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Full Length Research Paper

Elevation of Testosterone Level in Periwinkle Snail *Tympanotonus fuscatus var radula* Exposed to Bis-Tributyltin Oxide (TBTO)

Ogbomida, Emmanuel Temiotan [1*] and Ezemonye, Lawrence Ikechukwu [2]
[1]Ecotoxicology and Environmental Forensic Unit, National Centre for Energy and Environment, Energy Commission of Nigeria, University of Benin, Benin City, Nigeria.
[2]Department of Animal and Environmental Biology, University of Benin, Benin, Nigeria.
*Corresponding author's email: ogbomida2000@justice.com

Abstract
Bis (tri-n-butyltin) oxide (TBTO) is an organotin biocide used in marine antifouling paints to prevent encrustation of marine organisms on the hull of ships. The release of tributyltin (TBT) from hull of ships represents one of the main problems of direct, diffuse and continue contamination of the marine environment. To assess the effect of TBT pollution in Warri Harbour, Periwinkle snails, *Tympanotonus fuscatus var radula* an important shellfish in the Niger Delta ecological zone was exposed to sublethal concentrations (0.1, 1.0, 10 and 20µg/L) of TBTO using direct sediment toxicity bioassay to determine TBT bioaccumulation and its effects on testosterone levels. Tissue analysis for TBT was done using Solid Phase Extraction (SPE) and gas chromatography with flame ionization detection (GC-FID) after derivatization with Grignard reagent pentyl magnesium chloride. The study employed the Organization for Economic Development and Cooperation (OECD) Guideline 218 protocol. Tissue analysis of *T. fuscatus var radula* for TBT was found to be significantly low for TBT. Serum analysis of the Periwinkles snail after 10, 20 and 30days exposure period to varying TBTO concentrations showed increased significantly ($p<0.05$) free testosterone levels with increased concentration and exposure durations. TBT induced elevated testosterone in snails may be indicative of imposex expression an irreversible sexual abnormality in female molluscs. The observations in this study showed that *T. fuscatus var radula* is susceptible to imposex development and may affect their reproductive ability which may lead to population decline.
Key words: Imposex, Periwinkle Snail, Testosterone Elevation, Tributyltin Oxide

Introduction

During the last decades, scientists especially marine biologists have been concerned with new type of pollutants associated with antifouling paints known as organotin group (Meador, 1997; Gooding, 1999; Hall, 2000; Amr, 2004). Organotin compounds, such as tributyltin (TBT), have been extensively used as antifouling agents in paints applied to ships' hulls, boats and aquaculture nets. It is one of the most powerful antifouling biocide used to prevent biological fouling - a phenomenon which has considerable economic costs and environmental risks. It is one of the most toxic substances that have been deliberately introduced into natural waters (Goldberg, 1986; Terlizzi, 2004).

Aquatic pollution resulting from TBT usage has been of great concern due to their bioaccumulation potential, persistence in sediment up to several years, and highly toxic effects on non-target aquatic life. TBT is a widespread contaminant in aquatic environments and has been included in the group of Xenoestrogens or endocrine disrupters, due to its observed effects in natural ecosystems such as worldwide imposex (a

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pseudo-hermaphroditic) condition, characterized by the development of a penis, vas deferens, and seminiferous tubules in females) in marine gastropods (deFur, 1999; Alzieu, 2000). This pseudo-hermaphroditic condition usually lead to reduced fecundity, reproduction failure and population decline in a variety of aquatic organisms (Kishimoto, 1973; Matthiessen *et al.,* 1995; Nicholson and Evans, 1997).

Globally, decline of gastropod population has been recorded as a consequence of the induction of the imposex effect in female molluscs by TBT pollution (Chiavarini *et al.,* 2003). This pseudo-hermaphroditic condition occurs at environmentally relevant TBT concentrations as low as 1µg/L (LeBlanc and Bain, 1997; Bryan *et al.,* 1986; Linley-Adams, 1999; Nash *et al.,* 2004). In 1985, the world production of triorganotins with biocide properties was in the range of 8–10,000 tons annually (de Mora, 1996). Over recent decades, the use and the production of TBT-containing paints have been restricted, resulting in significant reductions in environmental concentrations (Alzieu, 1998). Despite these restrictive regulations, coastal TBT contaminations still reach up to 200ng TBT/l (Michel, 2001).

