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Assesment of organotin contamination at the Gadani Shipbreaking Yard, Pakistan

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Abstract

Imposex is a well-documented sexual abnormality in female gastropods caused by tributyltins and triphenyltins leached into the marine environment from antifouling paints applied on submerged marine structures such as fishing nets and bouys. In the present study two bioindicator species of gastropods, *Thais bufo* and *Thais rudolphi*,were examined from the Gadani shipbreaking yard, Pakistan. The indices of imposex intensity, i.e., relative penis size index (RPSI) was higher in *T. bufo* (50.60%) as compared to *T. rudolphi* (17.01%). The vas deferens sequence index (VDSI) and incidence of imposex were 4.0% and 100% respectivly in both species. Chemical analysis revealed higher bioaccumulation of total butyltins than phenyltins. The body burden of diphenyltin was greater than monophenyltins and triphenyltins. Similarly higher bioaccumulation of dibutyltin than tributyltin and monobutyltin was observed in both species with the exception of high content of tributyltin and monophenyltin in *T. bufo* male and imposex respectively. This study therefore demonstrates that the potential influence of organotin contamination due shipbreaking activities and caused high bioaccumulation of TBT and 100% incidence of imposex in both targeted species.

Key words : Shipbreaking Yard, Imposex, Bioaccumulation, Butyltins, Phenyltins, Tributyltin, TBT

INTRODUCTION

Superimposition of male sexual characteristics on female gonochoristics gastropods is defined as "imposex"[1]. The development of this sexual abnormality is mainly caused by TBT (tributyltin) used in antifouling paints. These paints are commercially beneficial to increase fuel efficiency and the speed of vessels by preventing fouling of different organisms such as barnacles, seaweeds and gastropods on the hulls of ships[2]. However, TBT has many deleterious effects on marine organisms such as deformation of oyster shells[3], endocrine disruption and mortality in *Mytilus edulis*[4]. In addition, imposex condition in over 268 species belonging to 11 different families of gastropods has been reported from different parts of the world[5]. The utilization of TBT as a biocidal agent, slimicide, stabilizing agent and catalyst has caused widespread contamination in marine environments[6]. TBT has also been reported to bioaccumulate in the tissues of marine animals and over time reaches to humans through the food chain [7].The use of organotin compounds were widely banned in the late 1980s in many countries and has been monitored by a number of agencies since then. Restrictions on the use of TBT were further strengthened by Marine Environmental Protection Committee (MEPC) of International Maritime Organization (IMO) in 2003 and 2008 [8]. Moreover, restrictions against application of TBT based paint has been implemented in almost 26 countries. However, there is no check on organotin released by recycling of old ships at ship breaking yards[9] which dispose off large amount of hazardous materials, including organotins, into the marine environment without any pretreatment[10]. Neogastropods have been widely used as sensitive biomonitoring markers for the assessment of organotin contaminations [11]. In Pakistan this genital abnormality has been documented in *T. carinifera, T. bufo*, *T. rudolphi, Babylonia spirata* and in *Morula. granulata* by morphological examination[12,13]. Thus, the present study aimed to assess the intensity of imposex as a biomonitoring tool and to examine the concentrations of butyltins (BTs) and phenyltins (PhTs) in the vicinity of Gadani shipyard using two species of neogastropods *T. bufo* and *T. rudolphi*.

MATERIALS AND METHODS

Sample collection

T. bufo (n=32) and *T. rudolphi* (n=25) were collected randomly by hand at low tides in the month of August 2011 near the Gadani ship breaking yard. The Gadani ship-breaking yard is situated at 25° 7'N and 66°43'E about 50 kilometer in the northwest of Karachi in the southern part of Balochistan along the Arabian Sea (Figure 1). The beach is sandy and separated by prominent rocky outcrop from the main coastline. The sampling station is near from main ship breaking area and gastropods are usually found in holes and crevices of rocks. Specimens were brought live to the laboratory and kept in glass aquaria filled with seawater.The shell length (SL) was measured nearest to 0.1 mm from apex to the tip of siphonal canal by using vernier caliper and the measurement of penis length was taken from its base to the tip using plastic coated mm graph paper. The samples were narcotized for 30 min in 7% $MgCl_2$ solution; each specimen was then cracked, opened and the soft tissue was excised with stainless steel scalpel blades. All the specimens were morphologically examined and some were lyophilized and stored at 20°C for organotin analysis.

