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The effectiveness of some compounds derived from antifouling paints in promoting imposex in *Nassarius reticulatus*

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Adult *Nassarius reticulatus* were exposed for two to three months to dissolved tributyltin (TBT), dibutyltin (DBT), triphenyltin (TPT) and diphenyltin (DPT) chlorides and to copper sulphate ($CuSO_4$) in order to study the effectiveness of these compounds to induce imposex (penis and vas deferens development). The dialkyltins and copper did not promote imposex under exposure to 500 ng Sn l^{-1} and 500 ng Cu l^{-1}. However, dissolved nominal concentrations varying between 100 and 500 ng Sn l^{-1} of TBT or TPT induced imposex in, respectively, 22 to 43% and 24 to 44% of the females. The testosterone titres of females increased significantly after two months' exposure to 250 ng Sn l^{-1} of TPT and TBT and the same happened to males exposed to 100 and 250 ng TPT-Sn l^{-1} and to 250 ng TBT-Sn l^{-1}.

INTRODUCTION

Tributyltin (TBT) antifouling paints were first introduced in the 1960s (Bennett, 1996) and became widely used due to their higher and longer-term antifouling performance in comparison to the conventional copper paints (Champ, 2000). Triphenyltin (TPT) is used as a cotoxicant with TBT in some antifouling paints but its major application is as a fungicide in agriculture (potato, celery, sugar beet and rice cultures) (Fent, 1996). The imposex (superimposition of male characters onto females of gonochoristic prosobranch species) (Smith, 1971) has been commonly observed since 1969 and now affects over 118 prosobranch species worldwide (Bettin et al., 1996). It is well established that imposex is universally caused by TBT compounds. In aquatic environments TBT is mainly released from TBT-antifouling coatings of ship hulls and from dockyard hydroblasting wastes. However, TBT may not be the only compound causing imposex. Copper induces imposex in *Lepsiella vinosa* (Lamarck) (Nias et al., 1993) whereas triphenyltin promotes imposex in *Thais clavigera* (Küster) (Horiguchi et al., 1997) and *Marisa cornuarietis* (Schulte-Oehlmann et al., 2000). Surprisingly, there is still a considerable lack of knowledge regarding the effects of other compounds than TBT on imposex induction in prosobranch species.

Nassarius reticulatus (L.) is an ubiquitous species of the European coast (Graham, 1988) that was proposed as a bioindicator of TBT pollution by Stroben et al. (1992a). This species has been successfully used in TBT monitoring programmes (Barroso et al., 2000; Barroso et al., in press; Bryan et al., 1993; Oehlmann et al., 1993; Barreiro et al., 2001). Its moderate sensitivity to TBT pollution and its ubiquity in the north-east Atlantic make this species a potential candidate for organotin monitoring programmes in the seacoast covered by the OSPAR Convention. However, it is of crucial importance to fully understand if other compounds derived from antifouling paints may also induce imposex in this species. While TBT, TPT and copper are leached directly from antifouling paints to the water, the degradation of TBT/TPT in the ecosystem involves a sequential debutylation via dibutyltin (DBT)/diphenyltin (DPT) and monobutyltin (MBT)/monophenyltin (MPT) to inorganic tin, with a progressive decrease of toxicity (Batley, 1996; Fent, 1996). The main objective of the present work is to examine the effects of TBT, DBT, TPT, DPT and copper on the induction of imposex in *N. reticulatus*.

MATERIALS AND METHODS

Experiments

Nassarius reticulatus specimens were collected from an inshore marine station at Torreira (north-west Portugal) in March and September 2000, using a fishing dredge operated by boat. At this site, which has been surveyed annually since 1997, females never showed imposex. Two series of laboratory experiments were performed and will be here designated experiment 1 and experiment 2. The first started in March and the second in September 2000. The general procedures were common to both series of experiments. After collection, the animals were reared in 120-l glass aquaria with artificial seawater SERA (35 psu) for three days. Dead or low condition animals were eliminated during this phase. A subsample of 40 females was narcotized in 7% $MgCl_2$ in distilled water, the shells were open and the individuals were dissected under a stereo microscope for imposex analysis. Only adult animals were used in the experiments. Prior to distributing the animals into the treatment aquaria, they were narcotized for 20 to 30 min in 7% $MgCl_2$ in distilled water for a quick identification of sex and to check for the absence of penis and initial vas deferens growth in females. One of the criteria to identify females was the presence of the ventral pedal gland. The animals were then reared in separate 60-l glass aquaria with the dissolved testing compounds tributyltin (TBT),

