International Journal for Environmental Rehabilitation and Conservation
ISSN: 0975 — 6272
XI (SP2): 357 — 371
www.essence-journal.com

Original Research Article

Imposex induction in stramonita haemastoma, A bioindicator for organotin contamination in coastal environments

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A R T I C L E I N F O

Received: 17 July 2020 | Accepted: 15 August 2020 | Published Online: 30 September 2020

EOI: 10.11208/essence.20.11.SP2.163

A B S T R A C T

Imposex is an imposition of male characters in female due to organotin compounds in Stramonita haemastoma (Neogastropoda: Muricidae). The assessment of this phenomenon was investigated as a suitable bioindicator of tributyltin (TBT) contamination in the Moroccan Atlantic coast by: 1. Imposex induction in healthy females (from the safe site) after inoculation with TBT in the laboratory; and 2. Determining the incidence of imposex in S. haemastoma collected from areas with various levels of TBT and determining the concentrations of this chemical compound in its tissues. In this context, the aim of this study was to determine the impact of TBT based on sexual indices (RPLI, RPSI, FPL, VDSI and percentage of imposex). Imposex intensities and organotin concentrations in tissues showed good correlation, indicating S. haemastoma as a reliable bio-indicator of TBT contamination in marine coastal waters. Body burden threshold of TB T for imposex induction was stimated to be 7-20 ng.g-1.

K E Y W O R D S

Organotins | Stramonita (Thaïs) Haemastoma | Bioassay | Imposex | TBT

C I T A T I O N

Mortaji, H. El.; Benhra, A.; Elkhiati, N.; Haimeur, B. EL; Bouhallaoui, M. and Kabine, M. (2020): Imposex induction in stramonita haemastoma, A bioindicator for organotin contamination in coastal environments. ESSENCE Int. J. Env. Rehab. Conserv. XI (SP2): 357 — 371.

https://eoi.citefactor.org/10.11208/essence.20.11.SP2.163

Introduction

Tributyltin (TBT) is an organotin compound widely used in the composition of antifouling paints intended for the protection of the hulls of boats, stabilization of PVC (Polyvinyl chlorid) and prevention of clogging of cooling systems (Borghiand *et al.,* 2002). Despite all these benefits, TBT molecule remains extremely dangerous for the environment and threaten animal, especially molluscs, communities in both marine and freshwater ecosystems (Sayer *et al*., 2006). Indeed, many species are sensitive to very low concentrations of this biocide and a concentration of only c.a 0.4 ng/l has been shown to disturb endocrine activity (Huet *et al.,* 2004).

Since the beginning of the 1980s, certain ecological disorders have been observed and His and Robert (1985) suggested that the weak recruitment of oyster spawn (in the Arcachon Basin, France) could be the consequence of strong larval mortality caused by the TBT. Alzieu *et al.,* (1981) highlighted the role of the TBT in disturbing shell formation in oysters *Ostrea edulis.* As a consequence of the TBT threat to shell fisheries, France was the first country to regulate the use of organotin-based antifouling paints (Alzieu, 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 and Bryan., 1986; Horiguchi *and al.,*1997; Depledge and Billinghurst, 1999). Imposex, has been observed in several areas of the globe where OT contamination is present (Mathiessen and Gibbs., 1998).

Correlations between dose or concentration and negative biological effects of TBT have been demonstrated in the laboratory (Bryan *et al.,* 1988). Transplantation experiments where gastropods were moved to a contaminated harbour area have clearly demonstrated initiation of the imposex phenomenon (Stewart and Thompson, 1994). Further, this stimulation of imposex is indicated to be an irreversible process for the gastropod (Gibbs and Bryan, 1987).