Identification of male, female and imposex

The females were recognized by the presence of a sperm ingesting gland and albumen gland and males were identified by the penis behind the right tentacle, prostate gland and vas deferens. The females with male sexual characters (penis and vas deferens) were designated as imposex[14].

Figure 1. Map showing sampling site.

Imposex indices

To measure the intensity of imposex, relative penis size index [RPSI=(mean female penis length)^3/(mean male penis length)^3×100][15,16]. and vas deferens sequence index [VDSI = average of VDS stages 0-6] [17].

To evaluate the tribytyltin (TBT) metabolizing capacity of targeted species, butyltin degradation index (BDI) was measured by using following formula[18][DBI = [DBT+MBT]/TBT] . The value of DBI<1.0 indicates fresh input of TBT, while DBI>1.0 represents efficient and gradual biodegradation of TBT into dibutyltin (DBT) and monobutyltin (MBT).

Chemical analysis

Extraction

Analysis of organotin compounds was carried out from the whole body tissues of both *T. bufo* and *T. rudolphi* by using the methodology as previously reported [19,20]with some modifications [21]. Initially the tissues were freeze dried, ground and placed in a glass centrifuge. Tripropyltin (300 ng) was added as an internal standard in each sample that was then extracted twice by sonication for 10 min with 15ml of toluene/glacial acetic acid (HOAc) (10:4). Extracts were collected in a separating funnel and then 10 ml of 0.5% ammonium pyrrolidine dithiocarbamate (APDC) and 60 ml of 20% NaCl (w/v) solution were added. This extraction process was carried out twice and each time an extract of the top/organic layer was collected in a conical flask, percolated through activated anhydrous Na_2SO_4 recovered and finally evaporated to a small volume (1-2ml) at 30°C by rotary evaporator.

Derivatization and clean up process

A grignarg reagent (i.e., n-pentylmagnesium bromide) at 2.0 ml was added to extracted samples and this reaction was allowed to occur by shaking for one minute and then by keeping the solution at 40°C in a water bath for 40 min. After the derivitization flask was placed in an ice bath and any excess amount of grignard reagent was neutralized by adding few drops of Milli-Q water and 10 ml of HCl (25%). Derivatized extract was then recovered and the aqueous phase was liquid-to-liquid extracted twice with 10 ml of 10% benzene/hexane. Derivatized extract was eluted with 30 ml of 10% benzene/hexane through a glass chromatographic column packed with 5.0 gm of activated florisil and 2.0 gm of activated anhydrous Na_2SO_4 and evaporated to 0.1 ml under a gentle stream of nitrogen.

Instrumentation

Samples were analyzed using an Agilent 7890 gas chromatograph coupled with a Flame Photometric Detector (GC-FPD) with a 610 nm cut-off interference filter for tin compounds. This system was equipped with a fused silica capillary column (HP-5 MS 30.0 m in length × 250 µm i.d × 0.25 µm film thickness) (J and W Scientific, Folsom, CA, USA) [21] for separation. Injection (2.0 µl) was performed in the split less mode and injector port and detector were both set at 250 °C. The column temperature was set for 1 min at 80 °C initially and reaches up to 5 °C/min to 190 °C, then increased to 280 °C at 10 °C /min, holding this temperature for 5min. The procedure was validated by using the certified reference material BCR 477 (mussel tissue). The mean recovery through the entire analytical procedure for MBT, DBT, TBT, MPhT, DPhT and TPhT were 101.1%, 109.5% and 87.3%, 114.5%, 106.5% and 92.2% respectively. The detection limits of the method for MBT, DBT, TBT, MPhT, DPhT and TPhT were 0.07, 0.05, 0.02,0.12,0.26 and 0.03 ng^{-g} dry wt.