dibutyltin (DBT), triphenyltin (TPT), diphenyltin (DBT) and copper. The organotins were used in the form of chlorides—TBTCl (Fluka, 97%), $DBTCl_2$ (Fluka, 97%), TPTCl (Fluka, 97%) and $DPTCl_2$ (Aldrich, 96%)—although their biological activity is known to be little influenced by their inorganic radicle (Bryan et al., 1988). Copper was used in the form of sulphate ($CuSO_4$) (Aldrich, 98%). Dimethylsulphoxide (DMSO) (Sigma, 99.5%) and distilled water were the solvents for the organotin and $CuSO_4$ solutions, respectively. Just before the start of the experiments, 20 μl of the solutions were added to the 55 l artificial seawater SERA (35 psu) of each aquarium. In order to minimize the decline of the concentration of the spiked chemicals over time, due to possible adsorption to aquaria surfaces, volumes of 15 l seawater were replaced twice a week in each aquarium and the correspondent aliquots (5.5 μl) of the solutions were added. Dimethylsulphoxide control aquarium received the same volume of DMSO as the other treatments. Furthermore, surfaces inside the aquaria were disturbed as little as possible and filtering was supplied at a minimum sustainable level. Hence, each 60-l aquarium was equipped with only an aerated tube and an internal 150 l h^{-1} power filter containing a small piece (80×80×5 mm) of sponge. The water temperature was approximately constant at 18°C (±0.5°C). The animals were fed once a week with frozen bivalves *Spisula* sp. and *Donax* sp. captured from the same site as the test species. Each month after the start of the experiments a group of females was definitively removed from each aquarium and immediately analysed. Males were also analysed at the end of the experiments.

In experiment 1 five groups of 45 females plus 20 males were exposed for three months to 500 ng Sn l^{-1} of TBT, DBT, TPT, DPT and to 500 ng Cu l^{-1}. Two control groups, with the same number of animals, were included in the experiment. One corresponded to DMSO and the other to seawater only. Therefore, a total of seven aquaria were used, each one corresponding to a specific treatment. The shell height of the animals was about the same in all treatments: mean ± standard deviation (SD) values of female and male shell heights were, respectively, 27.5 ± 1.0 and 26.3 ± 0.8 mm.

In experiment 2 five groups of 55 females plus 20 males were exposed for two months to different concentrations of dissolved TBT (100, 250 and 400 ng Sn l^{-1}) and TPT (100 and 250 ng Sn l^{-1}). Two control groups were exposed to DMSO and seawater, each with the same number of animals. Mean shell heights were approximately constant in all treatments (27.1 ±0.8 and 26.2 ± 0.7 mm for females and males, respectively).

Imposex analysis

Animals were narcotized using 7% $MgCl_2$ in distilled water. The shell height (distance from shell apex to lip of syphonal canal) 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 female penis length (FPL) was measured with a graduated eyepiece in a stereo microscope, providing an accuracy of 0.05 mm. Male penis length was measured using 1 mm graduated graph paper under a stereo microscope. The vas deferens sequence (VDS) was classified according to the scoring system proposed by Stroben et al. (1992a). This system includes the following sequence: growth of a tiny penis without a penis duct (stage 1); formation of a penis duct (stage 2); a vas deferens develops from the penis duct towards the vaginal opening (stage 3) and reaches the vulva (stage 4) or runs into the ventral channel of the capsule gland (stage 4^+). This sequence is accompanied by the growth of the penis (type a). However, the vas deferens may develop from the right ocular tentacle up to the vulva without the formation of a penis (type b) (Stroben et al., 1992a). Besides the measurement of the penis length and VDS, the percentage of females affected with imposex (%I) was also determined.

