Assessment of reproductive disorder (imposex) induced by tributyltins in marine gastropods

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Abstract: Imposex is a genital disorder characterized by imposition of male sexual characteristics in female gastropods due to exposure to tributyltin (TBT). TBT is used as biocidal agent in antifouling paints, applied on the ship hulls and marine submerged structures such as fishing gears and buoys. In the present study bioassay experiment was carried out to determine imposex inductive and endocrine disruptive effect of TBT in two species of gastropods of genus *Thais*. In this experiment normal specimens of *T. bufo* and *T. rudolphi* were exposed to three different concentrations (100, 500 and 1000ngl^{-1}) of TBTCl for four weeks in laboratory and at the end of experiment level of free testosterone and TBT body burden was estimated by radioimmunoassay and gas chromatograph coupled with a flame photometric detector respectively. In both tested species exposed to 500 and 1000ngl^{-1} of TBT imposex stages developed, while in 100ng l^{-1} and control groups showed no imposex condition. Elevation of free testosterone level in imposex females has also been observed. These observations indicate that the TBT act as potential imposex inducer and endocrine disruptor in the targeted gastropod species and these species can be used as sensitive biomonitoring tool for TBT contamination.

Keywords: Imposex, tributyltin, testosterone, endocrine disruptor, bioindicator.

INTRODUCTION

Tributyltin (TBT) is a ubiquitous pollutant in aquatic environment (Gooding *et al*., 2003) and its toxicity at bio-molecular, cellular, individual, population and community level has been identified after its introduction in marine environment largely as a result of boating and shipping activities (Kim *et al*., 1999; Cooke 2002; Shimasaki *et al*., 2002; Shim *et al*., 2003). TBT is also act as endocrine disruptor which may lead to masculinization phenomenon (imposex) in female gastropods (Shim *et al*., 2000; Titley-O'Neal *et al*., 2011). This imposex condition (pseudohermaphrodism) is a morphological change used as biomarker for the detection of organotin contamination particularly for TBT (Bryan *et al*., 1986; Morcillo and Porte 1998; Wilson *et al*., 1993). Imposex condition develops in female gastropods at concentration as low as >1 ngl^{-1} of TBT (Gibbs and Bryan, 1996). Therefore, gastropods are considered as extremely sensitive to the TBT (LeBlance and Brain, 1997) and this genital syndrome has now been successfully used to determine the presence and degree of environmental TBT pollution (Zeidan and Boehs, 2017). Moreover, one of the best examples of a particular dose-dependent biological effect caused by a pollutant is development Imposex condition in gastropods (Laranjeiro *et al*., 2018). Field investigations (Matthiessen and Gibbs, 1998; Sternberg *et al*., 2008) and bioassay experiments regarding the action mechanism of TBT have established a link between development of imposex and elevated level of free testosterone in gastropods (Bettin, 1996; Gooding *et al*., 2003). TBT bioassay experiments are limited to few species such as *T. clavigera* (Horiguchi *et al*., 1997), *Nucella lapillus* (Santos *et al*., 2005), *IIyanassa obsolete* (Gooding *et al*., 2003), *Buccinum undatum* (Mensink *et al*., 2002) as compared to field investigations which have been widely used to explain the toxicity of TBT pollution in over 200 species of gastropods (Shim *et al*., 2000). Moreover, bioassay experiments provide more precise understanding of the specific pollutants as compared to field investigations (Lima *et al*., 2011). Therefore, this study was designed to investigate the role of tributyltin (TBT) as an endocrine disruptor and imposex inducer in targeted species namely, *T. bufo* and *T. rudolphi* found along the coast of Pakistan.

MATERIALS AND METHODS

Sample collection

Two species of gastropods *T. bufo* and *T. rudolphi* were handpicked during low tide from the intertidal zone of Manora Rocky Ledge in January 2011. This site has no previous record of imposex incidence in any species of gastropod examined by Asfar (2009). The specimens of targeted species were brought live to laboratory and washed thoroughly with tap water to remove epifauna from outer surface of the shells. The specimens were then kept in well aerated glass aquaria filled with seawater. The water was changed daily and its temperature was maintained at 20±1^{o}C. The specimens were first of all

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narcotized with 7% $MgCl_2$ for 30 mins to relax their bodies and then sexes were quickly identified by presence and absence of penis in male and females as reported by Hourguchi *et al*., (1994) and Blackmore (2000). Specimens of targeted species were acclimatized for one week before start of the experiment. They were fed oyster meat during acclimatization and experiment.

