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***Stramonita haemastoma* as a bioindicator for organotin contamination in coastal environments**

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Abstract

Stramonita haemastoma was investigated as a suitable bioindicator of TBT and TPhT contamination in the tropical Atlantic Ocean by: 1. Imposex induction in healthy females after inoculation with TBT and TphT in the laboratory; and 2. Determining incidence of imposex in *S. haemastoma* collected from areas with various levels of tributyltin (TBT) and triphenyl tin (TphT) and determining the concentrations of these chemical in its tissues and that of its prey, the mussel *Perna pernas*. Imposex intensities and organotin concentrations in tissues showed good correlation, indicating *S. haemastoma* as a reliable bioindicator of TBT and TPhT contamination in coastal waters. Body burden threshold of TBT and TPhT for imposex induction was estimated to be 10-20 ng.g⁻¹.

Keywords: organotins, *Stramonita (Thais) haemastoma*, bioassay, Guanabara Bay

Introduction

Organotins (OT) are man-made organometallic compounds widely used as biocides, mainly as antifouling paints on ship hulls. TBT and TPhT (triphenyltin) liberated from the hulls in contact with seawater have been shown to be very toxic to the marine biota (Hoch, 2001; Champ, 2000). The most characteristic sub-lethal effect of organotins is hormonal disruption in gastropods, leading to the imposition of male sexual attributes in the females, i.e., imposex (Gibbs & Bryan, 1986; Horiguchi, Shiraishi, Shimizu & Morita, 1997; Depledge & Billingham, 1999). Imposex, has been observed in several areas of the globe where OT contamination is present (Mathiessen & Gibbs, 1998). The neogastropod *Stramonita haemastoma* is a muricid, closely related anatomically to other species of the genus *Thais*. This genus is widespread in tropical and temperate waters, including the Mediterranean (Rilov, Gasith, Evans & Benayahu, 2000), North America, the Caribbean and South America (Rios, 1994), Australia and Asia (Stewart, De Mora, Jones & Miller, 1992; Tan, 1997; Bech, 2002a), living even in cool waters in Korea and Japan (Horiguchi, Shiraishi, Shimizu & Morita, 1994; Shin, Kahng, Hong, Kim, Kim & Shim, 2000). The incidence and intensity of imposex in *S. haemastoma* varies inversely with the distance from OT sources, as for instance, harbors, marinas and shipyards (Spence, Hawkins & Santos, 1990; Castro, Cascón, & Fernandez, 2000; Rilov, Gasith, Evans & Benayahu, 2000; Fernandez, Límaverde, Castro, Terra & Wagener, 2002; Fernandez, Wagener, Limaverde, Scofield, Pinheiro & Rodrigues, 2005). Among species of the *Thais* genus, imposex was shown to occur in *T. orbita* (Stewart et al, 1992), *T. clavigera* (Horiguchi et al, 1994; Tan, 1997; Blackmore, 2000; Shin et al, 2000; Hung, Hsu, Mang & Chuang, 2001), *T. bronni* (Horiguchi et al, 1994), *T. bitubercularis* (Tan, 1997; Bech, 2002a), *T. distinguenda* (Tan, 1997; Bech, 2002a), *T. jubilacea* (Tan, 1997), and *T. rufotincta* (Bech, 2002a). A few authors used organotins in bioassays to induce imposex in *T. clavigera*: Horiguchi et al (1997) used injection of the toxicants, while Liu & Suen (1996) tested direct exposure to the toxicant in water. Bech (2002b) tested transplantation of *T. distinguenda* from non-contaminated to contaminated sites

coupled to body burden chemical evaluation. All studies gave positive results for imposex induction by organotins.

In Brazil, very little is known about the presence and threats imposed by organotins in coastal environments. Fernandez et al (2005) and Almeida, Wagener, Maia & Miekeley (2004) found high concentrations of TBT in sediments of Guanabara Bay, in Rio de Janeiro, and reported that TBT may be persistent in the anoxic sediments of the bay. Godoi, Montone & Santiago-Silva (2003) also reported high concentrations in sediments from the Santos Bay, where boat and ship traffic is very intense. As for biological studies, Fernandez, Nogueira, Miyamoto & Wagener. (1997), Magalhães, Struchi & Asbahr, (1997) and Castro et al (2000) reported the occurrence of imposex in *Stramonita haemastoma* in Rio de Janeiro, Santos and Recife. Imposex was also found in *Stramonita Rustica* in the Northeastern coast of Brazil (Camillo Jr, Quadros, Castro & Fernandez, 2004; Castro, Meirelles, Cascón & Fernandez, 2004), however, in most cited studies the cause of such an alteration has not been proven to be TBT by correlating, for instance, the anomaly intensity with chemical body burden.

Brazil, as most Latin American countries, does not regulate the use of organotins on vessels.

An International Convention under IMO authority is under way to globally ban the use of organotins as antifouling agents. However, these substances will be present in the marine environment long after banning, either released from ship hulls still carrying these substances, or from anoxic sediments where TBT may show a half-life of several years (Dowson, Bubb & Lester, 1996; Stewart & Thompson, 1997; Alzieu, 1998). Enforcing such a convention will imply monitoring environmental conditions, a process that may be tedious and expensive, especially for developing countries such as Brazil, with more than 7,000 km of coast.

The use of a reliable bioindicator as a tool for screening hotspots is a viable option to the expensive chemical analysis of samples from every site suspected of contamination (Axiak Vella, Micallef, Chircop & Mintoff, 2003, Fernandez et al, 2005). Having this in mind the present work

tests for a cause-and-effect relationship between TBT and TPhT exposure and the development of imposex in *Stramonita haemastoma*. Two approaches were taken: (1) a laboratory bioassay, or dosing experiment; and (2) a field investigation of imposex coupled to organotin determination in the gastropod as well as in its natural prey (*Perna perna*) in Guanabara Bay. Our goal was to validate the use of this widespread gastropod species, and of the mussel *Perna perna*, as sentinels for organotin contamination in coastal environments in Brazil, and in other areas where they occur.