Organotin and copper analysis

Tributyltin, DBT, monobutyltin (MBT), TPT, DPT and copper were measured in the whole tissues of pooled females. The analysis of organotins was performed by the Servicios Xerais de Apoio á Investigación (Universidade da Coruña). Simultaneous derivatization and extraction of organotin compounds was achieved following the method of Szpunar et al. (1996) with minor modifications. Briefly, two replicate samples of 0.1 g lyophilized tissue were digested with tetramethylammonium hydroxide by application of microwave power. After adjustment to pH=5, sodium tetraethylborate and isooctane containing tetrabutyltin as an internal standard were successively added. After microwave radiation treatment, the organic phase was recovered and analysed by gas chromatography–mass spectrometry (GC-MS). The methods rendered a quantification limit of around 20 ng Sn g^{-1} dry weight (DW) for the butyltins and 10 ng Sn g^{-1} DW for the phenyltins. The procedure was validated with a certified reference material—the Japanese NIES11 fish tissue (National Institute for Environmental Studies, Japan Environment Agency). Recoveries in routine samples were assessed by a standard addition method and results were accordingly corrected. The recoveries for the different organotins were about 90% (TBT), 95% (DBT), 85% (MBT), 75% (TPT) and 54% (DPT). The analysis of copper was made by the Chemistry Department of the University of Aveiro. Samples of 0.1 g lyophilized tissue were digested with nitric acid in a Teflon container (enclosed system) at 60°C and then placed in an oven (100°C) for one hour. After treatment with H_2O_2 at 80°C, the total copper was quantified by means of a graphite furnace atomic absorption spectrometer and using the method of standard additions.

Steroid extraction and radioimmunoassay

The testosterone and 17β-estradiol titres were measured in the whole tissues of females or males subjected to experiment 2 for two months. Additionally, these hormones were also measured before the start of the experiment. The hormonal analysis for a given treatment was performed in two to four replicates, each one constituted by three to four pooled specimens. For free steroids extraction the pooled specimens of each sex were homogenized in 2 ml ethanol and homogenates were extracted four times with diethyl

ether (1:1, v/v) for 5 min. The organic fraction was separated by centrifugation at 2000*g* (5 min). The upper diethyl layers were combined and evaporated under a stream of nitrogen at 30°C. After being redissolved in 2 ml 80% methanol, the residues were washed twice for 5 min with 5 ml petroleum ether in order to separate steroids from the lipid fraction, as described in Bettin et al. (1996). The washed methanol fractions were evaporated before dissolving them in 0.1% gelatine buffer (sodium azide 0.1% at pH 7.4) for radioimmunoassay. In order to obtain the extraction efficiency, homogenates (5 g of tissue in 2 ml of ethanol) were extracted after addition of either ^{3}H testosterone (5 μl equivalent to 104,000 cpm) or ^{3}H 17β-estradiol (5 μl equivalent to 115,000 cpm). The radioactivity was measured in a liquid scintillation counter BECKMAN LS 3801 (USA) for 2 min, after the addition of 2 ml of scintillate liquid INSTA-GEL II PLUS (PACKARD), and the calculated recovery mean values ± SD were 57.4 ± 3.3% for testosterone and 79.4 ± 7.6% for 17β-estradiol.

The testosterone and 17β-estradiol were quantified by solid-phase ^{125}I radioimmunoassay, using kits from Diagnostic Products Corporation. The sensivities of the standard curves were 40 and 8 pg per tube for testosterone and 17β-estradiol, respectively. The two antisera are highly specific and have an extremely low cross-reaction to other natural occurring steroids, including oestrone (<10%), oestriol (<0.3%), testosterone (<0.001%) for 17β-estradiol and androstenedione (<0.05%), estradiol (<0.02%), dihydrotestosterone (<5%) for testosterone. Gamma radiation was measured in a LKB-Wallac MiniGamma Counter 1275.

Statistical analysis

The software STATISTICA was used to compute descriptive statistics and to perform the Model I analysis of variance (ANOVA) followed by a post hoc least significance difference (LSD) test in order to determine significant (α=0.05) differences between means of the treatment groups vs the referred control group.

RESULTS

Imposex levels

Neither the 40 sub-sampled females collected at Torreira were affected with imposex nor the females introduced in the treatment aquaria presented penial or vas deferens growth. Hence, at the start of the experiment, the females were unaffected by imposex. The organotin body burden of the females collected at Torreira in March 2000 was below the detection limits, except for DPT that presented a concentration of 21 ng Sn g^{-1} DW.