Bioassay experiment

At the beginning of experiment *T. bufo* (n= 68) and *T. rudolphi* (n= 85) were distributed in four different treatment groups. The number of males and females in each group and their mean shell length (SL), shell width (SW) and aperture size (AS) are given in Table 1. The animals in groups 1 to 3 were injected with 5µl of 100, 500, and 1000ng l^{-1} of tribulytinchloride (TBTCl) (Merck, 97%) respectively and the group 4 (control) was injected with the same amount of 0.01 mM dimethyl sulfoxide (DMSO) solution. These doses were repeated on alternate days for four weeks. At the end of the experiment the snails were cracked, open and the morphological examination of the soft tissue was carried out for the assessment of imposex as describe as describe by Fioroni *et al*. (1991); Gibbs and Bryan (1994); Oelhlmann *et al*., (1996) and then half of the specimens were freeze dried for analysis of TBT body burden and the remaining samples were stored at -20^{o}C for testosterone analysis.

Butyltin analysis

Extraction: Analysis of TBT was carried out from the whole body tissues (n=4) of both *T. bufo* and *T. rudolphi* by using the methodology described in previous reports (Abalos *et al*., 1997; Diez *et al*., 2002; Wang *et al*., 2010). Initially the tissues were freeze dried, grounded 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 mins with 15 mL 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 (APDC) extraction process was carried out twice and each time extract (top/organic layer) was collected in a conical flask, percolated through activated anhydrous Na_2SO_4 recovered and finally evaporated to very small volume (1-2 mL) at 30^{o} C by rotary evaporator.

Derivatization and clean-up process*:* Grignard reagent 2 mL (n-pentylmagnesium bromide) was added to extracted samples and this reaction was allowed to occur by shaking for 1 min and then by keeping the solution at 40^{o}C in a water bath for 40 mins. After the derivatization flask was placed in ice bath and 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–liquid extracted twice with 10mL of 10% benzene/hexane.

Derivaitized extract was eluted with 30mL of 10% benzene/hexane through a glass chromatographic column packed with 5g of activated florisil and 2g of activated anhydrous Na_2SO_4 and evaporated to 0.1mL under a gentle stream of nitrogen. After that 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 is 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) for separation. Injection (2 µl) was performed in the split less mode and injector port and detector were both set at 250^{o}C. The column temperature was set for 1 min at 80^{o}C initially and which reaches up to 5^{o}C/ min to 190^{o}C, then increased to 280^{o}C at 10^{o}C/min, holding this temperature for 5 min. The procedure was validated by using the certified reference material BCR 477 (mussel tissue). The mean recovery through the entire analytical procedure for MBT (monobutyltin), DBT (dibutyltin) and TBT (tributyltin) were 101.1, 109.5 and87.3, respectively. The detection limits of the method for MBT, DBT, TBT, were 0.07, 0.05 and 0.02, ng g^{-1} dry wt.

Testosterone extraction and enzyme immunoassay

Extraction of free testosterone was carried out by homogenizing the whole body tissue in 5mL distilled water and then 1mL of homogenate was taken into another tube and 3mL of diethyl ether was added to it. This mixture was vortexed and allowed to stand for few minutes and their top layer was collected in a clean test tube. This was repeated twice and then the diethyl ether was evaporated under nitrogen and reconstituted with 500 µL of assay buffer. Testosterone standards (7.81 to 2000pg mL^{-1}) were prepared and analyzed along with the samples by following procedure described in testosterone EIA Kit *Enzo Life Sciences* (Catalog No. ADI-900-065).

STATISTICAL ANALYSIS

To compare the level of free testosterone and butyltins (TBT, DBT and MBT) body burden between control and treatment groups, one- way ANOVA has been carried out at $p<0.05$ probability levels by using MINITAB statistical software.