The underlying mechanism by which TBT causes imposex in gastropods has not been conclusively elucidated, the weight of evidence favours the aromatase-inhibition hypothesis (Matthiessen, 1995). This hypothesis states that higher levels of TBT in gastropods can inhibit competitively cytochrome P450–dependent aromatase (CYP19) which prevent the conversion of testosterone to 17b- estradiol (Bettin, 1996; Stroben, 1991) thereby result in increase in testosterone titre which is responsible for imposex development. It has been shown that elevated testosterone levels increased the incidence of imposex in female dogwhelks, *N. lapillus (*Bettin, 1996; Tanabe, 1999) and *Nassarius (Hinia) reticulates* (Bettin, 1996) mud snails *I. obsoleta* (Oberdörster, 2000) and ramshorn snails (*Marisa cornuarietis*).

Testosterone elevation is, therefore a useful biomarker of organotin pollution. Testosterone is typically viewed as a vertebrate-type sex steroid. However, mounting evidence suggests that testosterone has a role in gastropod reproduction and sexual differentiation. The use of testosterone biomarkers as biological indicator in the expression of imposex in marine snails has been considered ecological relevance, and this would play a leading role in environmental management (Axiak *et al.,* 2000; Zucker *et al.,* 1992). The present study evaluates the effects of Bis (tributyltin) oxide (TBTO) at environmentally relevant concentrations on the testosterone levels in the mud snail *Tympanotonus fuscatus var radula* considered responsible for imposex expression in gastropod.

Materials and Methods

Collection of test organism

Periwinkle snails *Tympanotonus fuscatus var radula* of similar sizes (shell length of 28 to 32 mm, diameter of aperture 0.7-0.9mm) were collected by handpicking into a bucket (12.6L) from Miller Creek at low tide, in Warri, Delta state. The periwinkles were always collected from the same site, in order to reduce variability in biotype. They were transported to the laboratory and kept in holding glass tanks (30 x 30 x 30 cm), which contained aerated brackish water (6L) at measured salinity. Individuals Periwinkle snails were washed on site with surface water to remove mud from the shells and then transported to the laboratory in plastic containers.

Test chemical

Bis (tri-n-butyltin) oxide (TBTO) (>96%) formulated antifoulant MA1161 was obtained from Sigma-Aldrich chemicals Germany through Rovet Scientific Limited Benin City, Nigeria.

Preparation of substrate

Sediment used for the toxicity test was collected from the same site were the

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periwinkle snails were collected. They were sun dried to standardize moisture content, and thereafter passed through a sieve (0.25mm) to obtain uniform substrate particles according to Tokolo (1988) and Otitoloju (2002). A pre-determined amount of 500g of sediment was weighed and introduced into the experimental tank. Brackish water was used as the diluents for the entire bioassay test. Sediments were spread to form a uniform layer in the bioassay tank and a pre-determined concentration of tributyltin oxide diluted in ethanol was added. The mixtures were gently mixed to achieve even distribution of the pesticide before the introduction of the test species. This procedure was adopted throughout the bioassay test.

Preparation of stock solution

The procedure for preparing test solution was based on the method of Parks *et al* (1999). The stock solution containing Bis-tributyltin oxide (TBTO) was prepared in two steps. One milligramme per litre (1mg/l) of TBTO was dissolved in 2ml of ethanol. A secondary water based stock solution was then prepared by adding 997ml of filtered brackish water to the primary stock solution to make 1000mg/l of TBTO. The solution was stirred and transferred into a lidded glass bottle and stored at 4^0C. Ethanol in this concentration has no deleterious effects on the test organisms (Peterle, 1991).

Sublethal toxicity test

The 30 day-direct sediment toxicity tests with *T. fuscatus var radula* species were carried out according to OECD Guideline 218 (OECD, 2002). Sediment samples were collected using a van veen grab and standardized as described by Tokolo (1988) and Otitoloju (2002). The prepared sublethal test concentrations (0.1, 1.0, 10 and 20µg/L) were added to the sediment, homogenized and allowed to settle for 2 to 3 hours. Ten (10) adult periwinkles *T. fuscatus var radula* were introduced into each glass tank containing 500g of sediment and 1000ml of test solution. The experiments were in three replicates per treatment. The control test containing ten (10) periwinkles samples received the same treatment as the exposed periwinkle, except that the TBTO/ethanol solution was exchanged with ethanol only. The TBTO/ethanol test solution was replaced continuously and stock solution was shielded as a precaution to maintain stable TBTO/ethanol test concentrations (Peterle, 1991). This is because TBTO is easily degraded by UV-light (WHO, 1980). After 30 days exposure period, tissue concentration for testosterone and TBT levels of the *T. fuscatus var radula* were determined.