RESULTS

Morphological examination of *T. bufo* and *T. rudolphi* revealed 100% incidence of imposex with stage 4 (penis with penis duct and vas deferens that is continuous from penis upto the valva) in all females. The stages ≥5 were not recorded in any female. Highly significant differences were found between the length of penis of both male and imposex females in *T. bufo* ($F=16.44$, $p<0.05$) and *T. rudolphi* ($F=13.61$, $p<0.05$). The RPSI was found to be higher in *T. bufo* (50.60%)as compared to *T. rudolphi* (17.01%) whereas, development of vas deferens only up to stage 4 in all specimens have represented that VDSI was 4.0 in both species The mean values of biometric measurements are summarized in Table 1. No significant difference between shell length (SL) of imposex females and males in *T. bufo* ($F=1.55$, $p>0.05$) was observed, while in *T. rudolphi* SL of imposex females was considerably higher than males ($F=13.61$, $p< 0.05$).A Chi-square test indicated that the sex-ratio between males and imposex females was not significantly different from the 1:1 theoretical ratio in both species (*T. bufo*: $X^2=0.04$, $p>0.05$; *T. rudolphi:*$X^2=0.13$,$p>0.05$) (Table 1) One-way analysis of variance (ANOVA) indicated that *T. bufo* and *T. rudolphi* had no significant difference for DBT ($F= 0.15$, $p>0.05$) and MBT ($F=0.14$, $p>0.05$) body burden while bioaccumulation of TBT ($F=5.70$, $p=0.05$) was different in the tested species but not highly significant. Similarly body burden of TPhT ($F=1.45$, $p >0.05$) and MPhT ($F=0.09$, $p>0.05$) showed no difference whereas, DPhT was significantly different ($F= 19.28$, $p <0.05$) in both species. One-way ANOVA was also used to compare the body burden of total butyltins ∑BT (TBT+DBT+MBT) and total phenyltins ∑PhT (TPhT+DPhT+MPhT) in both species, which showed significantly higher bioaccumulation of total butyltins than total phenyltins in both *T. bufo* ($F=37.80$ $p<0.05$) and *T. rudolphi* ($F=22.42$; $p<0.05$).In imposexed *T. bufo* TBT reflected lower

