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Imposex in *Stramonita haemastoma*: a preliminary comparison between waterborne and dietborne exposure

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Abstract

400 adult individuals of the *Stramonita haemastoma* were collected from a pristine beach in Ceara State, Brazil. These organisms were transplanted into a marina with intense shipping activities and were fed weekly with oysters obtained from the same beach from which they were collected, being exposed only to the bioavailable organotins in the environment, without accounting for diet (waterborne exposure). 30 individuals were analyzed every 2 weeks after transplantation to investigate the development of imposex. After 15 days, 90% of the females had developed imposex (VDSI I-III, RPLI 3.22), with 100% of the females developing imposex after 30 days; at the end of the experiment, the VDSI levels ranged between I-V. These results were compared with those of a previously published study performed simultaneously in which gastropods obtained in same area were fed in the laboratory with oysters from the same marina in which the gastropods were transplanted (dietborne exposure). Despite the higher levels obtained through exposure via water at the end of 90 days, both exposure routes induced imposex in 100% of the females of the *S. haemastoma*. However, aqueous exposure induced higher levels, possibly due to the constant contact between the organisms and contaminants.

Keywords: Dietborne; Imposex; Organotin contamination; *Stramonita haemastoma*; Waterborne

INTRODUCTION

Imposex is defined as a masculinization phenomenon or a pseudo hermaphroditic condition characterized by the development and superimposition of non-functional male sexual characteristics (penis, vas deferens and/or seminiferous tubules) in female prosobranch gastropods (Smith, 1971). The positive correlation between the occurrence of imposex and the proximity to potential pollutant sources, such as ports, marinas and yacht basins, was reported later (Smith, 1981) and actually, imposex is recognized as a truly global phenomenon, with at least 240 species of prosobranch gastropods affected (Titley-O'Neal *et al.*, 2011).

The chemical basis for imposex was obtained through environmental studies that established significant correlations between the incidence of imposex and the water TBT concentrations (e.g., Stroben *et al.*, 1995), with the imposex intensity varying according to sensitivity of the species considered. Thus, there is ample evidence that imposex is strictly linked to exposure to TBT, even in *Stramonita haemastoma* (Toste *et al.*, 2013).

Although the TBT released by antifouling paints is presumed to be the main inducer of imposex in gastropods, the route of entry into the organisms is poorly understood. Additionally, once released into the marine environment, the TBT sorptive equilibrium between the dissolved and solid phases may cause it to become bioavailable to the biota through a combined exposure to these different phases (Coelho *et al.*, 2002a). Several reviews have demonstrated that marine organisms accumulate contaminants from the

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seawater, sediment and contaminated food (Langston & Burt, 1991, Alzieu, 1996, Coelho *et al.*, 2002a, 2002b). In addition, it is known that TBT exposure to organisms usually occurs through contaminated water or food. However, there is no information about the organotin uptake routes in *S. haemastoma*. This gastropod is widely distributed in the Brazilian coastal areas and is easily found on the bedrock of most Brazilian beaches. Transplant (Queiroz *et al.*, 2007, Castro *et al.*, 2012a) and biomonitoring studies based on *S. haemastoma* imposex incidence have been performed (e.g. Fernandez *et al.*, 2005, Castro *et al.*, 2007, 2012b), demonstrating the sensitivity of the species to organotins. *S. haemastoma* (Kool, 1987) is a relatively large predatory whelk (up to ~ 80 mm shell length), feeding preferentially on filter-feeding bivalves, such as oysters and mussels (Butler, 1985). Oysters are relatively ubiquitous, and TBT accumulation has been shown to be positively related to seawater TBT concentrations (Langston et al., 1991).

Based on the above, the present work aims to verify whether there are differences in the imposex development parameters in *S. haemastoma* exposed to two different environmental conditions. An understanding of the major organotin assimilation route in this important TBT biological indicator species is extremely important because provides basic information that may be useful for studies about the mechanism of imposex development and impact of TBT on aquatic communities.

Thus, two approaches were taken in this study: (1) in the field conditions where the organisms were presumably exposed to possible contaminated water and received a diet without contamination (waterborne exposure) and (2) in the laboratory by exposing the organisms to a contaminated diet (dietborne exposure) (data published in Lima *et al.*, 2006). The both experiments were conducted jointly in July 2004 and will be compared in the present study.