Methods

Sampling

For the bioassay, gastropods, and mussels used as feed, were collected in January 2001, from the Pontal do Atalaia in Arraial do Cabo, Rio de Janeiro. This is a pristine area where *Stramonita* populations were healthy and showed no evidences of imposex (Ribeiro, 2002).

For the survey in Guanabara Bay samples of *Stramonita haemastoma* and *Perna perna* were collected in September, 2000 and in October-December, 2001, from rocky substrates in sites at different distances from known sources (see Figure 1) of organotins, so as to obtain an imposex gradient in the field. From each site a minimum of 20 individuals were collected (Ellis & Pattisina, 1990). Sexual maturity of the gastropods was inferred from shell size that was, in general, close to 40 mm, and the presence of marked ribs at the shell edge, near the operculum (Minchin & Davies, 1999; Ellis & Pattisina, 1990). During transportation to the laboratory all organisms were kept in aquaria equipped with an air pump.

Bioassay

From the total of 400 collected individuals measuring 40 ± 5 mm of shell length (measures were taken with vernier calipers), 25 were randomly picked and examined for confirmation that the sampled population was imposex free. The assay was performed in 8 aquaria containing 42 gastropods and 10 mussels each. The aquaria were filled with 35 L of

seawater, collected from the sampling site given above, and equipped with air pumps, and activated charcoal filters. Five days were given for adaptation of the organisms to the new conditions prior to starting the inoculations. Two of the 8 aquaria were used as controls. Animals from one of the control batches were inoculated with ethanol (Merck, P.A.), the solvent used for diluting TBT and TPhT standards. The remaining aquaria were divided in two groups of three aquaria; the first group was used for inoculation of the organisms with increasing concentrations of TBT (aquaria TBT-1, TBT-2, TBT-3), and the second group was used for inoculation of TPhT aquaria TPhT-1, TPhT-2, TPhT-3). To ensure better control of the desired dose, the relation between shell length (SL) and fresh weight of soft parts (FW) was tested in a separate batch of organisms ($SL_{(mm)} = 3,46 \times FW_{(g)} + 29,97$; $r = 0,725$). This relation was thereafter used to estimate the animal mass from the shell length (for example, an organism of 40 mm shell length has an average weight of 2.9g). Based on the work of Horiguchi et al (1994) the applied dose was of the order of 10-20 $ng.g^{-1}$. Solutions of tributyltin chloride 96% and triphenyltin chloride 95% (Aldrich Chem. Co.) were then prepared by diluting the standards with ethanol (Merck, P.A.). Inoculation was performed by injecting 1 μL (containing the following amounts of organotin as Sn: TBT-1 = 14.9 ng; TBT-2 = 29.9 ng; TBT-3 = 59.6 ng; TPhT-1 = 12.9 ng; TPhT-2 = 25.9 ng; and TPhT-3 = 51.8 ng) of the diluted solution in the animal foot with the aid of a 5 μL chromatographic syringe. An air-solution-air sandwich was used to ensure that the desired volume was fully injected. Elapsed 14 days (Group1) from inoculation, 15 organisms were removed from each aquarium for determination of the imposex frequency and intensity. The same evaluation was applied to the remaining organisms 28 days after the inoculation (Group 2). A previous transplantation test had shown that this time was sufficient for imposex development in this species (Ribeiro, 2002).

During the 4 weeks of the bioassay duration, water in the aquaria was exchanged weekly. Temperature and salinity were always kept at the initial conditions (20° C and 34.5 $g.kg^{-1}$, respectively). Living mussels were also weekly replaced by freshly harvested animals, and those

by chance dying during the experiment were immediately removed from the aquaria.

Field Gradient Study

Animals were sampled at five sites in Guanabara Bay (Figure 1) previously studied for imposex intensity by Fernandez (2001). Sampling stations were selected as to possibly provide animals with a broad range of imposex intensity (from an expected maximum in the near of the Rio de Janeiro Yacht Club, in Urca wave breaker, to a minimum in the less impacted area of the Cagarras Islands, located outside the bay). From each site at least 20 individuals were collected (Ellis & Pattisina, 1990). The sexual maturity of the gastropods was inferred from the shell size (40.0 ± 5.0 mm), and the presence of marked ribs at the shell edge, near the operculum (Ellis & Pattisina, 1990; Minchin & Davies, 1999). During transportation to the laboratory all organisms were kept in air-pump-equipped aquaria.

Imposex intensity was determined in the gastropods from each station. Thereafter, these animals, along with a sample of 20 mussels *Perna perna* per station, were separated for chemical analysis.

Imposex intensity evaluation

Sex of the animals was determined by the presence of the prostate gland in males, and the albumen, sperm-ingesting and capsule gland in females (Gibbs & Bryan, 1987, 1994; Horiguchi et al, 1994). Penis length in males and in imposexed females was also measured with calipers (Axiak et al, 1995), and vas deferens development was observed under a binocular microscope.

Imposex response in the populations was evaluated by using the RPLI (relative penis length index), RPSI (relative penis size index) and VDSI (vas deferens sequence index), according to the procedures given under Gibbs & Brian, 1986; Gibbs, Bryan, Pascoe & Burt, 1987; Fioroni, Oehlmann & Stroben, 1991; Stroben, Schulte-Oehlmann, Fioroni & Oehlmann, 1995. This last index was determined using a six-stage scale similar to the classic Gibbs scale for *Nucella lapillus* (Gibbs & Bryan, 1994) adapted for *Stramonita haemastoma* by Fernandez et al. (2002). In this scale, grade 0 is attributed to normal females. Grade I includes females presenting

a small hump or pre-penis behind the right tentacle. In Grade II, a small penis is visible. In grade III, the penis is bigger, and an incomplete vas deferens could be observed in the palial cavity floor. In grade IV, the vas deferens is complete and female penis reach a size greater than half of the male's mean size. Grade V is characterized by vulva blockage and Grade VI by aborted egg mass in the capsule gland. After narcotization with a 7% MgCl₂ in a 1:1 solution of distilled water:local seawater (Huet et al, 1995) shells were crushed using a small vice. Narcotization was required to obtain precise measurements of penis size, since the unanesthetized organism retracts after shell removal rendering manipulation and anatomic examination very difficult.