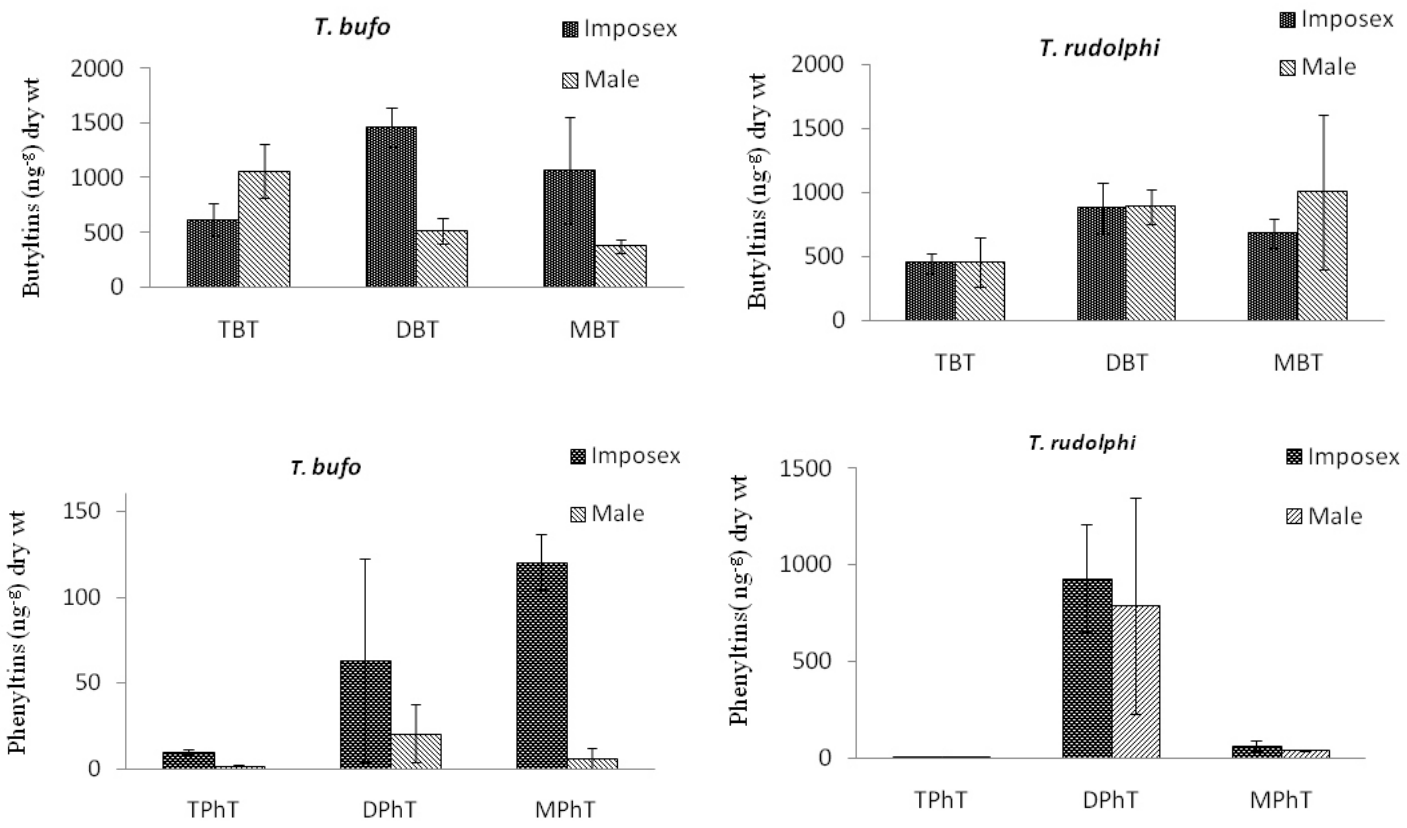

Figure 3. Percentage composition of butyltins and phenyltins in imposex and male *T. bufo* (A) and *T. rudolphi* (B).

Table 1: Morphometric measurements and butyltin degradation index (BDI) of *T. bufo* and *T. rudolphi*. N- sample size, SL- shell length, PL- penis length, VDSI- vas deferens index

Species	Sex	N	SL (Mean±SD) mm	PL (Mean±SD) mm	RPSI	VDSI	BDI
T. bufo	Imposex	15	37.28 ± 3.12	10.26 ± 2.6	50.60	4	4.00
	Male	17	37.25 ± 2.48	12.87 ± 2.36			0.80
T. rudolphi	Imposex	13	45.25 ± 5.95	13.16± 4.28	17.01	4	3.46
	Male	12	41.33 ± 4.63	23.75 ± 7.08			4.14

proportion (19.71%) of total butyltin than DBT (46.45%) and MBT (33.83%), while in males it constituted major content accounting (54.67%) of total butyltin as compared to DBT (26.58%) and MBT (19.33%). In male *T. rudolphi* the TBT constituted (19.44%), DBT (37.89%) and MBT (42.75%) of butyltins and imposex showed 33.89% MBT, 43.72% DBT and 22.37% TBT (Figure 3).In *T. bufo* bioaccumulation of DPhT (71.41%) in male and MPhT (62.2%) in imposex represented the highest fraction of total bioaccumulated phenyltins, whereas TPhT in male (6.95%) and imposex (5.13%) constituted its lower concentration. Phenyltin distribution pattern was also observed in *T. rudolphi* with high DPhT content in male (94.05%) and imposex (83.98%) as compared to MPhT and TPhT in both sexes as shown in Figure 3.

Butyltin degradation index (BDI) was also calculated to define TBT metabolizing capability in both species. The BDI of *T. bufo* in males was 0.8 and in imposex it was (4.0).Whereas, BDI in *T. rudolphi* was 3.4 and 4.1 in imposex and males, respectively (Table 1).

DISCUSSION

Previously, morphologically examined species of neogastropods have indicated presence of imposex condition from the site which experiencing boating and shipping activity. However, the sites which exposed to open sea and experiencing no shipping activity here no such incidence of imposex has been observed [22,23]. Similarly, in the present study the incidence of imposex in *T. rudolphi* and *T. bufo* was 100% at Gadani ship-breaking yard where large number of ships are recycled and broken down. Therefore, these observations suggest that the TBT released from antifouling paints during ship-breaking and ship trafficking could possibly be the causative agent for sexual abnormality as reported by ealier workers [8, 24].