MATERIAL AND METHODS

Organisms. A population of *Stramonita haemastoma*, with no signs of imposex development, was obtained at Caponga Beach, Ceara State, Brazil (Castro *et al.*, 2000) (Figure 1). In this population, 600 adult specimens (shell length between 20 and 40 mm) were collected, kept in 60 L aerated aquaria filled with natural seawater (salinity 35) and maintained in the laboratory at 25°C for a week to acclimate. A total of 30 individuals were then analyzed to confirm the absence of imposex at the collection site. The remaining animals were divided into two groups: the first was transplanted directly into the contaminated environment (waterborne exposure), and the second remained in the laboratory and was fed with possibly contaminated oysters (dietborne exposure).

Waterborne exposure. The animals were placed in cages and transplanted into a small marina inside the Indústria Naval do Ceara repair shipyard (Figure 1). This site was previously analyzed for the occurrence of imposex, and high imposex levels were detected in the resident gastropods (Castro *et al.*,

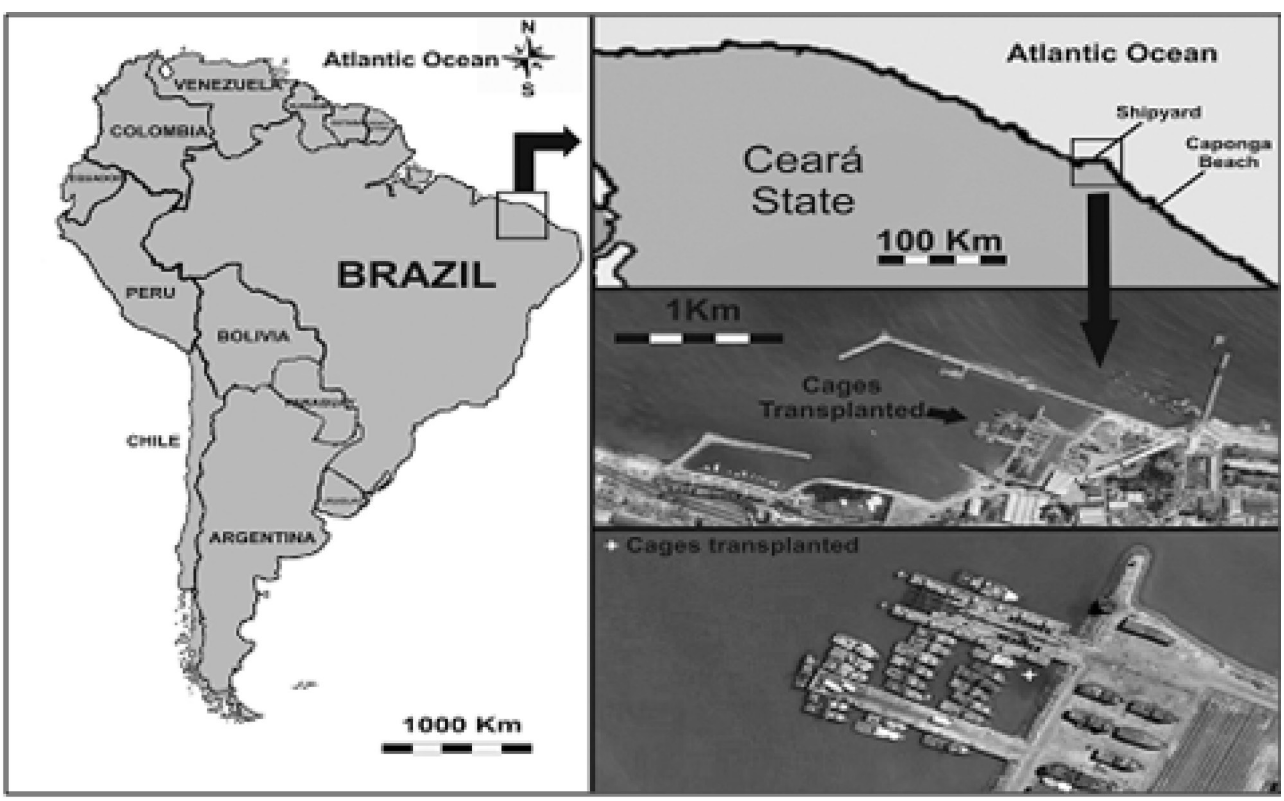

Figure 1 - Local of the transplantation from the uncontaminated site at Caponga Beach to the contaminated site at the small marina inside the Indústria Naval do Ceara repair shipyard. The local collection and control of the contaminated oysters are also shown.