Sample pre-treatment for chemical analysis

Separate composite samples of female and male gastropods from each station were prepared for chemical analysis. Soft tissues selected for chemical analysis were homogenized in an Ultra Turrax and duplicate sub-samples of each homogenate were stored at -20°C in clean glass vessels until analysis. Mussel's soft parts were removed from the shells with the aid of a stainless steel blade. A pooled sample (20 individuals) from each station was homogenized in an Ultra Turrax. Duplicate sub-samples were stored at -20°C in clean glass containers until analysis. Similarly, pooled samples of males and females from each aquarium of the bioassay were prepared and analyzed in duplicate. Gastropods were fed during the bioassays with mussels collected weekly; therefore, organotins were analyzed in sub samples of each batch of harvested mussels.

Analytical Procedure

Analytical protocol was based on Bayona, Fernandez, Porte, Tolosa, Valls & Albaiges (1991) and adapted as described in Fernandez et al. (2005) to the determination by gas chromatography with pulsed flame photometric detection GC/PFPD. The analytical procedure including extraction, clean-up, derivatization and GC/MS determination is described in length in Limaverde (2001). In brief, after spiking 2g of tissue homogenate with tripropyltinchloride (TPrTCl 98%, Merck) and tricyclohexyltinchloride (TcyTCl 97%, Aldrich), samples were treated

with methanol in HCl and extracted with tropolone solution in 3:2 ethyl ether/hexane and sonication. Derivatization proceeded with PeMgBr in diethyl ether under N_2 atmosphere. Clean-up was done in glass columns filled with activated silica and anhydrous Na_2SO_4 . After elution with ethyl ether in hexane and volume reduction under N_2 stream, tetrabutyltin was added as internal standard.

A Varian 3600CX gas chromatograph fitted with an 8200 auto sampler, a pulsed photometric detector (Varian) and a DB-17 (50%-phenyl-methylpolysiloxane) fused silica column (J&W Scientific) were used. The detailed chromatographic conditions are given under Fernandez et al (2005). The determinations were made in triplicate. Quantification was performed by internal standard procedure, using TeBT added after derivatization. Procedural blanks were carried out for every batch of samples.

The analytical procedure was validated by analyzing the reference material NIES-11 (National Institute for Environmental Studies- Environmental Agency of Japan) certified for TBT. The values found for TBT ($512 \pm 58 \text{ ng.g}^{-1}$ as Sn; $n=6$) and TPHT ($1384 \pm 260 \text{ ng.g}^{-1}$ as Sn; $n=6$) are in good agreement with the certified value for TBT ($475 \pm 36 \text{ ng.g}^{-1}$ dw as Sn) and the suggested value for TPHT (1942 ng.g^{-1}), respectively. The calibration curves gave correlation coefficients ($n=6$) of: 0,999 (TBT, DBT, MBT, TCyT), 0,986 (TPhT and DPhT), 0,979 (MPhT) and 0,995 (TeBT). The concentration of butyltins and phenyltins were corrected for recovery of TPrT ($73 \pm 5 \text{ ng.g}^{-1}$). The losses involved in the overall process were estimated by analyzing a sample low in organotins, spiked with a mixture of the standards (TPrT, TBT, DBT, MBT, TPhT, DPhT, MPhT and TCyT) of known concentrations. Equilibration was allowed for 2 hours before extraction. The average value found was $75 \pm 12 \%$ of the initial concentration (TBT gave $91 \pm 18\%$, and the lowest value, not included in the average, was of $60 \pm 2\%$ for MPhT). Mean relative standard deviations for $n=5$ were: 12.2% (TPrT), 3.5% (TBT), 15.0% (DBT), 14.8% (MBT), 27.4% (TPhT), and 36% (DPhT). Detection limits given as [Sn] for the various organotins are: TBT = 0.39 pg; DBT = 0.66 pg; MBT = 0,96 pg; TPhT = 0.90 pg; DPhT =

0.72pg.

Results and Discussion

Table 1 shows the results for the bioassay. Because of the small penis development in imposex females, RPLI and RPSI were not calculated, except for females harvested 28 days after TBT inoculation, which displayed penis of more than 1 mm (RPLI = 17,1; total number of females was equal to 7 from which 5 (71%) showed imposex). The average penis length found in females 28 days after inoculation of 20 ng.g⁻¹ TBT (TBT-3) was 1.5 ± 0.9 mm. Horiguchi et al (1994) and Bech et al (2002b) also found for gastropods of the same genus as *S. haemastoma* that TBT concentrations of 10-20 ng.g⁻¹ of fresh tissue induced development of imposex. Sampling 14 days after inoculation of TPhT was not carried out because of the high mortality observed. In this case, all survivals were harvested 28 days after inoculation, and among those only one female presented a measurable penis size (2 mm). As for the VDSI, no significant differences were observed among organisms 14 days after inoculation with different TBT doses; all altered organisms were classified as in level 1 of the applied VDSI scale. Two animals in the control group, inoculated with ethanol, developed imposex. A good correlation was found between frequency of imposex incidence and inoculated doses (see Figure 2).