The gastropod *Stramonita haemastoma* was selected as the biological indicator species for evaluation of pollution by TBT on the Moroccan Atlantic (El Mortaji *et al.,* 2011) and Mediterranean coasts (Lemghich and Benajiba, 2007). This species was selected because it is common in this region (EL Mortaji *et al.*, 2011; Lemghich and Benajiba, 2007) and it has a strong tendency for imposex development (SPENCE *et al.,* 1990, Terlizzi *et al.,* 1997). Furthermore, the incidence of imposex in this species is in known to be closely linked with TBT (Liu and Suen, 1996; Horiguchi *et al.,* 1997, Rilov *et al.,* 2000, Chiavarini *et al.,* 2003). A few authors used organotins in bioassays to induce imposex in ***Thaïs*** genus: Horiguchi *et al.,* (1997) used injection of the toxicants, while Liu and Suen (1996) tested direct exposure to the toxicant in water. Bech (2002) tested transplantation of ***Thaïs*** genus from non-contaminated to contaminated sites.

In Morocco, very little is known about the presence and threats imposed by organotins in coastal environments. Lemghich and Benajiba (2007) and Mortaji *et al*., (2011) reported imposex in *Hexaplex trunculus*, *Bolinus brandaris* and *Stramonita haemastoma* along the Mediterranean and

Atlantic coast of Morocco. 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 *et al.,* 2003; Fernandez *et al.,* 2005). Having this in mind the present work tests for a cause-and-effect relationship between TBT exposure and the development of imposex in *Stramonita haemastoma*. Two approaches were taken: 1. Imposex induction in healthy females (from the safe site) after inoculation with TBT in the laboratory; and 2. Determining the incidence of imposex in *S. haemastoma* collected from areas with various levels of TBT and determining the concentrations of this chemical compound in its tissues.

Materials and Methods

Sampling: For the bioassay, gastropods, and mussels used as feed, were collected in July 2012, from Dar Bouaaza, Casablanca. This is a pristine area where *Stramonita* populations were healthy and showed no evidences of imposex (Mortaji *et al.,* 2011). During transportation to the laboratory all organisms were kept in aquaria equipped with an air pump.

For the survey in Moroccan Atlantic coast samples of *Stramonita haemastoma* was collected in July, 2012, from rocky substrates in sites at different distances from known sources (Figure 1). From each site a minimum of 20 individuals were collected (Ellis and Pattisina, 1990; Limaverde *et al,.* 2007). Sexual maturity of the gastropods was inferred from shell size that was, in general, close to 40 mm (Ellis and Pattisina, 1990). These animals were kept in plastic boxes containg seawater from the respective sites and they were led to the laboratory.

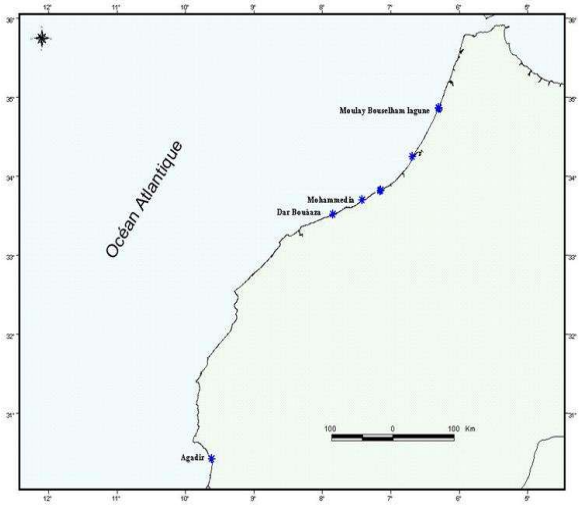

Figure 1: Sampling stations in Atlantic coast of Morocco in this study of organotins, so as to obtain an imposex gradient in the field.