Tissue TBT Analysis

TBT concentration was analyzed following the method described by Omura . (2004). Periwinkle tissues were homogenized and extracted twice with 40ml of 0.1% tropolone–acetone and 10ml of 1M HCl. The combined extracts were transferred to 100ml of 0.1% tropolone–benzene and 500ml of hexane washed water. After shaking and partitioning, the organic layer was eluted through a glass column packed with 35g of anhydrous Na_2SO_4 to remove moisture. The benzene extract was rotary-evaporated at 40°C almost to dryness and the volume was made up to 5ml with hexane. The extract was derivatized by the addition of 5ml of pentyl magnesium chloride (in diethyl ether) as the Grignard reagent. The excess Grignard reagent was decomposed with 20ml of 1N H_2SO_4 and the derivatized extract was passed through a 20g Florisil®-packed dry column to remove lipids and then cleaned by eluting through a 6g Florisil®-packed wet column. Sample extracts were analyzed for TBT using gas chromatographic system equipped with flame ionization detector (GC/FID) (Hewlett-Packard 6890 Series, Little Falls, Delaware, USA) according to the method of Bortoli (2003). Sets of blanks and standards were also processed following the same method as for samples. The injection mode was split less and the temperature was set at 250°C. The column

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was a 30m x 0.32mm i.d. x 0.25µm film thickness HP-5 (Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1ml/min. The temperature was programme at 55°C for 1 min, then 20°C/min to 200°C, which was held for 5 min. The oven was programmed from 40°C (1 min) at 20°C/min to 220°C (3 min) and the carrier gas was at a constant pressure of 80kPa.

Tissue preparation for testosterone quantification

In preparation for testosterone analysis, periwinkle tissues were homogenized with a Tearor homogenizer (Biospec Products, Bartlesville, OK) in ice-cold 25mM Tris-HCl with ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 x g (for 20 min at 4°C), and the supernatant was collected and stored at -20°C until analysis. Levels of testosterone were determined using an RIA Kit (Cayman, Ann Arbor, MI, USA). For each treatment, periwinkles were randomly collected from the bioassay experimental tanks on day 10, 20 and 30 for analysis.

Testosterone quantification

Serum testosterone concentration was determined by radioimmunoassay (RIA) as described by Addo *(*1989) and Parks (1999). Snail tissue was homogenized for 30 seconds in 1.0ml ethanol using a Tissue Tearor homogenizer (Biospec Products, Bartlesville, OK). Homogenate was stored at -20°C for at least 24 hours. Testosterone and its metabolites were extracted from the tissues with 3.0ml ethyl acetate. Sample extract was divided into two equal volumes. Aliquots were evaporated under nitrogen, and one aliquot was resuspended in 250µl 0.05M phosphate buffer (pH 7.6) for free (unesterified) testosterone quantification. Total (unesterified + esterified) testosterone was determined by saponification of the second aliquot in 1.0ml methanol containing 1.0% potassium hydroxide (Addo, 1989). Samples were incubated for 3 hours at 45°C in an atmosphere of nitrogen. Following saponification, 4.0ml deionized water was added to each tube and the sample was extracted with 3.0 ml methylene chloride. Extracts were evaporated under nitrogen, resuspended in 1.0ml 0.05M phosphate buffer (pH 7.6), and measured for testosterone by radioimmunoassay (RIA) (Diagnostic Products Corp., Los Angeles, CA). Testosterone was measured in the samples according to the protocol provided with the RIA kit. The RIA was validated for use in snail extract using criteria of parallelism as described by Parks (1999). Standard curves were routinely generated using both the standards provided in the RIA kit and standards prepared in the phosphate buffer. Testosterone-fatty acid ester in tissue samples was calculated as total testosterone minus free testosterone.

Statistics

Differences in testosterone levels between treatment and control groups were evaluated by ANOVA and Dunnett's Multiple Comparison test following log transformation when variances associated with the experimental groups were not homogenous. Statistical analyses were performed using SPSS statistical software version 16.

Results

The results of the testosterone levels after 10, 20 and 30 days exposure to TBTO are shown in Figure 1. The results showed that testosterone levels in *T. fuscatus var radula* exposed to varying concentrations (0.1, 1.0, 10 and 20µg/L) of TBT increased with increased TBT concentrations and exposure durations Fig 1. The highest testosterone level was observed in day 30 while the least was recorded in day 10. There was significant difference ($p \leq 0.05$) between the control and the treated. The concentrations levels of free testosterone increased significantly indicative of imposex expression in the periwinkle.