In the present study the incidence of imposex was 100% and all the imposex showed well developed penis and VDSI was 4.Likewise,100% imposex incidence and stage 4VDSI has also been reported in females of *T. clavigera*, *M. granulata,* and *T. tuberosa* with high TBT body burden from coast of Taiwan[25,26].Therefore, VDSI and percentage of imposex incidence could not be the precise measure of species specific imposex intensity, while difference in RPSI in both *T. rudolphi* (17.01%) and *T. bufo* (50.60%) demonstrate species selective response for organotin contamination[27].

No alteration in sex ratios was observed in both tested species, which may be due to the vas deferens development that reaches only stage 4, which is considered as the last fertile stage[28]. However, in *N. lapillus*[29],*T. biserialis*[30]and *T. clavigera*[31,32,33] populations, females gastropods lose their reproductive ability due to development of sterile stages (blockage of vulva and aborted egg capsules) leading to alteration in sex ratios as a result of continuous exposure to organotin compounds[34].

TBT mainly act as imposex inducing agent as reported in *N. lapillus*[35, 29]. Beside this,TPhT is also classified as an endocrine disruptor that induced imposex in *T. clavigera*[36,17]and its concentration is generally lower or sometimes negligible in biota, sediments and water with most probable assumption of low proportion of TPhT utilization as co-biocidal agent in antifouling paints and major usage as fungicide[37]. Similarly, in the present study significantly lower concentration of ∑TPhT as compared to ∑BT in *T. bufo* and *T. rudolphi* also indicate less utilization of TPhT in antifouling paints. Furthermore, at the sampling site, there is no influx of pollution from agriculture, domestic sewage, industrial waste and boating activities such as from marinas and harbours which are non-existent in the area. The main source of pollution is the ship breaking industry at the study site. Low TPhT body burden has also been observed indifferent gastropods [38, 39] such as *Hexaplex trunculus* from lagoon of Venice[40]and *T. gradata* from Southern Coast of Peninsular Malaysia[41].

In the present study dibutyltin (DBT) and diphenlytin (DPhT) were the major compounds among butyltins and phenyltins, respectively. This could be explained by three different factors, first, the accumulation of organic matter favors the accumulation of TBT in sediment and leads to resuspension which enhances the mobility of DBT and increases the accumulation from water in organisms including gastropods ; second, gastropods have capability to efficiently metabolize TBT into its derivative products, MBT and DBT as reported in *N. nitidus*[42]; third, DBT is widely used as PVC stabilizer, catalysts for polyurethane foams and silicon resins [43], therefore this could originate in the aquatic environment also through degradation/ breaking of ship accessories such as plastics pipes, furniture and foams. Furthermore, butyltin degradation index (BDI) >1 was observed in both males and imposex of *T. rudolphi,* whereas, in *T. bufo* was >1 in imposex and <1 in males. BDI >1 indicate that the TBT has been dealkylated efficiently into DBT and MBT under the metabolizing process in organisms [42]. But in male *T. bufo* BDI <1 and exceptionaly indicate fresh input of TBT [18]or high content of TBT as compared to DBT and MBT which may be due to involvement of food content hydrological [44], ecological [45] and physiological (TBT metabolization process) factors [42]. Moreover, imposex *T. bufo* showed higher value of RPSI and greater BDI value than *T. rudolphi*, indicating faster degradation which may be responsible for stronger imposex reponse in *T. bufo* than *T. rudolphi.* Although no information is available in literature on the relationship of butyltin degradation index and species-specific imposex response such as VDSI or RPSI. However, correlation in body burden of TBT and RPSI have been observed in both species as reported by from Hong Kong[46].

In the targeted species *T. bufo* and *T. rudolphi* >400 ng $g^{-1}$of TBT body burden as well as >200ng g^{-1} and in *T. clavigera* and 40-530 ng Sn/g in transplanted mussels from ship yard[24,32] indicate that the ship breaking yards are also contributor of organotin contamination mainly through paint leachate during recycling process of old ships coated with antifouling paints[47,43].Moreover, present results have indicated that both selected species of gastropods can be used as useful bioindicator.

CONCLUSION

This work has clearly indicated that shipbreaking yard is contaminated with organotin compounds despite of global ban on the application of antifouling paints since 2008. This contamination is mainly due to recycling process of old ships already coated with TBT based paints. Therefore, to prevent organotin comination not only the replacement of organotin based paint but also management of waste such as pre treatment of solid waste during ship breaking is needed.

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