI, and considering that no significant differences between female's shell length was observed between both surveys (which could affect penis size), a significant decrease in the female penis length was registered in Sts. 2, 3 and 5 between 2003 and 2005. This evolution over a such short time period may reveal a decrease in TBT pollution and therefore lower levels of imposex due to population renovation or to a limited degree of imposex reversibility, a phenomena already reported in previous studies [16, 17].

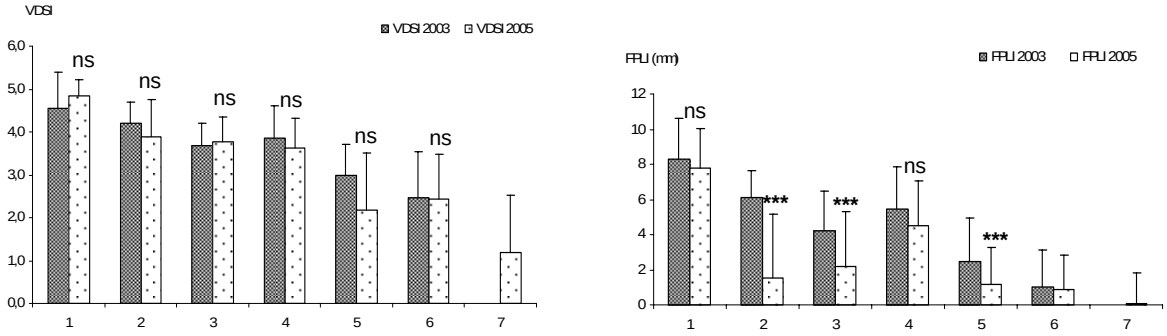

Figure 2. Temporal comparisons of VDSI and FPLI imposex indices between 2003 and 2005 at the different sampling locations. Bars represent standard error. ns: not significant; ***: *p<0.001*

Relationship between TBT exposure and imposex in laboratory experiments

Female penis length variation after 30 days of exposure is shown in Fig. 3. The female's shell length in the control group was not significantly lower than the rest of TBT treatments, therefore the differences in penis size are not an outcome of differences of the animal's shell length.

ANOVA showed that there was a significant effect of imposex induction (measured as female's penis length index) by TBT ($F= 14.26$; $P<0.001$). The post-hoc analysis revealed the existence of significant differences between each of the TBT treatments and the seawater control (see Figure 3), though no significant differences were observed between groups of TBT treatments ($\alpha=0.05$).

The results clearly show that TBT causes the development of imposex in this species, as already mentioned for many other species, reinforcing the robustness of imposex as a fairly specific response to this contamination.

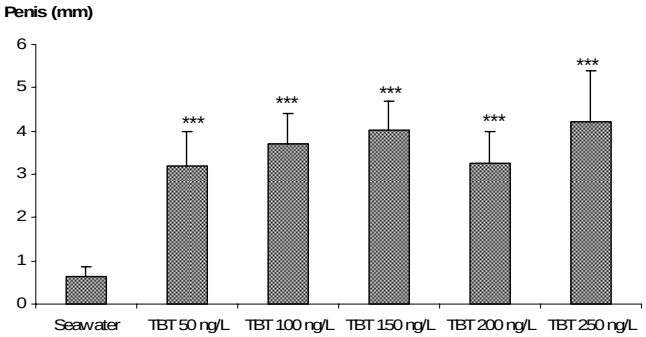

Figure 3. Female penis length variation during 30 days in laboratory experiments. ***: *p<0.001*.

The observed reduction of FPLI between 2003 and 2005 is most probably a consequence of the decreasing levels of TBT pollution derived from the implementation of the ban. Reduction in FPLI can therefore be used in future monitoring surveys to study the reduction of TBT levels in the environment.

Acknowledgments

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