The groups of organisms sampled 28 days after inoculation with 59.6 ng of TBT (as Sn) and 51.8 ng of TPhT (as Sn), respectively, had significant VDSI levels: 2.3 (n=6) for TBT inoculation, and 1.4 (n= 5) for TPhT while the control group was imposex free. The X² test and contingency tables were used to check for possible independence of F (healthy females) and I (imposexed females) factors from organotins inoculation dose. For TBT the test was applied first separately to the results for 14 (Group 1) and 28 (Group 2) days of experimentation, and then to the entire data set. The dependency relation found between F/I and the injected dose for Group 1 ($x_0 = 12.72$; $x = 9.23$, d.o.f. = 5, $p < 0.05$) is more evident for Group 2 data since the independence hypothesis was rejected with a value well above the rejection threshold ($x_0 =$

35.81; $x = 9.23$, d.o.f. = 5, $p < 0.001$). Similar relations were obtained for TPhT data in Group 2.

Chemical analysis revealed DBT ($7.38 \pm 2.53 \text{ ng g}^{-1}$ ww as Sn for females and $13.70 \pm 2.06 \text{ ng g}^{-1}$ ww as Sn for males) contamination in the gastropods freshly harvested from the sampling site, and the presence of DBT (5.20 to 6.94 ng.g^{-1} ww as Sn) and MBT (0.54 to 1.05 ng g^{-1} ww as Sn) in the mussels. In the control batches, DBT and MBT concentrations increased over the 28 days of experiment, resulting in a DBT background value (as Sn) of $20.4 \pm 0.6 \text{ ng.g}^{-1}$ in the gastropods. This is an indication that seawater from the area, taken as pristine, contained traces of organotins, possibly derived from eventual navigation activities (pleasure and fishing boats). Although the contamination source for DBT was not clearly identified here, based on the observations of Horiguchi et al (1997) and in the present observations it can be assumed that the background DBT concentration in the gastropods played no role in the development of imposex. The presence of MBT and MPT concentration in excess of the background in females after 28 days of inoculation is evidence of parent compound metabolism.

Table 2 shows organotin concentrations in the animals of the bioassay, and Figure 3 displays the mass balance obtained by using the inoculated dose, the background concentration, and the measured body burden after 14 and 28 days of inoculation. This balance provides an estimate of the average organotin fraction accumulated in the organism. TPhT concentrations in females was 30-40% higher than for TBT, and evidently the accumulation in males is remarkably smaller than in females, especially for TBT, suggesting a more effective biotransformation mechanism in males. The generally higher TPhT persistence in tissues, also observed by Horiguchi, Shiraishi, Shimizu, Yamazaki & Morita (1995), suggests a slower metabolism of this compound in this species.

The field studies showed that organotin concentrations in gastropods (see Table 3) from Leme, Cagarras Islands, Vermelha Beach, Cotumduba and Urca are of the same order of those reported for gastropod of the same genus from other contaminated areas (Horiguchi et al, 1995).

A single study by Rilov et al (2000) along the coast of Israel reports imposex development as well as organotin concentrations in *S. haemastoma*. TBT, DBT and MBT concentrations of 447.7 ng.g⁻¹, 913.6 ng.g⁻¹ and 111.3 ng.g⁻¹, respectively, were found in animals from Herzelia marina, heavily polluted by organotins. Mean VDSI was 4 and some sterile females were identified. At Ha Bonin, a natural reserve and reference area, concentrations were 18.9 ng.g⁻¹ TBT, 51.2 ng.g⁻¹ DBT and 12.2 ng.g⁻¹ MBT, while 25% of females displayed imposex and RPSI was of 0.1. At Vermelha beach, some 4-5 km away from the main organotin sources in Guanabara Bay, TBT, DBT, MBT and TPhT average concentrations were 20.3 ng.g⁻¹, 36.9 ng.g⁻¹, 4.6 ng.g⁻¹ and 7.45 ng.g⁻¹, respectively. Mean VDSI of 3 and RPLI of 30.7 for *S. haemastoma* in Vermelha Beach indicated a more severe response at comparable body burdens. The presence of TPhT, which also induces imposex in this species, may account for the apparent higher sensitivity of animals in Vermelha Beach.

As for the mussels, butyltins were at concentrations comparable with those reported for mussels of the same genus (*Perna viridis*, L.) from contaminated areas of Thailand (Kan-Atireklap, Tanabe, Sanguansin, Tabucanon & Hungspreugs 1997), for *Mytilus trossulus* from Vancouver Harbour (Horiguchi et al, 2003), and for mussels from Danish coastal waters (NERI 2000 *apud* Strand & Jacobsen, 2005). Concentrations in the gastropod were, in general, in the following order: DBT>TBT>MBT. The same order was observed in the mussel tissues, except for animals from Urca where TBT>DBT>MBT. TPhT was the most important phenyl species in both cases.

Berg, Arnold Müller, Mühlemann & Schwarzenbach (2001) and Langston & Pope (1995) showed that TBT sorption on particulate organic matter is fast and reversible; therefore besides TBT in the dissolved phase, particulate organotins are also potentially available to filter feeders (De Mora, 1996). From all observed sites, Urca, located in a highly eutrophic embayment and in the near of an important leisure boat anchoring area, is the most likely to contain a significant fraction of organotins adsorbed on organic particles. These set of conditions may explain the

high absolute and relative TBT concentrations in the organisms from this site.

Bryan et al (1989) estimated that in carnivorous gastropod organotins body burden seem to result from equal contributions from diet and the surrounding environment. The bioconcentration (BC; uptake from water or particulate phase via body surface) in mussels *Mytilus edulis* reported by Laughlin et al (1986) is of the order of 5000 while the biomagnification factor (BM; through algae ingestion) was equal to 2. By using such a BC it is possible to roughly estimate that in the sampled sites butyltin concentration in the water column (assuming that biomagnification is of lesser importance) ranged from 1.5 ng L⁻¹ in Arraial do Cabo to 33 ng L⁻¹ in Urca. Such concentrations are of special concern since detrimental effects of TBT on plankton and oysters have been reported at the concentration range from 0.5 to 50 ng.L⁻¹ (Alzieu, 1996). In the study by Stroben, Schulte-Oehlmann, Fioroni & Oehlmann, (1995), relating imposex level in gastropods to TBT concentrations in water, sterility in *Nucella lapillus*, the only muricid tested that showed this phenomenon, commenced at 4 – 6 ng.L⁻¹ TBT and was widespread in populations exposed to 20 - 30 ng L⁻¹ TBT levels. These levels are similar to the estimate in Urca, where females of *S.haemastoma* could not be found. Because imposex development in this gastropod species can cause female sterility, which leads to profound alteration in populations, the absence of females in Urca seems to concur with the estimated level of 33 ng.L⁻¹ TBT in water.