RESULTS

Imposex induction

Morphological examination before bioassay experiment showed that the all selected animals were normal male and female gastropods. However, at the end of four weeks of experiment, 20% females of *T. bufo* have showed imposex condition after exposure to 500ng^{-1} TBTCl, of which 10% acquired 1b stage of imposex while remaining 10% developed 3b imposex stage. In 1000 ngl^{-1} of TBTCl treatment 22% females changed to imposex out

of which 11% females developed stage 1a having a tiny penis (bud like formsation) which corresponds to initial growth of the penis and in the remaining 11% female 1b stage developed (table 1). Likewise, in *T. rudolphi* after exposure to 500ngl^{-1} of TBTCl 1b VDS (Vas deferens) stage developed in 10% females, whereas in 1000ngl^{-1} treatment group 22% females became imposexed, half of which showed VDS stage 1b and those remaining were in stage 3b. Samples exposed to 100ngl^{-1} TBTCl and DMSO control group showed no sign of imposex in both species (table1 and fig. 1).

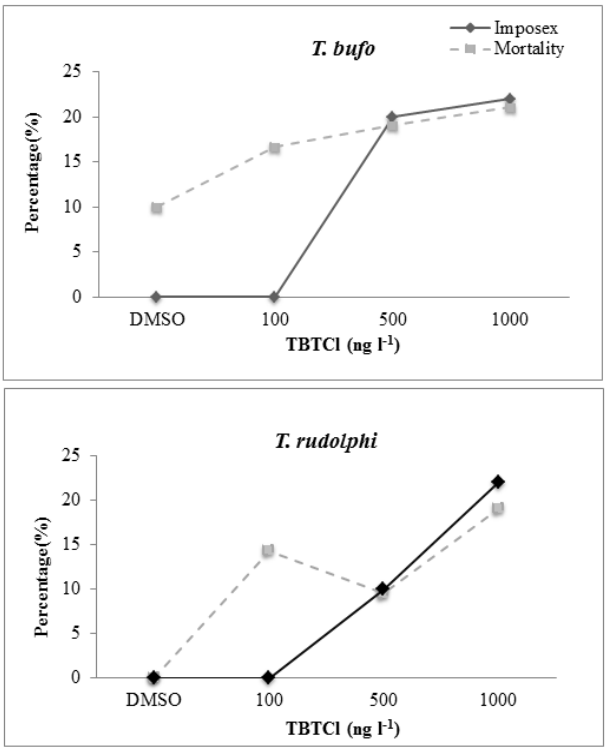

Fig. 1: Concentration response relationship for mortality and imposex after TBTCl (ng l^{-1}) exposure in *T. bufo* and *T. rudolphi*

Mortality In *T. bufo* mortality were 16.66, 19.04 and 21.05% in 100, 500 and 1000ng l^{-1} treatment groups, respectively and in DMSO control group mortality of only one gastropod was recorded (fig 1). In *T. rudolphi* 19% mortality observed in 1000ngl^{-1} TBTCl treatment group as compared to 9.52 and 14.28% mortality in 500ng l^{-1} and 100ng l^{-1} treatment groups respectively, while, in DMSO control group mortality was zero in this species.

Butyltins body burden The assessment of butyltins body burden in specimens exposed to different concentration of TBTCl (ng l^{-1}) and control showed that in *T. bufo* treated with 1000ngl^{-1} of TBTCl mean values of TBT (1138.65±127.53ng g^{-1}), DBT (535.75±23.96ng g^{-1}) and MBT (183.48±13.76ng g^{-1}) were high as compared to 500ng l^{-1} treatment group in which TBT, DBT and MBT concentrations were 358.19±160.56, 135.35±48.63 and 51.12±23.07ng g^{-1} respectively. While in the 100ngl^{-1} of TBTCl treatment group the concentration of TBT (249.03±6.83ng g^{-1}) and MBT (47.77±1.31ng g^{-1}) were low compared to the 500ng g^{-1} of TBTCl group but the concentration of DBT (153.95±21.90ng g^{-1}), in whole body tissues was slightly high. In DMSO control group also very low levels of TBT (154.06±60.67ng g^{-1}), DBT (90.33±28.91ng g^{-1}) and MBT (54.04±5.91ng g^{-1}) were observed (fig. 2).