Bioassay: From the total of 240 collected individuals measuring 44 ± 3 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 5 aquaria containing 43 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. Five days were given for adaptation of the organisms to the new conditions prior to starting the inoculations. Two of the 5 aquaria were used as controls (positive and negative). Animals from one of the control batches (positive control) were inoculated with ethanol, the solvent used for diluting TBT standards. The remaining aquaria were used for inoculation of the organisms with increasing concentrations of TBT (aquaria 1, 2, 3). Based on the work of Limaverde *et al.,* (2007) and the applied dose was of the range of 14-59 ng. Solutions of tributyltin chloride 96 % was then prepared by diluting the standards with ethanol. Inoculation was performed by injecting 1µl (containing the following

amounts of organotin as Sn: Aq-1 = 15 ng; Aq-2 = 30 ng and Aq-3 = 60 ng) of the diluted solution in the animal foot with the aid of a 5µl chromatographic syringe. 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; Limaverde *et al.,* 2007).

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.

Biological Monitoring: Animals were sampled at four sites in Moroccan Atlantic coast (Figure 1) previously studied for imposex intensity by El Mortaji *et al.* (2011). Sampling stations were selected as to possibly provide animals with a broad range of imposex intensity (from an expected maximum in the port station (Agadir) and in the near of the Mohammedia industry, to a minimum in the less impacted area of Moulay Bousselham beech and Dar Bouaaza uncontaminated area). Imposex intensity was determined in the gastropods from each station. Thereafter, these animals, were separated for chemical analysis.

Imposex Intensity Evaluation: The molluscs were narcotized with $MgCl_2$ (7 % w/v) (Fernández *et al.,* 2007). Subsequently, length of each snail was measured from the apex to the distal end of the siphonal canal using callipers. The shell of the animals was crushed with a clamp, soft parts were placed in a Petri dish, and examined using a binocular microscope.

Sex of the animals was determined by the presence of the prostate gland in males, and the albumen and capsule gland in females (Gibbs and Bryan, 1987, 1994; Horiguchi *et al.,* 1994; El Mortaji *et al.,* 2011). Penis length in males and in imposexed females was also measured with a millimetric slide (De Castro *et al.,* 2007), and *vas deferens* development was observed under a binocular microscope. The degree of imposex in females was estimated using the following indexes:

$$\% I = \frac{\text{Number of females with imposex}}{\text{Total number of females}} \times 100$$

The imposex quantification was usually made by three indexes: the RPLI (relative penis length index), RPSI (relative penis size index) and VDSI (vas deferens sequence index) (DE Castro *et al.*, 2007; Fernandez *et al.*, 2002). The RPLI is an index that quantifies the degree of imposex in the population and is obtained from the equation: (Mean length of female penis) / (Mean length of male penis) X 100. This index is better applied in low contaminated areas (Fernandez *et al.,* 2002). The RPSI quantifies the degree of imposex in the population by the equation: (Mean length of female penis)^3/(Mean length of male penis)^3 X 100. This cubical index is better applied in highly contaminated areas, when the length of the female penis approaches the length of the male penis (De Castro *et al.,* 2007). The VDSI was determined using a six-stage scale similar to the classic Gibbs scale

for *Nucella lapillus* (Gibbs and Bryan, 1994) adapted for Stramonita haemastoma by Fernandez *et al.,* (2002). In this scale, grade 0 is attributed to normal females, stage 1 = proximal section of vas deferens is formed, stage 2 = initiation of penis development and further development of vas deferens, stage 3= formation of a small penis and development of the distal section of the vas deferens, stage 4= involves mainly fusion of the vas deferens, stage 5= overgrowth of vas deferens on the genital papilla and hence female sterilely, and stage 6= aborted egg capsules can be seen in the capsule gland. The vas deferens sequence index (VDSI) defined as the sum imposex stage in given samples of females (LAHBIB *et al.,* 2011). This index was calculated using the following formula:

$$VDSI = \frac{\Sigma \text{ imposex stage in given samples of females}}{\text{Total number of females}}$$

Sample pre-treatment for chemical analysis: Five males and five females (50–60 mm in SL) were randomly selected from each station were prepared for chemical analysis.; the operculum was removed and the soft parts were finely ground in glass bottles using a T18 basic Ultra-Turrax® disperser at 6,000 rpm. Thereafter, tissues were freeze-dried, weighed, and maintained at −20 °C in the dark until analysis. The chemical analyses were made in a Tunisian laboratory managed by Professor El Menif.