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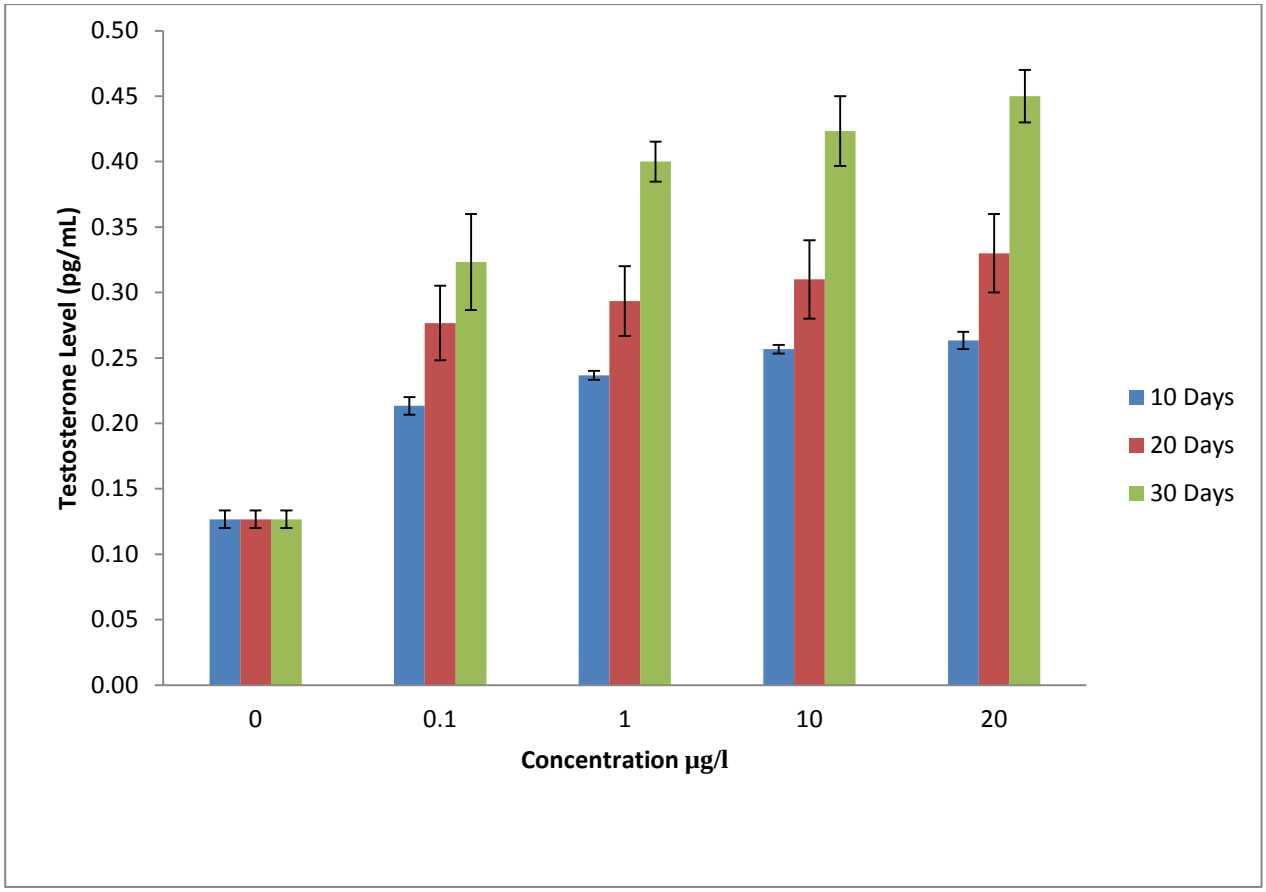


Fig 1. Testosterone levels after 10, 20 and 30 days exposure to TBTO. (Error bars represent Standard error on the mean of two replicates).