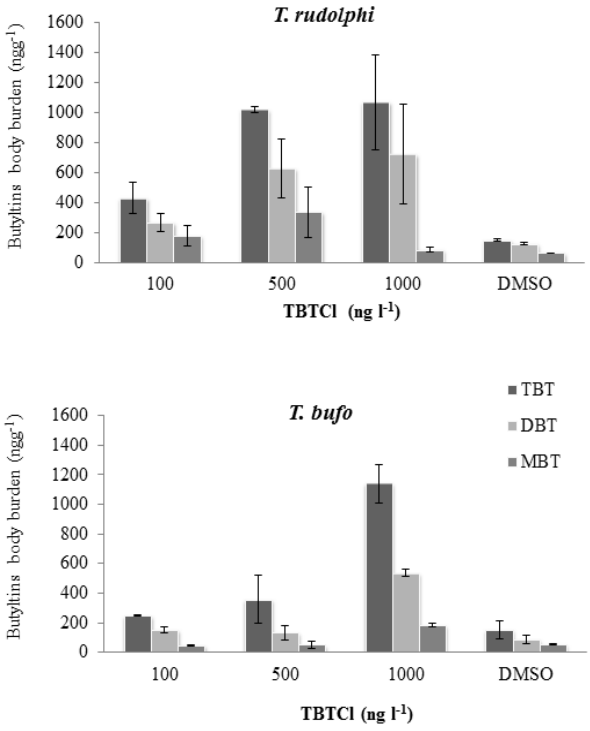

Fig. 2: Butyltin body burden in *T. bufo* and *T. rudolphi* after exposure to different concentrations of TBTCl (ng l^{1}) and in DMSO control.

Likewise, in *T. rudolphi* exposed to1000ng l^{-1} of TBTCl higher body burden of TBT (1068.57± 314.52ng g^{-1}), DBT (719.58±332.21ng g^{-1}) and MBT (83.26±14.89ng g^{-1}) were found as compared to 500 ngl^{-1} treatment group in which concentration of TBT (1018.65±23.08ng g^{-1}), DBT (625.35±198.95ng g^{-1}) was low but MBT (335.99±166.82ng g^{-1}) was high. Whereas in the 100ng l^{-1} treatment group low bioaccumulation of TBT (428.01±103.93ng g^{-1}), DBT (265.04±58.05ng g^{-1}) and MBT (178.12±66.78ng g^{-1}) were observed. In DMSO control group also low concentration of TBT (147.27±8.06ng g^{-1}), DBT (123.64±8.77ng g^{-1}) and MBT (61.04±2.5ng g^{-1}) were detected (fig. 2).

In *T. bufo* one way ANOVA for bioaccumulation of TBT (F= 34.92; p<0.05), DBT (F= 74.57; p <0.05) and MBT (F= 42.20; p <0.05) showed a significant difference among all four treatment groups. Similarly, in *T. rudolphi* TBT (F= 14.01; p <0.05) and MBT (F= 9.23; p <0.05)

levels were significantly different while DBT (F= 4.69; p >0.05) showed no statistically significant difference among the treatment groups (table 2).

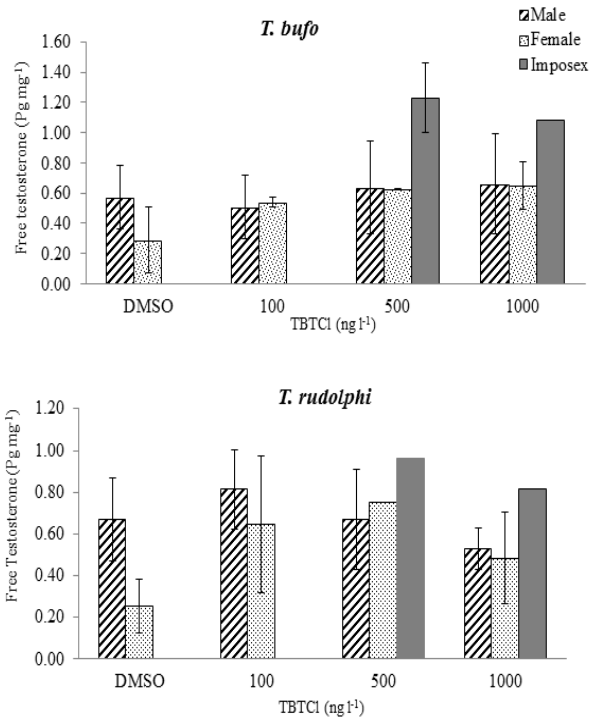

Fig. 3: Level of free testosterone in males, females and imposex of *T. bufo* and *T. rudolph*i after 4-weeks exposure to TBTCl (ng l^{-1}) *T. bufo* and *T. rudolphi.*