Experiment 1

The imposex levels and the organotin body burden residues of the females are summarized in Table 1 and Figure 1A. In the DMSO control, after three months, only one animal showed a small excrescence near the right ocular tentacle, corresponding to the initial growth of a penis. The TBT and TPT were shown to be potent promoters of imposex and induced both types (a and b) of VDS. In the first month imposex was only promoted

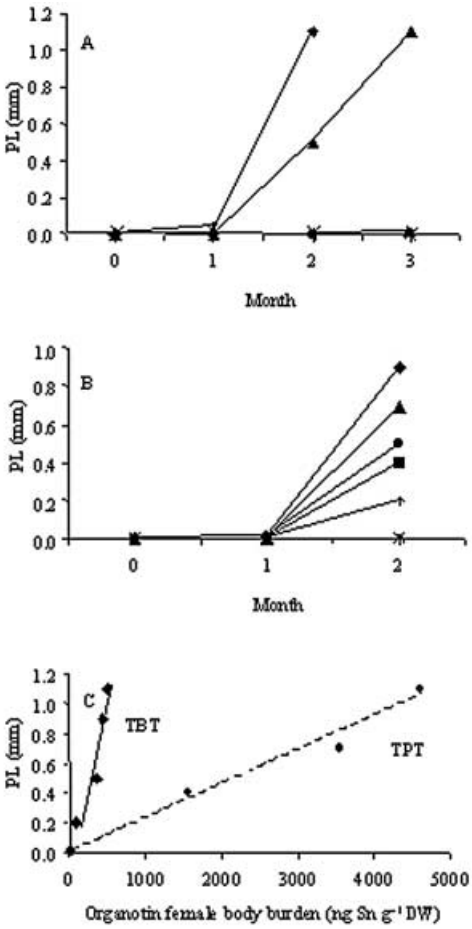

Figure 1. *Nassarius reticulatus*. Mean penis length (PL) of females subjected to the following treatments: (A) 500 ng Sn l^{-1} TPTCl (▲), $DPTCl_2$ (+), TBTCl (◆), $DBTCl_2$ (×), 500 ng Cu l^{-1} $CuSO_4$ (–), seawater (control 1) (*) and DMSO (control 2) (●); (B) 100 (■) and 250 (▲) ng Sn l^{-1} TPTCl, 100 (+), 250 (●) and 400 (◆) ng Sn l^{-1} TBTCl, seawater (control 1) (×) and DMSO (control 2) (–); (C) relationship between TBT and TPT whole female body burden and penis development (lines adjusted by eye).

by TBT. Two months' exposure to 500 ng Sn l^{-1} of TBT and TPT induced imposex in 43 and 27% of the females, respectively. The corresponding TBT female body burden was 498 ng Sn g^{-1} DW. Between the second and the third month of TBT exposure a high mortality caused the end of this test. Three months of exposure to 500 ng TPT-Sn l^{-1} induced imposex in 27% of the females, which presented a TPT residue of 4623 ng Sn g^{-1} DW (Table 1). Imposex development was not observed under the dibutyltin chloride, diphenyltin chloride and copper sulphate exposures. The female mean residues of DBT and DPT after the third month were, respectively, 240 and 151 ng Sn g^{-1} DW; these values are close to those derived from debutylation of TBT and TPT in treatments 1.3 and 1.5. The copper female body burdens after the third month in treatments 1.1 and 1.7 were, respectively, 153 and 445 μg g^{-1} DW. The mean male penis length did not present significant differences between the DMSO control and the other treatments (α=0.05) (Table 1). Mortality did not exceed 14% of the animals per aquarium after the three month period, except in the case of TBT exposure (35% after two months).

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Table 1. Nassarius reticulatus *mean female penis length (FPL), vas deferens sequence index (VDSI), percentage of females affected with imposex (%I) and mean male penis length (MPL) determined for samples of a given number of animals (N) exposed (experiment 1) for one to three months to dissolved nominal water concentrations of 500 ng Sn l^{-1} TPTCl, $DPTCl_2$, TBTCl, $DBTCl_2$, of 500 ng Cu l^{-1} $CuSO_4$ and to seawater (Control 1) and DMSO (Control 2). The same parameters were also determined for animals exposed (experiment 2) for one to two months to dissolved nominal water concentrations of 100 and 250 ng Sn l^{-1} TPTCl, of 100, 250 and 400 ng Sn l^{-1} TBTCl, and to seawater and DMSO. Mean female whole tissue body burden for the organotins and copper (see text) was determined at the end of the experiments for the concerned treatment.*