Approximately 200 mg of freeze-dried ground tissue was used to quantify contents of TBT, dibutyltin (DBT), and monobutyltin (MBT). The tissues were digested using a tetramethylammonium hydroxide solution (25%) in deionized water. Samples were simultaneously derivatized and extracted using sodium tetraethylborate and n-hexane. The derivatization reaction was controlled using a tripropyltin standard solution. The isolated hexane was concentrated by evaporation under a gentle stream of pure nitrogen. Samples were then cleaned up using solid-phase extraction florisil cartridges and eluted with isooctane. A known volume of tetrabutyltin standard solution was added to verify the gas chromatography flame photometric detection performance throughout the analyses. The same procedure was applied for preparing external standards of TBT, DBT, and MBT. The quantity and speciation of butyltins in each sample were determined using a Varian 3400 gas chromatograph. The system was equipped with a CP Sil 5 CB capillary column (inner diameter 320 μm, length 25 m, film thickness 0.25 μm) and a modified commercial flame photometric detector (by addition of a quartz burner to increase Environ Monit Assess sensitivity). The sample was injected under splitless injection mode. The column temperature was maintained at 80 °C for 2 min and then increased to 230 °C at 8 °C.min^{-1}. Both injector and detector were maintained at 240 °C. Hydrogen was used as the carrier gas at a flow rate of 12 ml.min^{-1}. The flame photometer was equipped with a 610 nm filter selective for tin-containing compounds and operated using a hydrogen–air–nitrogen flame.

The detection limits were 0.8 ng Sn g^{-1} dry weight (dw) for MBT, 0.7 ng Sn g^{-1} dw for BT, and 1.0 ng Sn g^{-1} dw for TBT. Analysis of a certified reference material (mussel tissue BCR 477, six replicates) using this procedure resulted in the following concentrations (as mg Sn g^{-1} dw) 1.03 ± 0.04 for MBT, 0.75 ± 0.03 for DBT, and 0.86 ± 0.04 for TBT. The

certified values were 1.01 ± 0.19, 0.79 ± 0.06, and 0.90 ± 0.08, for MBT, DBT, and TBT, respectively. This procedure does not allow for the quantification of phenyltins that are not eluted at the florisil purification stage.

Results and Discussion

The percent of imposex, sex-ratio, and the length of the penis and vas deferens of females and males of *S.heamastoma* during this bioassay is presented in tables (1 and 2).

	A						B					
	SR	VDSFσ	VDSMσ	%I	FPL	IS	SR	VDSFσ	VDSMσ	%I	FPL	IS
Cn	0,45	0	15.0±1.4	0	0	0	0,9	0	23,2±1,9	0	0	0
Cp	1,5	0	17.3±1.5	0	0	0	0,73	0	24±5,6	0	0	0
Aq-1	1,14	2±5,8	20,8±5,6	85,7	0	I	1,3	8,54±2,8	22,4±2,6	100	0,68±0,5	II andIII
Aq-2	0,5	2±1,5	22,3±6,2	70	0	I	0,6	13,9±4,2	22,9±1,8	100	2,84±6,7	II-VI
Aq-3	0,66	7,7±5	23,1±4,4	100	3,3±4,5	I-V	0,38	14,8±4,8	23,9±2	100	4,98±1,2	II-VI

Table 1: Biological results of the bioassay. A = results for sampling 14 days after inoculation; B= results for sampling 28 days after inoculation; FPL = The length of the penis of females; SR- Sex-ratio; VDSF- The length of the vas deferens of females; VDSM - The length of the vas deferens of males; %I – percent of imposex; IS- Imposex stages; Cn- negative control ; Cp- positive control ; Aq-1- 15 ng; Aq-2- 30 ng; Aq-3- 60 ng; σ- Standard deviation