Free testosterone level In the present study *T. bufo* females exposed to 500ng l^{-1} TBTCl developed imposex and showed higher level of free testosterone (1.23±0.23pg mg^{-1}) as compared to males (0.66±0.37pg mg^{-1}) and females (0.60±0.05). Similarly, in 1000ng l^{-1} TBTCl exposure group the level of testosterone was higher in imposex (1.08±0.00pg mg^{-1}) than females (0.66±0.16pg mg^{-1}) and males (0.65±0.33pg mg^{-1}). In 100ng l^{-1} TBTCl treatment group mean values of testosterone were 0.53±0.03pg mg^{-1} and 0.51±0.21pg mg^{-1} estimated in females and males respectively. However, in DMSO control group lower level of testosterone (0.29±0.22pg mg^{-1}) was in females than males (0.57±0.21pg mg^{-1}) (fig 3).

In *T. rudolphi* the 1000ngl^{-1} TBTCl treatment group showed elevated level of testosterone in imposex (0.82±0.00pg mg^{-1}) as compared to females (0.49±0.22pg mg^{-1}) and males (0.53±0.10pg mg^{-1}). Similarly, in the 500ng l^{-1} TBTCl treatment group the level of free testosterone was higher in imposex (0.96±0.00pg mg^{-1}) than males (0.74±0.29pg mg^{-1}) and females (0.64±0.16pg mg^{-1}). Whereas, in the 100ngl^{-1} treatment group females (0.64±0.33pg mg^{-1}) had lower level of testosterone than males (0.81±0.19pg mg^{-1}) and DMSO control group also showed low concentration of testosterone (0.25±0.13pg mg^{-1}) in females than males (0.66±0.20pg mg^{-1}) (fig 3).

Moreover, one way ANOVA for level of free testosterone showed significant difference among treatment groups in both *T. bufo* (F= 3.88; p <0.05) and *T. rudolphi* (F= 2.90; p =0.05).

DISCUSSION

In the present work *T. bufo* and *T. rudolphi* both were selected for bioassay experiments and collected from Manora Rocky Ledge as this site has been reported to be free of TBT contamination and no incidence of imposex have been documented from here in any examined gastropod species (Asfar, 2009). Similarly, in the present study no incidence of imposex was observed during morphological examination of targeted species before conducting bioassay experiments. The bioassay experiments showed that the specimens have acquired different stages of imposex during TBTCl exposure via injection and indicates that both species of neogastropods are sensitive to TBT as reported during field investigations from Manora Channel (Asfar, 2009).

During the experimentation, TBT exposure trials showed that 20-22% *T. bufo* and 10-20% *T. rudolphi* females developed sexual abnormality with a and b type of imposex stages after exposure of 500 and 1000ngl^{-1} TBTCl. While in 100ng l^{-1} TBTCl and control group (DMSO) development of imposex condition was not detected in any specimen. Previously, in other bioassay experiments different stages of imposex have also been reported at different concentrations and at different duration of TBT exposure such as gastropod *Nassarius reticulatus* exposed to 250 and 400ng Snl^{-1} of TBT developed a and b type of VDS after one month of exposure and no imposex female was found in the control group (Barroso *et al*., 2002), in *Nucella lapillus* imposex phenomena was observed in 20-50% females after exposed to 50ng$l^{-1}$of TBT for three months(Santos *et al*., 2005). Increased imposex frequency has also been reported in *I. obsoleta* when exposed to 1 and 10ng l^{-1} TBT for six months (Gooding *et al*., 2003). In *T. clavigera* exposed to 1µg and 0.1 µg g^{-1} of body weight of TBT for one month showed promotion in imposex via increasing penis length in female gastropods (Horiguchi *et al*., 1997). These studies indicate that the development of imposex depends on the different factors such as concentration, duration of exposure, bioavailability and sensitivity of the species to TBT as reported by Rudel (2003).