Some authors have studied the biomagnification of organotins in food webs (Laughlin, French & Guard, 1986; Strand & Jacobsen, 2005). Mensink, Boon, ten Hallers-Tjabbes, van Hattun & Koeman, (1997) calculated for a North Sea whelk a BM of 8 in relation to *Mytilus edulis*, and TBT concentrations in snails lower than in mussel by a factor of 7. Stäb, Traas, Stroomberg van Kesteren, Leonards, van Hattum et al (1996) also report no biomagnification in the food web of fresh water systems in Netherlands. In the present work, BM (BM = [OT]_{gastr.}/[OT]_{mussel}, OT = organotins) values for TBT ranged from 0.5 to 2.7 (n = 6 observed sites); for DBT the range was from 0.9 to 1.6; for MBT 1.2-3.5; and for TPhT 1.9 to 7.6 (n = 4

sampling sites). Data were not normalized to lipid content because of the higher affinity of the studied compounds to proteins. For the butyltins the linear plot ($[BT]_{\text{gastropod}} = 13.91 + 0.74[BT]_{\text{mussels}}$; $r = 0.953$; $p = 0.0009$, BT = butyltins) of concentrations in the gastropods versus concentrations in the mussels show that, in the low concentration range ($[BT]_{\text{gastropod}} \leq 50 \text{ ng g}^{-1}$ ww as Sn, BT = butyltins), the calculated BM > 1, decreasing smoothly above this limiting concentration. If it is considered that biomagnification does not occur, this maybe the threshold value above which bioelimination mechanisms assume higher weight than bioaccumulation processes. The relatively high biomagnification for TPhT may imply that biotransformation mechanisms are less efficient for this compound.

TBT contribution to the BT body burden was of 37 to 64% in gastropods and of 37 to 67 % in mussels. There was no relation between the fraction of TBT in gastropods and in the mussels. Only mussels from Urca showed TBT at higher levels than DBT, indicating that recent inputs of antifouling overwhelm on going degradation processes.

The following pairs of data were tested for correlation: (1) VDSI levels and TBT concentration; (2) VDSI level and total butyltins; (3) VDSI level and the sum of all organotins. Because no females were found in Urca and there were no significant differences in organotin concentration between females and males, a tentative VDSI level for females, when existing in Urca, was obtained from the organotin concentration in males from this station using the linear equations ($y = 0.004x + 0.145$; $r^2 = 0.885$ for OTs versus VDSI, and $y = 0.046x + 0.148$; $r^2 = 0.903$ for butyltins versus VDSI) obtained for data from all other sites. The obtained results of 6.2 (OTs) and 6.4 (butyltins), included in the above correlation tests, may explain the absence of females there, since at these imposex levels possible lethal alterations are expected, as for instance blockage of the vulva and presence of dark decaying tissue in the capsule gland. Some populations in heavily impacted areas are composed mostly of males, because of selective female mortality (Gibbs & Bryan, 1994). Figure 4 shows the plot of VDSI versus butyltins and the good fit of the bioassay values (VDSI = 2.3 and butyltins = 28.2 ng.g^{-1} , 28 days after TBT

inoculation) to the curve.

There was excellent correlation between the data obtained for mussels collected in the same sites and dates as the gastropods, and imposex levels in the later ($r = 0.934$; $p < 0.05$). Because concentrations in mussels are closely related to that of contaminants in the water column, the above correlation suggests organotin induction of imposex in *Stramonita haemastoma* under local conditions in the observed sites.

Conclusions

The bioassay demonstrated that TBT and TPhT are capable of inducing imposex in *S. haemastoma*. This evidence coupled to the field studies demonstrate that this species is an adequate bioindicator of organotins contamination, either by means of monitoring the body burden of OTs or/ and determining imposex incidence. The inclusion of mussels in the field studies was useful as an indicator of the presence of organotins in the water column and demonstrated that this species is also functional in monitoring organotin contamination. Mussels can be useful in evaluating whether anomalies in gastropod population distribution (as in the case of Urca where only male gastropods were found) and linked to higher levels of organotin contamination in the environment. The results from the field studies allied to the bioassay confirmed the reliability of the VDSI scale proposed by Fernandez (2001) for *S. haemastoma* as demonstrated by the excellent correlation found between VDSI level and organotin concentrations in tissues.

The chemical analysis of animals used in the bioassay indicated metabolization and excretion of TBT and TPhT by females and males of *S. haemastoma* with indications of a faster elimination in male gastropods.

As for concentrations of organotins in snails measured in the field study, those found for TBT in Vermelha Beach and in Urca are above the inoculated concentrations in the bioassay that caused imposex development. These areas evidently show contamination levels that pose a threat

to molluscs and other organisms at the lower trophic levels, which appear to be at higher risk from exposure to such substances.

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Table 1- Biological results of the bioassay. A = results for sampling 14 days after inoculation; B = results for sampling 28 days after inoculation; C = final results of the bioassay after each treatment; F- healthy females; I – imposexed females; M – males; T- total number of animals; VDSI – level of VDSI in imposex females; % - imposex frequency; D* - average dose (ng Sn / g of fresh tissue); Mort. – mortality; F-T – total number of healthy females; I –T –total number of imposexed females; % T – total imposex frequency; nr – not retrieved.