Aquaria	Number of Imposexed females	headcount of stage						VDSI
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	
Initial Positif control	0	~	~	~	~	~	~	~
Negatif control 1	0	~	~	~	~	~	~	~
Positif control 1	0	~	~	~	~	~	~	~
15ng	6	6	0	0	0	0	0	0,85
30ng	7	7	0	0	0	0	0	0,7
60ng	9	4	2	1	1	1	0	2,22
Negatif control 2	0	~	~	~	~	~	~	~
Positif control 2	0	0	~	~	~	~	~	~
15ng	10	0	7	3	0	0	0	2,3
30ng	15	0	4	8	4	0	1	3,33
60ng	18	0	2	7	3	3	3	3,88

Table 2: Vas deferens stages (VDS) in female *S. haemastoma* according to the duration of exposure to TBT and VDSI index

About 80% of the *S. haemastoma* females showed initial imposex stages (IS: I) within the first 14 days after inoculation with 15 ng and 30 ng for TBT, indicating a rapid induction. But at 60ng, 100% of the females had developed imposex (fig2), reflecting the different stages of imposex (IS: I-V). After 28 days, 100 % of the females had developed imposex for Aq1 (15ng) and Aq2 (30 ng) (Fig. 2)

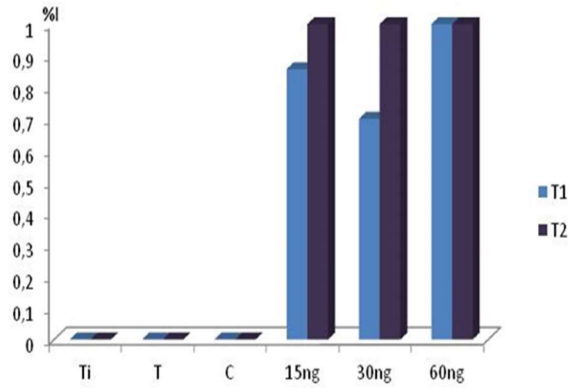

Fig. 2: Imposex percent (I%) in female S. haemastoma according to the duration of exposure and the TBT concentration inoculated. A frequency maintained for Aq3 (60 ng) with the females showing imposex stages from II to VI.

A significant increase in the length of the female penis was also noted in the period exposure. This was also observed by others (Bech, 2002; Horiguchi *et al.,* 1994; Lemghich *et al.,* 2007; Limaverde *et al.,* 2007; QUEIROZ *et al.,* 2007). Despite not having been observed statistical differences for VDSI (P = 0.735), were observed significant differences for the imposex % (P< 0.001); the FPL (P<0.05) and RPLI (P<0.05) induced in the exposure.

In this study the appearance of the penis for imposex females it is recorded only at the level of the concentration 60ng after 14 days of inoculation with a RPLI > 10. After 28 days of inoculation, we recorded the significant increase in the RPLI increasing inoculated TBT concentration, they are respectively 2, 9.7 and 17.2 (fig 3).

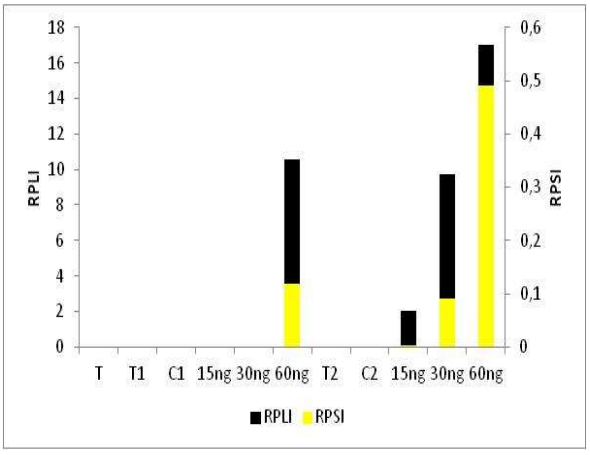

Fig. 3: Variations in the levels of RPSI and RPLI observed in individuals of S. haemastoma during the two periods of exposure.