2008). Additionally, this site is presumed to be an organotin-contaminated area due to the ship maintenance, repair and construction operations performed at the installations. The animals were fed weekly with *Crassostrea rhizophora* oysters collected from Caponga Beach (the same site where the snails were collected).

Dietborne exposure. In the laboratory, the animals (second group) were distributed into 7 tanks with filtered seawater at a salinity of 35 and constant aeration. One of the tanks was considered the Control group, which received oysters from Caponga Beach as food. The remaining tanks were fed *ad libitum* weekly with oysters obtained from a site of the Ceara shipping industry (Figure 1 – the same region to which the snails were transplanted for the waterborne exposition). Due to its suspension-feeding habit, oysters possibly assimilate and bioaccumulate organotin compounds dissolved in the water, sediment bound or in food (e.g., phytoplankton). The water in the aquaria was changed twice a week using water obtained at Caponga Beach.

The results of this exposure route (dietborne) were published previously by Lima *et al.*, (2006) and will be used in this article to compare the different routes of exposure.

Imposex determination. The removal of sub-samples of 30 organisms from both exposure experiments (waterborne and dietborne) was performed at intervals of 15 days, until day 90. Each sub-sample was taken to the laboratory, and the imposex indexes were analyzed as follows. The organisms were narcotized in 3.5% $MgCl_2$, and the shell length from the spiral tip to the end of the siphon channel of each animal was measured using a caliper (0.05 mm). The soft tissues were extracted, and the sex was determined by the presence (or absence) of the sperm-ingesting gland in females and the prostate in males. Females with a penis and sperm-ingesting gland were considered imposexed. The penis lengths were measured to the nearest millimeter. The imposex indexes was calculated as suggested by Gibbs & Bryan (1987): RPLI (Relative Penis Length Index) = (mean Female Penis Length / mean Male Penis Length) X 100. The VDSI (Vas Deferens Sequence Index) was inferred by a comparison of the observations in the animals with the scale proposed by Gibbs *et al.* (1987) and modified by Fernandez *et al.* (2002) for *Stramonita haemastoma*.

Statistics. Homogeneity of variances was assessed by Levene's test and normality by Shapiro-Wilks. The Pearson and Spearman correlations between waterborne and dietborne results were used according with the assumptions. Statistical significance when comparing the time of exposition in water was determined by Kruskal- Wallis nonparametric ANOVA and distribution free multiple comparisons, because the data not present normal distribution and containing heterogeneous variances. T-test was done to compare the waterborne and dietborne results. All tests were performed in R, version 3.0.1 (R Core Team, 2013) and were accomplished with a significance level of 0.05.

RESULTS AND DISCUSSION

Several studies have shown that the TBT accumulation from both waterborne and dietborne sources are important for many organisms, such as clams, mussels, crabs, carnivorous gastropods and fish. However, the patterns of TBT accumulation, distribution and elimination are completely different for these two routes of exposure (Rouleau *et al.*, 1999, Coelho *et al.*, 2002a, 2002b).

The accumulation from seawater would be the preferred route for filter-feeding organisms, such as clams (*Ruditapes decussatus*) and mussels (*Mytilus edulis*) (Laughlin et al., 1986, Coelho et al., 2002a). However, for such carnivorous organisms as crabs (*Chionoecetes opilio*) (Rouleau *et al.*, 1999), the main route would be via a dietary exposure, as the most important pathway for organisms of higher trophic levels.

In the transplant experiment (waterborne exposure – Figure 2 A, B and C), 90% of the *Stramonita haemastoma* females showed initial imposex stages (VDSI I - III) within the first 15 days after transplantation to the marina environment, indicating a rapid induction. In this sampling, the RPLI was low (< 5) reflecting the low average VDSI (0.93). After 30 days, 100% of the females had developed imposex, a frequency maintained until day 90. The VDSI increased significantly until day 90 (Kruskal-Wallis, H: 64.73, $p < 0.001$) however with less intensity between days 60 and 90. At the end of the transplantation experiment (i.e., after 90 days), the average VDSI was 3.4, with the females showing indexes from I to V. The final RPLI was 41.18, and significant increase (Kruskal-Wallis H: 61.49 and, $p < 0.0001$) in the average of the Female Penis Length was already noted within 30 days of the transplantation, reaching 3.51 cm after 90 days (Figure 3). Thus, it was possible to verify that, in only 90 days, the transplanted organisms reached almost the same imposex levels observed in native *S. haemastoma* from this area by Castro *et al.* (2008) (VDSI = 4.3 and RPLI = 58.6). These results highlight the rapid development of high imposex levels in a relatively short time, possibly due to the high sensitivity of this species and high contamination of the site.