Aquaria	A							B							C			
	F	I	M	T	VDSI	%	D*	F	I	M	T	VDSI	%	D*	Mort	F-T	I-T	%T
Control	7	0	8	15	0	0	0	13	0	12	25	0	0	0	2	20	0	0
Control + ethanol	6	2	7	15	1	25	0	15	0	10	25	0	0	0	2	21	2	8,7
TBT 1	3	3	9	15	1	50	5	9	3	12	24	1	25	5	3	12	6	33,3
TBT 2	3	5	7	15	1	63	10	6	5	14	25	1	45	12	2	9	10	52,6
TBT 3	2	6	7	15	1	75	24	1	6	5	12	2,3	86	20	15	3	12	80,0
TPT 1	nr	nr	nr	-	-	-	-	3	1	5	9	1	25	5	33	3	1	25,0
TPT 2	nr	nr	nr	-	-	-	-	2	3	3	8	1	60	8	34	2	3	60,0
TPT 3	2	5	8	15	1	71	23	8	5	10	23	1,4	38	28	4	10	10	50,0

Table 2. Results of organotin determination (ng g^{-1} ww as Sn) in gastropods from aquaria TBT-3 and TPhP-3 of the bioassay. Inoculated doses in ng g^{-1} ww as Sn: (a) 24.7; (b) 24.9; (c) 20.0 and (d) 20.0; (e) 22.8; (f) 23.5; (g) 28.3; (h) 25.3.

Samplings Compound	14 days		28 days	
	Females	Males	Females	Males
TBT-3	(n = 8) ^a	(n = 8) ^b	(n = 7) ^c	(n = 8) ^d
TBT	5.21± 0.13	<dl	< dl	< dl
DBT	29.20±5.26	19.77±3.61	16.00±1.17	22.59±2.20
MBT	< dl	2.21±0.68	12.35±0.49	3.06±0.03
TPhT-3	(n = 7) ^e	(n = 8) ^f	(n= 13) ^g	(n = 10) ^h
TPhT	16.08±0.75	4.31±0.74	14.14±2.03	8.23±1.85
DPhT	1.09±0.09	< dl	0.71±0.21	< dl
MPhT	< dl	1.14±0.67	< dl	4.10±1.77
(DBT)	12.51±1.04	20.86±6.12	20.89±6.72	23.97±3.17

Table 3. Imposex development expressed by VDSI (Vas Deferens Sequence Index*), organotin concentrations (ng g⁻¹ ww as Sn) in *S. haemastoma* (S.h.), and organotin concentrations in the mussel *P. perna* (P.p.). AC stands for Arraial do Cabo, sampling site for the biassay; 1-5 refer to sampling stations in Guanabara Bay shown under Figure 1.

	Sampling Sites													
	AC		5		4		2 (2000)		2 (2001)		3		1	
	S.h.	P.p.	S.h.	P.p.	S.h.	P.p.	S.h.	P.p.	S.h.	P.p.	S.h.	P.p.	S.h.	P.p.
VDSI	0	-	1.78 ± 0.43	-	2.28 ± 0.69	-	3.33 ± 0.49	-	2.71 ± 0.45	-	3.00 ± 0.82	-	6*	-
TBT	< dl	< dl	14.48 ± 1.31	5.44 ± 2.27	16.44 ± 2.38	13.03 ± 2.63	18.39 ± 2.39	34.29 ± 1.18	22.23 ± 3.27	35.89 ± 8.60	33.74 ± 3.45	19.62 ± 2.48	62.69 ± 1.84	110.08 ± 10.22
DBT	7.38 ± 2.53	6.77 ± 0.33	7.33 ± 0.28	7.85 ± 0.14	26.15 ± 3.61	20.88 ± 0.31	41.38 ± 2.08	38.13 ± 1.18	32.46 ± 4.56	36.05 ± 2.19	30.90 ± 4.59	19.25 ± 0.96	71.45 ± 4.42	54.13 ± 2.11
MBT	< dl	0.69 ± 0.10	0.87 ± 0.01	0.76 ± 0.03	2.02 ± 0.78	1.00 ± 0.02	7.18 ± 0.55	5.91 ± 2.56	1.97 ± 0.35	1.41 ± 0.21	4.14 ± 2.57	1.17 ± 0.06	3.84 ± 0.28	< dl
TPhT	3.30 ± 0.11	< dl	0.25 ± 0.05	0.53 ± 0.05	2.47 ± 0.29	0.53 ± 0.05	10.20 ± 1.04	< dl	4.69 ± 0.16	1.03 ± 0.09	4.37 ± 0.64	0.58 ± 0.01	10.87 ± 0.75	5.86 ± 0.94
DPhT	< dl	< dl	0.79 ± 0.04	< dl	0.96 ± 0.12	< dl	< dl	< dl	< dl	< dl	0.58 ± 0.05	< dl	< dl	< dl
MPhT	< dl	< dl	< dl	< dl	2.75 ± 0.73	< dl	2.79 ± 0.26	< dl	1.53 ± 0.27	< dl	< dl	< dl	< dl	< dl
Σ BT	7.38	7.46	22.69	14.05	44.61	34.91	66.95	78.33	56.67	73.36	68.78	40.05	137.97	164.21
Σ PhT	3.30	< dl	1.03	0.83	6.17	0.523	12.88	< dl	6.21	1.03	4.95	0.58	10.87	5.86
Σ OTs	10.68	7.46	23.72	14.88	50.80	35.44	79.94	78.33	62.89	74.39	73.74	40.62	148.84	170.07

Figure 1: Sampling stations in Guanabara Bay.