Treatment (code)	Months	N♀	FPL (mm) (Mean/SD)	VDSI (Mean/SD)	%I	N♂	MPL (mm) (Mean/SD)	Female body burden ng Sn g^{-1} DW (Mean/SD)				
								TPT	DPT	TBT	DBT	MBT
Control 1	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.1)	2	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
	3	20	0.0/0.0	0.0/0.0	0.0	17	13.9/1.2	–	–	–	–	–
Control 2	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.2)	2	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
	3	19	0.01/0.02	0.1/0.2	5.6	17	13.6/1.1	20/2	25/1	nd	nd	nd
TPT 500	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.3)	2	11	0.5/1.8	0.3/0.5	27.3	–	–	–	–	–	–	–
	3	17	1.1/2.6	0.5/1.1	26.7	18	13.7/2.1	4623/142	876/121	nd	nd	nd
DPT 500	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.4)	2	11	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
	3	19	0.0/0.0	0.0/0.0	0.0	16	13.3/1.5	30/7	151/2	nd	nd	nd
TBT 500	1	11	0.05/0.15	0.09/0.30	9.1	–	–	–	–	–	–	–
(1.5)	2	16	1.1/2.5	0.4/0.5	42.9	16	12.8/2.5	43/2	37/9	498/20	317/37	78/5
DBT 500	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.6)	2	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
	3	20	0.0/0.0	0.0/0.0	0.0	17	13.5/1.4	20/1	29/2	nd	240/22	47/3
$CuSO_4$ 500	1	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(1.7)	2	10	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
	3	19	0.0/0.0	0.0/0.0	0.0	17	13.7/0.9	–	–	–	–	–
Control 1	1	15	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(2.1)	2	35	0.0/0.0	0.0/0.0	0.0	18	11.5/2.1	30/7	28/2	nd	nd	nd
Control 2	1	15	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(2.2)	2	35	0.0/0.0	0.0/0.0	0.0	17	12.8/3.0	21/5	25/2	nd	nd	nd
TPT 100	1	15	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(2.3)	2	37	0.4/1.6	0.3/0.6	24.3	17	14.5/1.5	1566/117	181/23	nd	nd	nd
TPT 250	1	14	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(2.4)	2	36	0.7/2.3	0.7/1.0	43.8	17	14.4/1.5	3568/117	469/53	nd	nd	nd
TBT 100	1	15	0.0/0.0	0.0/0.0	0.0	–	–	–	–	–	–	–
(2.5)	2	37	0.2/0.6	0.6/1.0	32.4	18	15.0/1.9	23/4	33/1	76/4	67/10	nd
TBT 250	1	15	0.01/0.03	0.1/0.4	13.3	–	–	–	–	–	–	–
(2.6)	2	38	0.5/1.4	0.5/1.1	21.7	16	14.3/1.0	28/1	57/2	359/42	207/2	68/9
TBT 400	1	13	0.01/0.04	0.1/0.4	15.4	–	–	–	–	–	–	–
(2.7)	2	35	0.9/2.2	0.5/1.0	23.7	16	14.2/1.7	nd	23/2	439/28	246/37	68/9

–, not performed; nd, not detected.

Experiment 2

The imposex levels and the organotin residues of the females are summarized in Table 1 and Figure 1B. No females with imposex were observed in either control groups. This experiment confirmed that triphenyltin and tributyltin are very effective in promoting imposex in *Nassarius reticulatus*. As in experiment 1, these compounds induced both a and b types of VDS development. In the first month imposex was only promoted for concentrations of 250 and 400 ng Sn l^{-1} of TBT. Two months' exposure to TBT concentrations of 100, 250 and 400 ng Sn l^{-1} induced imposex in, respectively, 32, 22 and 24% of the females. The same time exposure to TPT at concentrations of 100 and 250 ng Sn l^{-1} promoted imposex in 24 and 44% of the females, respectively. The mean FPL values were even higher under TPT exposure, when compared to similar nominal seawater concentrations of TBT (Figure 1). However, and despite the fact that only nominal concentrations are known in this study, the bioconcentration factors for the two compounds appear to be very different, as it was also observed in experiment 1. Water concentrations of 100, 250 and 400 ng TBT-Sn l^{-1} corresponded to female residues of 76, 359 and 439 ng TBT-Sn g^{-1} DW, respectively. In the case of TPT, for water concentrations of 100 and 250 ng Sn l^{-1} the female residues were, respectively, 1566 and 3568 ng TPT-Sn g^{-1} DW (Table 1). The mean male penis length did not present significant differences between the DMSO control and the other treatments (α=0.05) (Table 1). Mortality did not exceed 15% of the animals during the two-month period.