A significant increase in the length of the female penis was also noted in the dietborne exposure (Lima *et al.*, 2006). Despite not having been observed statistical differences for the imposex % (T-test, $p = 0.363$), were observed significant differences for the VDSI (T-test, $p = 0.004$) and RPLI (T-test, $p = 0.006$) induced by the water and diet exposures.

In addition, different from moderate correlation founded between the imposex incidence and duration of dietborne exposure reported by Lima *et al.* (2006), the results of the present study showed that the imposex development in *S. haemastoma* is strongly correlated with exposure time in the transplant experiment (Spearman correlation, $R = 0.802$, $p = 0.03$, Figure 2A). These measurements are regarded as the two main indexes for the evaluation of the imposex levels

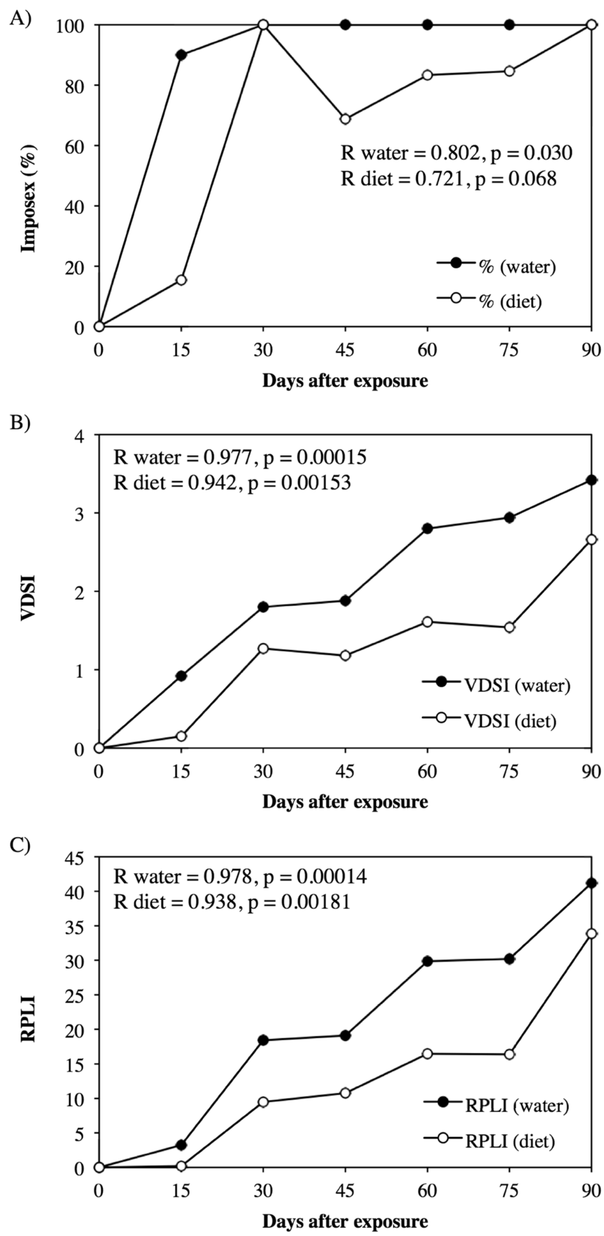

Figure 2 - Comparison between the (A) induction of imposex (%I); (B) VDSI and (C) RPLI after 90 days of waterborne and dietborne exposure. Results of Pearson correlations for %I and VDSI, and Spearman correlations for RPLI.

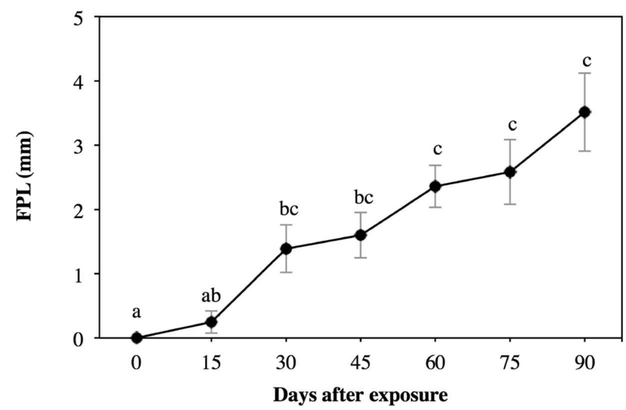

Figure 3 - Female penis growth in *S. haemastoma* for 90 days of waterborne exposure. Error bars represent SEs (n = 10-20 female snails). Letters shown the results of Kruskal-Wallis ($p \leq 0.05$).

in *S. haemastoma*, and have been reported strong positive correlations between RPLI and/or VDSI with environmental TBT concentrations in many contaminated areas (Rossato *et al.*, in press, Fernandez *et al.*, 2005).