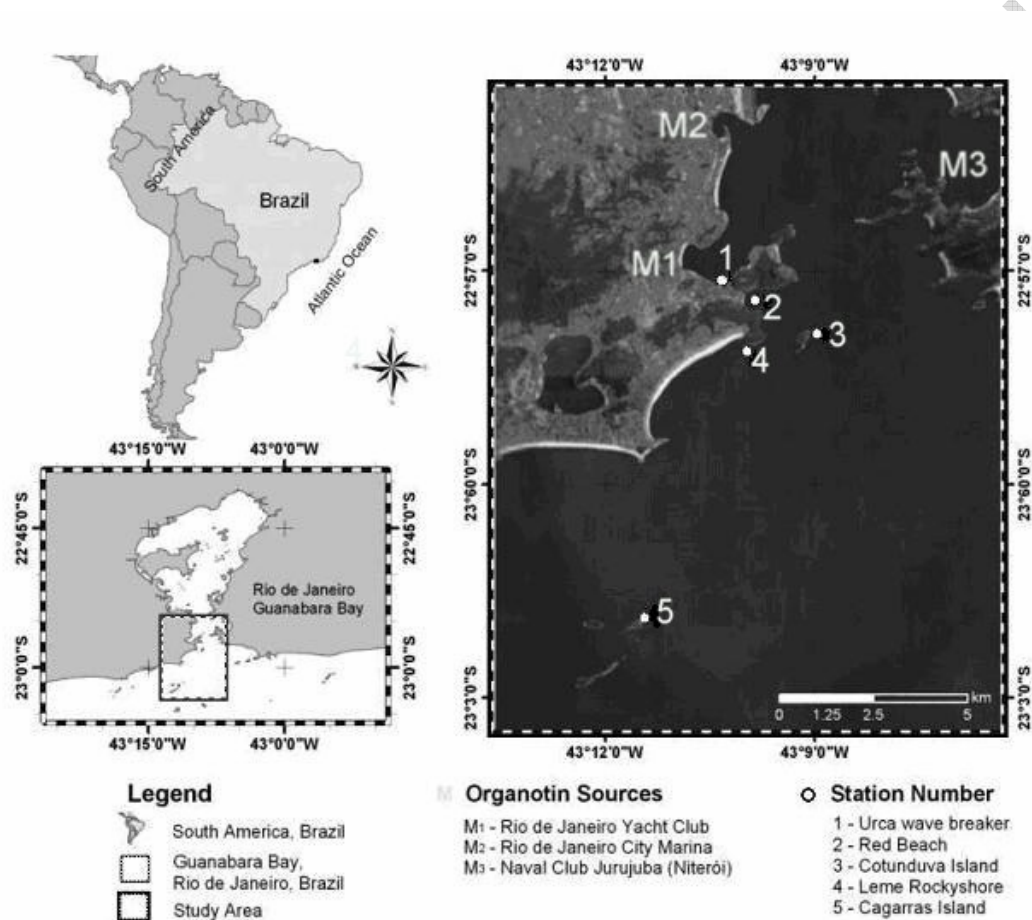


Figure 2: Imposex frequency and inoculated TBT concentrations in the different aquaria: 1 - control aquarium; 2 - control aquarium + ethanol; 3 - TBT 1 ($41.5 \text{ ng}\cdot\mu\text{L}^{-1}$); 4 - TBT 2 ($82.9 \text{ ng}\cdot\mu\text{L}^{-1}$); 5 - TBT 3 ($165.9 \text{ ng}\cdot\mu\text{L}^{-1}$)

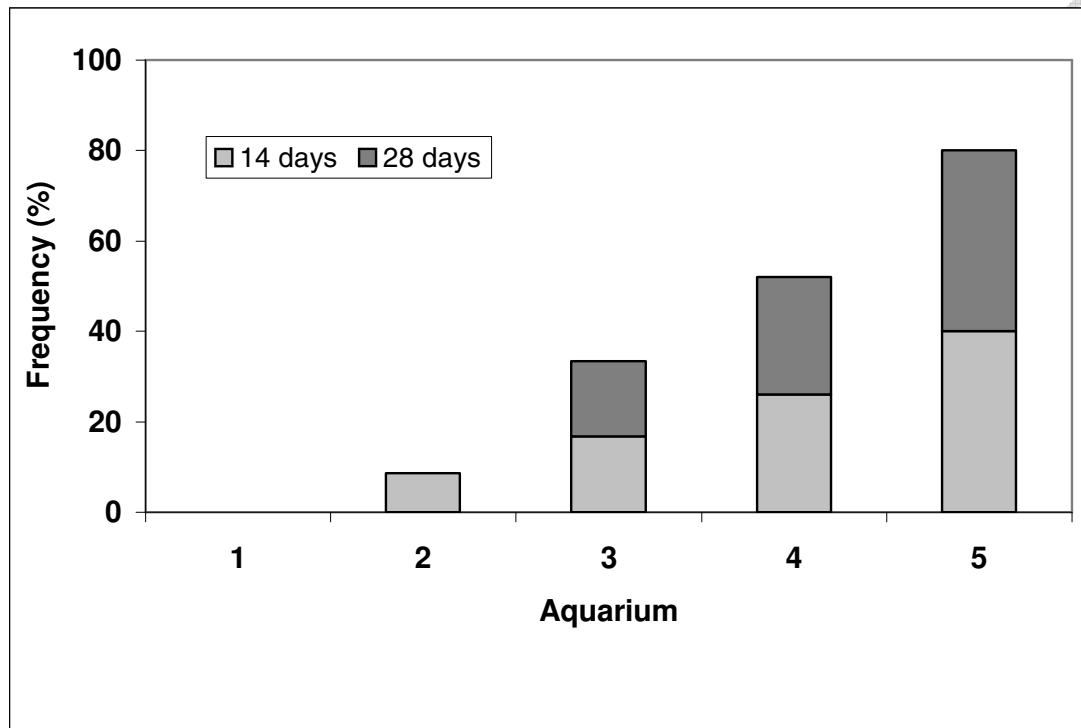


Figure 3. Calculated mass balance of organotin in the bioassay (ng g^{-1} ww as Sn). Key of abbreviations: I = inoculated dose of TBT or TPhT; B = DBT background concentration found in freshly harvested gastropods; A = total organotin body burden; E = excretion (calculated by difference, speciation not determined in water). OTs = Total sum of butyltin and phenyltin derivatives. Codes in the abscissa: B = butyltin (TBT-3 aquarium), M = males, F = females, 14 and 28 days after the injection; Ph = phenyltin (TPhT-3 aquarium).