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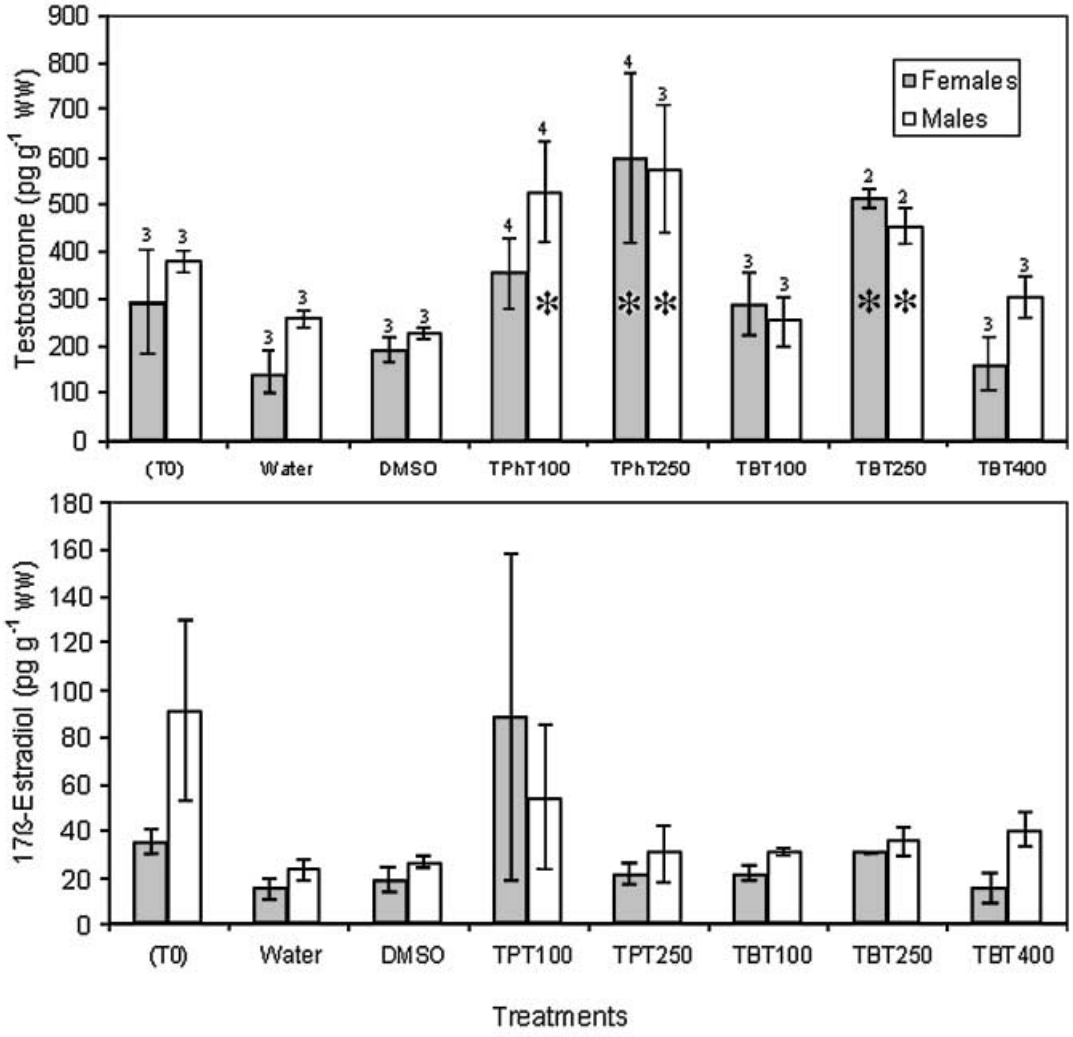

Figure 2. *Nassarius reticulatus* endogenous titres (mean ± SE) of testosterone and 17β-estradiol after two months of exposure to 100 and 250 ng Sn l^{-1} TPTCl, to 100, 250 and 400 ng Sn l^{-1} TBTCl, and to seawater and DMSO controls. Significant differences between means (ANOVA; α=0.05, after log transformation) of each organotin treatment vs mean of DMSO control is indicated by "*". Numbers above bars indicate number of replicates used for hormone analysis.