Considering that the oysters used as food organisms in the laboratory dietborne exposure were obtained from the same potentially contaminated marina where the transplant experiment was performed, it was assumed that the exposure concentrations were similar and showed the efficiency of a waterborne exposure to induce imposex, reflecting the increased of TBT bioavailability in environmental conditions, excepting the diet.

In general, TBT present hydrophobic characteristics, and its seawater solubility is low and related to temperature, ionic strength and pH (Fent, 1996). Moreover, the high lipid solubility the due to high octanol-water (K_{ow}) partition coefficient, contributes to TBT fast bioaccumulation in marine organisms (Maguirre, 2000). Thus, tributyltin is preferentially accumulated in the digestive and reproductive tissues, due to the higher lipid contents than the remaining tissues (mainly muscle) (Wang *et al.*, 2010). Moreover, as described by Coelho *et al.* (2002a, 2002b), the environmental conditions are very important in determining the bioavailability of TBT. Many biotic and abiotic factors may act together in altering the importance of different routes of assimilation. Considering this information, we stress that, in this work, the food of the organisms in both experiments was controlled and offered at the same amount and frequency, thereby ensuring that all of the organisms had the same food supply. However, despite the controlled laboratory conditions, it is impossible to assert that the organisms in the two exposure treatments (waterborne and dietborne) were under the same environmental conditions.

S. haemastoma is carnivorous, therefore it was expected that the imposex development would be more pronounced in the dietborne exposure because the lipophilic TBT is preferentially accumulated in the digestive and reproductive tissues (Wang *et al.*, 2010). However, imposex parameters (I%= 69 and VDSI 1.18) presented by the females in the dietary exposure (data published by Lima *et al.*, 2006) was lower than that observed in females transplanted at the marina (waterborne) (I%=100 and VDSI 1.9) after 45 days. This pattern was maintained until the end of the experiments (day 90) and was also observed by Wang *et al.* (2010), who carried out a similar study and reported that the *Thais clavigera* females exposed via dietborne (VDSI 1.35) exhibited lower imposex levels those exposed via waterborne (VDSI 1.87). The VDSI values (1.9) obtained through the waterborne exposure were similar to those reported by Wang *et al.* (2010) (1.87) after 45 days of water exposure. However, in the dietborne argued in the present study, the imposex development was lower than obtained by Wang *et al.* (2010) (1.18 and 1.35, respectively). Moreover, the low VDSI obtained by Lima *et al.* (2006) may be a consequence of a limiting food supply, as the animals were fed only weekly with whole oysters, whereas the *T. clavigera* whelks in the study of Wang *et al.* (2010) were fed daily and only with the digestive tissue of previously contaminated oysters (oysters accumulate ~ 90% uptake of TBT). On the other hand, the high relative imposex incidence after the waterborne exposure may be due to the fact that the transplanted organisms remained in contact with the contaminant for 24 hours, whereas the animals were only exposed to the contamination during the feeding period in the dietborne exposure.

Although higher imposex levels have been obtained during waterborne exposure at the end of 90 days, both exposure routes (waterborne and dietborne) induced imposex in 100% of the females. Therefore, we conclude that both routes are effective in inducing imposex. However, the water route induced more significant levels, possibly due to continuous organisms exposure to the contaminants in environmental conditions. These results corroborate other field and laboratory studies performed with several gastropod species, including the muricids *N. lapillus* (Davies, 2000, Quintela et al., 2000), *T. clavigera* (Shim *et al.*, 2000, Wang *et al.*, 2010) and *T. distingueda* (Bech *et al.*, 2002). Furthermore, the relative contribution of each route would depend on the prevailing ecological and physiological conditions and the spatial and temporal variations in TBT environmental levels. Accordingly, the extrapolation of the laboratory results to the natural environment should be undertaken with caution.

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