The first sign of imposex in *S. heamastoma* was the development of a section of the *vas deferens* half-way between the right ocular tentacle and the vagina. This observation is similar to those reported by Lima *et al.* (2006), Fernandez *et al.* (2005) and Rossato *et al.* (2014) in *S. heamastoma* and by Lahbib *et al.* (2008) and Abidli *et al.* (2009) in *Hexaplex trunculus.*

According to Fernandez and al. (2002), the average values of VDSI were taken into consideration, corresponding approximately to the total VDSI value of the females of each aquarium. We found an increase in the average VDSI after 28 day of inoculation (VDSI> 2,3, with the females showing VDS stages between 0 and 6), relative that one observed after 14day of inoculation (VDSI < 2,3, with dominance of initial stage of imposex (IS:1)), despite the fact that there was no statistical differences of the VDSI (P > 0,1) between two periods of inoculation (fig 4). The average penis length found in females 28 days after inoculation of 59 ng of TBT was 1.5 ± 0.9 mm (Limaverde *et al.,* 2007). Horiguchi *et al.* (1994), Bech (2002) and Limaverde *et al.,* (2007) found for gastropods of the same genus as *Stramonita* that TBT concentrations of 10-20 ng.g^{-1} of fresh tissue induced development of imposex. In the present study,

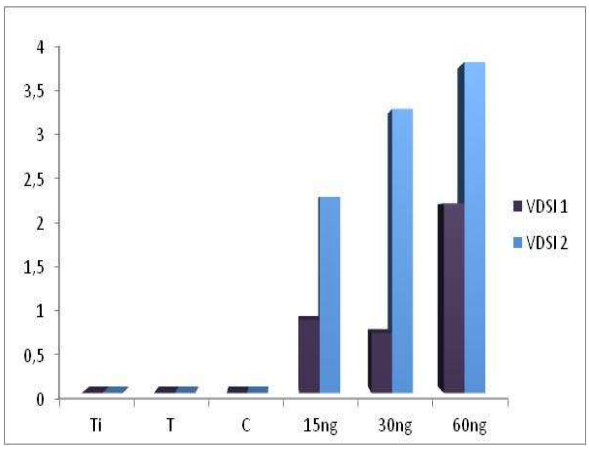

Figure 4: VDSI index in S. haemastoma between the two periods of exposure after inoculation

we have shown that after 14 days of inoculation with 60 ng of TBT, we have the penis development in *S. Haemastoma* with a gradual increase between 15 ng and 60 ng after 28 days of inoculation.

To compare the values RPLI, RPSI, VDSI and % of imposex, we have a gradual increase of the indices in increasing concentrations inoculated of TBT and the length of exposure (Fig. 2,3,4).

In addition, a good correlation was found between the imposex incidence and exposure duration reported by Lima *et al.* (2006) and Limaverde *et al.* (2007), the results of the present study showed that the imposex development in S. haemastoma is strongly correlated with exposure time in this bioassay ($R = 0.959$, $p < 0.001$). These measurements are regarded as the two main indexes for the evaluation of the imposex levels in *S. haemastoma,* and have been reported strong positive correlations between RPLI and/or VDSI with environmental TBT concentrations in many contaminated areas (Lima *et al.,* 2006, Fernandez *et al.,* 2005, El Mortaji *et al.,* 2011).