Imposex/organotin body burden relationship

Figure 1C represents the relationship between TBT and TPT female body burden and penis development, compiled for both experiments. Despite the fact that not all data were obtained for the same time of exposure (the value of the highest TPT concentration was observed after three months of exposure while the remainder correspond to a two-month period), the figure suggests that higher levels of TPT are necessary to trigger the development of imposex, in comparison with TBT.

Testosterone and 17β-estradiol levels

The endogenous testosterone and 17β-estradiol titres of females and males at the start (T0) and at the end of experiment 2 are shown in Figure 2. The comparison of the mean hormone titres observed in different treatments was performed by an ANOVA followed by an LSD test after log transformation of the data. In comparison with the seawater control, DMSO exposure had no significant effects on the hormonal levels in males and females. The comparison of the organotin treatments with the DMSO control reveals the occurrence of a significant increase of testosterone in females exposed to 250 ng Sn l^{-1} of TPT and TBT as well as in males exposed to 100 ng TPT-Sn l^{-1}, 250 ng TPT-Sn l^{-1} and 250 ng TBT-Sn l^{-1}. Surprisingly, testosterone titres did not rise for nominal concentrations of 400 ng TBT-Sn l^{-1}, which is inconsistent with the imposex levels induced by this treatment. The triorganotins had a negligible effect on the 17β-estradiol titres.

DISCUSSION

In the present work DMSO was tested as a solvent for organotins. It provided a very good dissolution of the organotin chlorides and demonstrated a very high solubility in seawater. Moreover, it was an alternative to the most frequently used solvent, ethanol, which has been shown to cause hormonal disorders in prosobranchs (Stroben et al., 1992b; Deutsch & Fioroni, 1996). It was observed that only a minor induction of imposex occurred in the DMSO control group of experiment 1, after three

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months of exposure. However, DMSO did not induce imposex in DBT and DPT treatments nor in the control group of experiment 2. Therefore, at least for short-term exposure, this solvent seems to have a negligible effect on imposex induction.

When controls are compared with treatments 1.3, 1.5 and 2.3 to 2.7, it becomes evident that triphenyltin and tributyltin strongly induced imposex development in *Nassarius reticulatus*. In experiment 1, exposure to 500 ng Sn l^{-1} of dibutyltin and diphenyltin chlorides, as well as to 500 ng Cu l^{-1} of copper sulphate, did not promote penis or vas deferens development in this species within the three-month period. Thus, imposex development was induced by the triorganotins but not through their natural direct degradation products DBT and DPT. In fact, treatments 1.6 and 2.7 showed similar DBT female residues of about 240 ng Sn g^{-1} DW, but imposex was only promoted in the case where TBT was present. Similarly, the DPT female residue in the second month of treatment 2.3 (181 ng Sn g^{-1} DW) was comparable to that of treatment 1.4 (151 ng Sn g^{-1} DW) but imposex was only induced when TPT was present. A background female TPT residue of about 20 to 43 ng Sn g^{-1} DW was observed in all treatments, except in 2.7 where it was not detected. The origin of this background level remains to be explained but it is not effective to induce imposex in the short-term. Schulte-Oehlmann et al. (2000) reported that *N. reticulatus* did not develop imposex under TPT exposure via sediments (nominal concentrations of 50–500 μg TPT-Sn Kg^{-1} DW) for up to three months, although it caused marked impairment of gametogenesis. This suggests that the mode of TPT exposure (sediment or water) influences the bioavailability and bioaccumulation of this compound and that high levels of TPT body burden may be necessary to trigger the development of imposex in this species.