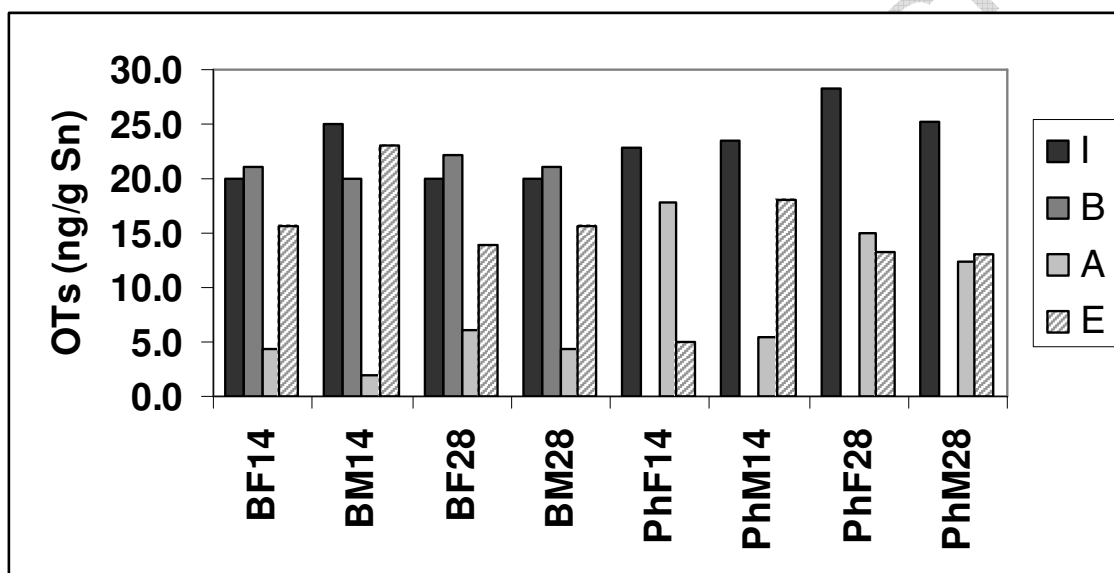
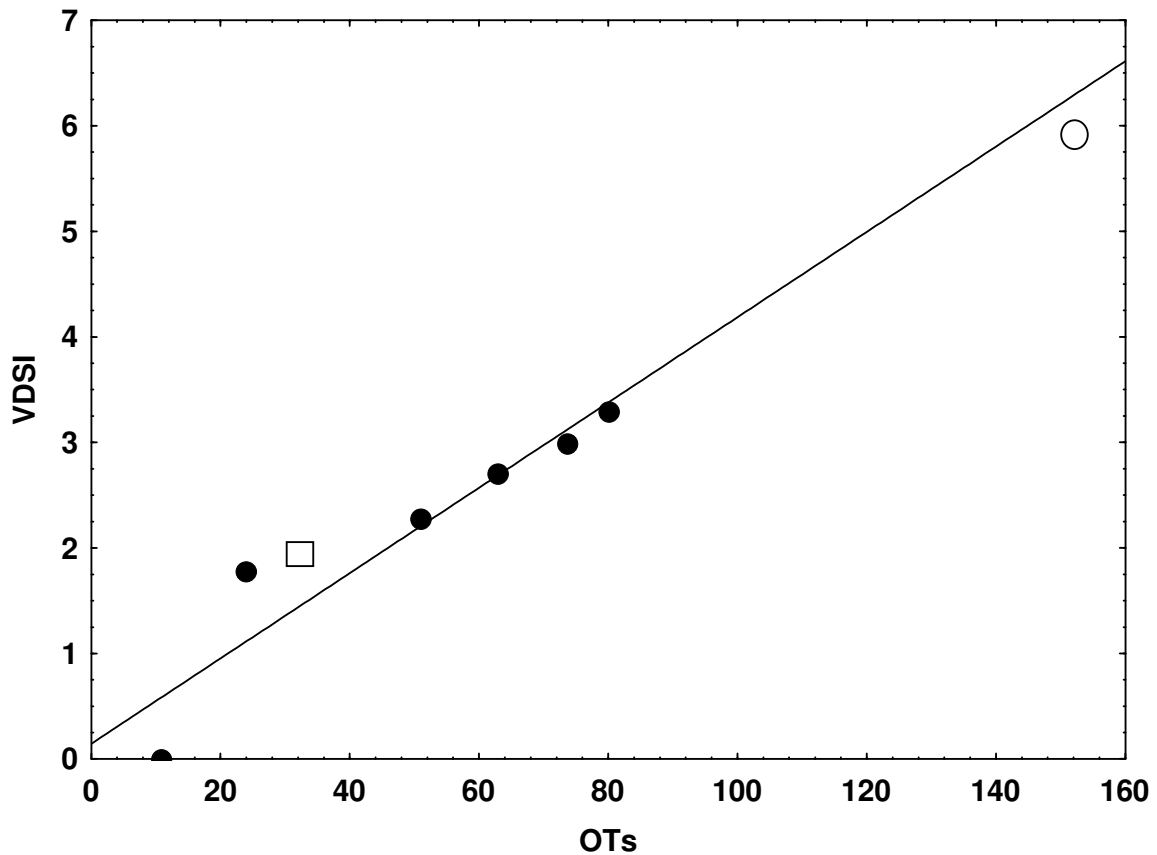


Figure 4. Correlation (linear fitting $r = 0.941$; $p < 0.005$; $VDSI = 0.145 + 0.04 \text{ OTs}$) between field data for imposex development expressed by the VDSI (Vas Deferens Sequence Index) and OTs (total organotins) concentration, ng g^{-1} as Sn (black dots). Results for the bioassay (open square) and concentrations in males from Urca (open circle) were interpolated.



ACCEPTED