The evolution of imposex according to the TBT concentration (15, 30 and 60 ng), allowed us to confirm the imposex effect induced by this pollutant, with the imposex frequency evolving from 70 % to 100 % after only 14 days of inoculation, this frequency is maintained after 28 days of inoculaion for the three Aq (Aq1, Aq2, Aq3). On the other hand, Limaverde *et al.* (2007) have carried out a similar study and reported that the *S. heamastoma* females exposed via inoculation (% I vary between 25 % and 86 % after 28 days of inoculation) had lower levels of imposex compared to those presented by this study. While the percent of imposex obtained through the dietborne exposure were similar to those reported by this study – 100 % after 30 days of exposure (Lima *et al.,* 2006; Rossato *et al.,* 2014). A significant increase in the length of the female penis was also noted in the waterborne exposure (Rossato *et al.,* 2014), with 100 % of imposex after 3 months of exposure at 50ng TBT/L and 6 months of exposure at 5 ng/L. Apparently, *S. heamastoma* is among the best bio-indicator species to monitor environmental pollution by TBT. Indeed, the imposex incidence in *Nucella lapillus* ranged between 20 and 50 % after three months of exposure to 50 ng TBT (Santos *et al.,* 2005).

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 (Kow) partition coefficient, contributes to TBT fast bioaccumulation in marine organisms (Maguire, 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,b), 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, we realized an evaluation of the effect of TBT on ground in parallel to the bioassay in controlled conditions.

The field study has shown that the phenomenon of imposex was widespread in the studied populations and all the specimen females from the 4 sites showed signs of imposex. Only in the ‘clean’ area on Dar Bouazza was the imposex undetectable.

The evaluation of the RPLI, RPSI and VDSI from along the Atlantic fluctuated from a low value (RPLI = 5.07; RPSI=0.01) at Agadir to a high value (RPLI = 66.66; RPSI = 29.62) in Mohammadia. On the other hand, the VDSI extended from 0.47 at Moulay Bousselham up to ≈ 2 in Agadir (table3).

site	sex-ratio	FPL/ σ	% I	RPLI	RPSI	VDSI	IS
My boussslhame	2,4	13,5±1,38	9,52	56,25	17,81	0,47	I,II,IV
Dar bouaaza	1,26	0	0	0	0	0	0
Mohemmedia	2,6	16,83±1,58	22,22	66,66	29,62	1,03	I,II,III,IV
Agadir	2,33	1,07±1,56	33,33	5,07	0,01	1,52	I,II,III,IV

Table 3: Biometric index comparison of imposex in the Atlantic coast during this study SR: sex ratio; σ: Standard deviation; IS: Imposex stages.

Table 3 and 4 have reported the results for imposex development as well as organotin concentrations in *S. haemastoma* along the Atlantic coast of morocco. TBT, DBT and MBT concentrations of ≈18 ng.g^{-1}, ≈17 ng.g^{-1} and ≈13 ng.g^{-1}, respectively, were found in animals from Mohammedia and Agadir, heavily polluted by organotins. At dar bouaaza, a natural reserve and reference area, concentrations were 0.4 ng.g^{-1} TBT, 6.11 ng.g^{-1} DBT and 5,37 ng.g-1 MBT, while 0% of females displayed imposex and VDSI was of 0. At Moulay bousselhame beach, some 4-5 km away from the main organotin sources in Moulay bousselhame lagune , TBT,

Les sites	TBT	DBT	MBT	Buts	BDI
Mohammedia	17.17	16,87	9,67	43,71	1,54
Dar Bouâaza	0.4	6,11	5,37	11,88	28,7
Moulay Bousselham	0	1,8	5,78	7,58	-
Agadir	15.01	11	13,19	39,2	1,61

Table 4: Results of organotin determination (ng g^{-1} ww as Sn) in *S. haemastoma* from different studied stations

DBT and MBT average concentrations were 0 ng.g^{-1}, 1.8 ng.g^{-1}, and 5.78 ng.g^{-1}, respectively. Mean VDSI of 0.47 and %I of 9.52 for *S.haemastoma* in Moulay bousselhame beach indicated a more severe response at comparable body burdens.