It has been reported for *N. reticulatus* and other prosobranchs that imposex results from elevated testosterone titres caused by TBT exposure (Bettin et al., 1996; Matthiessen & Gibbs, 1998). The present study shows that triphenyltin has a similar effect on this species, although the potential of these two triorganotins for endocrine disruption remains to be further compared. The stronger increase of testosterone induced by TPT exposure (Figure 2) may result from a much higher tendency for its bioaccumulation than TBT. The present work also shows that the effect of organotin exposure on male steroid titres is comparable to those found in females, indicating that common mechanisms are involved in the endocrine disruption. However, the increase of testosterone in males did not affect significantly their penis length.

The TBT nominal aqueous concentrations tested in the experiments correspond to values frequently observed in natural waters in the vicinity of hotspots. In fact, typical dissolved TBT levels in large marinas (especially before legislation restrictions) or large dockyard and ports are in the range of 100 to 200 ng Sn l^{-1} and occasionally as high as 600 ng Sn l^{-1} (Batley, 1996). There are very scarce data regarding concentrations of TPT in natural waters but they seem to be lower than those of TBT. In the Côte d'Azur the sub-surface waters near marinas presented a mean concentration of 4.5 ng TPT-Sn l^{-1} (Tolosa et al., 1996) although concentrations of up to 190 ng TPT-Sn l^{-1} have been reported for freshwater harbours in Switzerland (Fent, 1996). Nevertheless, female organotin body burdens may be more meaningful as a measure of dosage since they integrate several possible sources of contamination (water, diet and crawling surface). The female TBT residues observed at the end of the experiments (76 to 439 ng Sn g^{-1} DW) are in the range of values that occur in natural populations of *N. reticulatus* at moderately polluted sites in the European coast (Stroben et al., 1992a; Bryan et al., 1993; Barroso et al., in press), and so they can be considered 'close to real' doses. In what regards triphenyltin, a recent survey of the Portuguese coast revealed that TPT female whole body residue in the same species did not exceed 260 ng Sn g^{-1} DW (Barroso et al., in press) whereas Barreiro et al. (2001) found a maximum value of 125 ng Sn g^{-1} DW in north-west Spain. Compared to these values, the TPT female residues observed in the experiments ($\geqslant$1566 ng Sn g^{-1} DW) are extremely high. However, in Japan, natural populations of the prosobranchs *Thais clavigera* (Küster) and *T. bronni* (Dunker) presented TPT whole body burdens of up to 1200 ng Sn g^{-1} and up to 1700 ng Sn g^{-1} WW (wet weight), respectively (Horiguchi et al., 1994). The concentrations of TPT in *Mytilus edulis* (L.), collected in Japanese harbours, ranged from 210 to 1400 ng Sn g^{-1} WW (Shiraishi & Soma, 1992). In freshwater systems the levels of TPT in the zebra mussel *Dreissena polymorpha* (Pallas) has been reported to be of up to 3200 ng Sn g^{-1} DW in the Netherlands (Stäb et al., 1995).

It was known that *N. reticulatus* imposex is induced by TBT, either administered by injection, by aqueous and sediment exposure or through the diet (Stroben et al., 1992b; Bettin et al., 1996; Pope, 1998). However, the present results reveal that TPT is also a potent endocrine disruptor and a strong inducer of imposex in the species under acute exposure. Nevertheless, in natural populations TBT may play a more important role than TPT on the development of imposex in this gastropod, since environmental levels of triphenyltin in the European coast appear to be lower than those of tributyltin. In the western Iberian Peninsula the TPT whole body burden of *N. reticulatus* females, when detected, was on average less than 18% relative to TBT (Barreiro et al., 2001; Barroso et al., in press). Moreover, under acute exposure, imposex seems to be triggered at higher levels of TPT, in comparison to TBT, although this needs to be fully established in future research. The impact of TPT in *N. reticulatus* may not be as high as in *Thais clavigera*—the only marine gastropod for which TPT was previously reported to induce imposex—since TBT and TPT have similar effects on the level of imposex development in this latter species; besides, its Asian populations present very high TPT body burdens (Horiguchi et al., 1994, 1997; Shim et al., 2000). Nevertheless, the findings obtained from the current work provide additional experimental evidence about the consequences of using TBT and TPT as biocides in antifouling coatings. This gives further support to the 'International Convention on the Control of Harmful Antifouling Systems on Ships', adopted by the International Maritime Organization in October 2001, according to which the application of any organotin antifouling system will be banned from all ships after 1 January 2003.

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