Butyltin analysis in soft tissues of targeted species showed that the body burden of TBT in *T. rudolphi* was 1290.97-354.52ng g^{-1} and in *T. bufo* it was 1228.83-230.98ng g^{-1}. However, the incidence of imposex at the

Table 1: Morphometric measurements (mean± SD), mortality and imposex stages in *T. bufo* and *T. rudolphi.*

Species	Treatment groups (TBTCl ng l1)	Male (n)	Female (n)	SL (mm)	SW (mm)	AS (mm)	Mortality (%)	Imposex stages
T. bufo	100	9	9	43.72±3.21	31.72±2.44	35.61±3.01	16.66	
	500	11	10	43.75±3.80	32.40±3.34	36.21±3.11	19.04	1b , 3b
	1000	10	9	43.45±4.82	31.55±4.85	35.50±4.62	21.05	1a, 1b
	DMSO	4	6	43.90±3.01	31.80±3.04	35.60±3.23	10.00	
T. rudolphi	100	11	10	44.19±9.0	26.66±5.51	33.61±6.35	14.28	
	500	11	10	43.85±6.86	28.38±4.76	32.71±4.91	9.52	1b
	1000	12	9	44.23±7.38	29.71±4.22	34.76±4.76	19.00	3b, 1b
	DMSO	12	10	39.52±5.69	25.82±3.49	30.52±3.23	0.00	

1a =incipient penis represented by a ridge behind the right ocular tentacle;1b = initial development of penis duct; 3b = well developed penis duct. SL-shell length; SW- shell width, AS- aperture size, n- numbers.

Table 2: ANOVA testing for TBT, DBT and MBT body burden among treatments groups (100, 500, 1000ng l -1 TBTCl and DMSO) of *T. bufo* and *T. rudolphi.*

Species	Source	Variables	F-ratio	DF	P-values
T. bufo	Treatments	TBT	34.92	3	0.00*
		DBT	74.57	3	0.00*
		MBT	42.20	3	0.00*
T. rudolphi	Treatments	TBT	14.79	3	0.01*
		DBT	4.69	3	0.08
		MBT	9.23	3	0.02*

(F-ratio; Fisher ratio, DF; Degree of freedom, P-value; Probability, * significant difference)

end of the laboratory trials was low as compared to field study carried out at Gadani Shipbreaking Yard where it was 100% in both *T. bufo* and *T. rudolphi* (Hassan *et al*., 2014). This could be due to the selection of adult specimens for the experiments which are considered as less sensitive to TBT as compared to juveniles (Gooding *et al*., 2003). Less duration of exposure could also be the reason for low incidence of imposex in laboratory experiments. Moreover, bioaccumulation of TBT in both species was found to be dose dependent as observed in the ramshorn snail *Marisa cornuarietis* in laboratory experiments (Oehlmann *et al*., 1995). While low TBT body burden in DMSO control groups in the present trials indicate that the sampling site may have a low level of TBT contamination but not sufficient enough to induce imposex in selected specimen used for bioassays.

Earlier reports have indicated that TBT has a potent role in endocrine disruption and causes an alteration in the level of steroid hormones in the affected population of *I. obsoleta* during field and bioassay experiment (Gooding *et al*., 2003). Higher level of testosterone in imposex females as compared to normal females in *Hinia reticulata* has also been observed during exposure experiment (Bettin *et al*., 1996). Similarly, present results also confirm that the exposure of TBT causes the elevation of free testosterone in target species. In *T. bufo* imposex females level of free testosterone was (>1pg mg^{-1}) while in normal females it was (<1pg mg^{-1}) and *T. rudolphi* represents ~1pg mg^{-1} and <1pg mg^{-1} level of testosterone in imposex and normal females, respectively. However, the level of testosterone analyzed during this bioassay experiment was low as compared to in field investigations of the same species (Hassan *et al*., 2014). Similar variability in levels of free testosterone has also been reported in *I. obsoleta* in which a low testosterone level (1.8-3.1pg mg^{-1}) was observed after exposure experiments which were conducted for a short period (Gooding *et al*., 2003).Whereas, high levels of free testosterone (5-45pg mg^{-1}) was recorded in samples collected during field investigations(Gooding and LeBance 2004). This variation in the level of testosterone could be due to changes in the reproductive status of the organisms and also may be due to alteration in testosterone-fatty acid esterification (Gooding and LeBance 2004).

CONCLUSION

Present study has indicated that the TBT is potent imposex inducer in *T. bufo* and *T. rudolphi* and causes steroid imbalance with an increase of free testosterone in imposex female as compare to normal female and male. This study demonstrates the link between exposure of TBT and imposex development through endocrine disruptive mechanism.

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