The field study showed that organotin concentrations in gastropods (see Table Y) from Agadir, Mohammedia, Moulay bousselhame beach and Dar Bouaaza are of the same order of those reported for gastropod of the same genus from other contaminated areas (Horiguchi *et al.*, 1995; Lima *and al.*, 2006; Limaverde *and al.*, 2007). Strand *et al.* (2009) reported TBT, DBT, and MBT levels in gastropods from the Caribbean Virgin Islands in the following ranges, respectively: 3.8 – 13; 6.1 – 29; <1 – 20 ng (Sn) g^{-1} dw (dry weight) in Purpura patula; 3.5 – 14; 3.2 – 17; 2.8 – 13 ng (Sn) g^{-1} dw in *Thais rustica*; 8.1 – 101; 9.6 – 78; 12 – 62 ng (Sn) g^{-1} dw in *Thais deltoidea*; 17 – 119; 36 – 226; 12 – 111 ng (Sn) g^{-1} dw in *Cittarium pica*. In general, the estimated levels of BTs in *Odontocymbiola magellanica* (< MDL (detection limit) – 70.2 for TBT; < MDL – 34.3 for DBT; and < MDL – 184.5 ng (Sn) g^{-1} dw for MBT) were in the same order of magnitude of those found for species from the Caribbean Virgin Islands. Considering that the foot of *O. magellanica* is consumed in the region, the risk related to its consumption is relatively low. Even

considering the most contaminated site of Golfo Nuevo (LPH (Luis Piedrabuena Harbour)-16.29 ng g-1 of TBT considering the conversion factor of 2.74 from Sn), an average person of 70 kg would need to consume about 3.2 kg of fresh foot (meet) per day (or an equivalent of 57 feet) to exceed the European Food Safety Authority (EFSA) recommended Tolerable Daily Intake (TDI) of 250 ng/kg body weight of organotins compounds in food (EFSA. 2004). However, this analysis did not take into consideration any other source of contamination, very common in every harbour with high marine traffic, which could influence the total intake. In any case, this should be monitored over time. The Index RPLI is strongly correlated with RPSI (R = 0.938) and VDSI fairly well correlated with the percentage of imposex (R = 0.999). These indexes are highly correlated with the chemical results (R = 0.774) obtained by tissue analysis of the studied samples. The highest degree of imposex observed was clearly in the harbour areas: Agadir. The negative effects were also observed in the areas with the industrial activities the high annual activity of artisanal fishing boats: Mohammedia and Moulay Bousselham. A Dar Bouazza coast is not impacted and the imposex phenomenon was not detected in the specimens collected from this site.

Conclusions

The bioassay demonstrated that TBT is 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 results from the field studies allied to the bioassay confirmed the reliability of the VDSI scale proposed by FERNANDEZ *et al.* (2002) for S. haemastoma as demonstrated by the excellent correlation found between VDSI level and organotins concentrations in tissues. As for concentrations of organotins in snails measured in the field study, those found for TBT in Agadir and in Mohammedia are equal the first 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. Imposex appears to be widespread phenomenon affecting the mollusc's species along the Atlantic and Mediterranean coast of Morocco. The incidence and levels of imposex differs between the studied sites. Serious symptoms of this phenomenon were recorded near harbour of Agadir and in Mohammedia industry on the Atlantic coast of Morocco. Sexual alterations leading to imposex in females of S. haemastoma were observed at these sites. This reproductive anomaly is mainly attributable to TBT in seawater (Fernandez *et al.*, 2002; Trigui El-Menif *et al.*, 2007). Although laboratory and field studies have established a quantitative relation between imposex level and marine environment contamination by antifouling paint. In this respect, controls by the Moroccan legislation may be necessary (Jelic-Mycelic *et al.*, 2006).

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