Table 1: Bioaccumulation, Bio-concentration Factors, Toxicity Index Value and the hazard Quotient of *T. fuscatus var radula* exposed to sublethal concentrations (µg/l) for 30 days

Concentrations (µg/l)	Bioconcentration			Bioconcentration Factors (BCF)			Toxicity Index Value (TIV)			Hazard Quotient (HQ)		
	10	20	30	10	20	30	10	20	30	10	20	30
0.1	N.D	N.D	0.002 ± 0.00122	N.D	N.D	0.002 ± 0.01732	N.D	N.D	0.0001 ± 0.00006	N.D	N.D	0.0769 ± 0.06659
1.0	0.002 ± 0.001	0.002 ± 0.00105	0.0046 ± 0.00167	0.0020 ± 0.001	0.003 ± 0.001	0.0043 ± 0.00153	0.0000 ± 0.00003	0.0001 ± 0.00003	0.0002 ± 0.00005	0.0769 ± 0.03845	0.1154 ± 0.03845	0.1667 ± 0.05877
10	0.0033 ± 0.00153	0.004 ± 0.00186	0.0066 ± 0.00151	0.0003 ± 0.00015	0.0006 ± 0.0001	0.0007 ± 0.00015	0.0001 ± 0.00009	0.0002 ± 0.00004	0.0003 ± 0.00006	0.1282 ± 0.05876	0.2308 ± 0.03845	0.2821 ± 0.05877
20	0.0050 ± 0.0010	0.006 ± 0.00147	0.0090 ± 0.00122	0.0002 ± 0.00005	0.00004± 0.00005	0.0005 ± 0.00005	0.0002 ± 0.00004	0.0003 ± 0.00004	0.0003 ± 0.00004	0.1923 ± 0.03850	0.3077 ± 0.03850	0.3462 ± 0.0845

Discussion

Global concerns have been raised in recent years over possible adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system (Courant *et al*., 2007). Wildlife and human health effects of endocrine disruptors were first proclaimed by Rachel Carson in 1962, and based on growing body of knowledge, those concerns have increased. Since the early eighties the development of male sexual characteristics (imposex condition) has been reported for females of some marine neogastropod snails. This phenomenon has been primarily attributed to the contamination of coastal areas with tributyltin (TBT) as described in Smith (1981) and Bryan *et al.* (1987). Species inhabiting rocky shores, as well as soft bottoms of peripheral seas, are reported to be affected (Fioroni *et al.*, 1991, Ten Hallers-Tjabbes *et al.*, 1996).

The mechanism of toxicity in *N. lapillus* is described by Spooner and Goad (1990) as a result of increased testosterone level in treated female snails. The mechanism of toxicity in snails is described as a result of increased testosterone level in treated female snails (Spooner and Goad, 1990). It has also been postulated that TBT inhibit Cytochrome-P450-aromatase, which normally metabolizes testosterone to 17β-estradiol in females. Mollusks contain vertebrate-like steroids, and molluscan steroid-genesis is also similar to the vertebrate plan. It is hypothesized that TBT causes imposex and intersex in gastropods by interfering with steroid biosynthesis.

Results of this study showed that *Tympanotonus fuscatus var radula* exposed to different sub-lethal concentrations of Bis-tributyltin oxide (TBTO) significantly showed increased free testosterone levels and low fatty acid ester. Several hypotheses have been proposed to show how TBT causes the imposition of male phenotypic characteristics in some species of female gastropods. The hypothesis regarding the elevation of testosterone by TBT which has been widely touted as a result of the association between exposure to TBT and increased free testosterone titers in imposexed female gastropods (Spooner *et al.*, 1991; Bettin *et al.*, 1996; Ronis and Mason, 1996; Gooding *et al.*, 2003; Santos *et al.*, 2005) is fully established in this study. *Tympanotonus fuscatus var radula* exposed to TBTO elevates testosterone which then initiates some unknown biochemical signaling pathway with the ultimate outcome being the development of imposex.

This upsurge in testosterone level may lead to imposex expression in periwinkles. The results obtained were in conformity with that reported by Bettin, (1996), Oehlmann, (1993) and Spooner, (1991) that increase in immune-detectable testosterone levels after TBT exposure may lead to imposex. The significance of this is that many mollusks experience endocrine disruption when exposed to TBTs, and there is evidence that this is the consequence of elevated levels of testosterone (Matthiessen and Gibbs 1998; Morcillo, 2004). This condition is typically associated with the development of a penis by female snails Matthiessen (1995) and Blaber (1970). Although more severe cases can involve the development of a vas deferens or a convolution of the oviduct that resembles the seminal vesicles of males (Jobling *et al.*, 2002; Stroben , 1991).

Previous studies by Bettin (1996) and Spooner (1991) showed that TBT induced elevated testosterone levels in *N. lapillus* and *Hinia reticulata*. Bettin (1996); IMO (2001) and Tanabe (1999) demonstrated that *N. lapillus* and *Hinia reticulate* exposed to TBT concentrations showed elevated testosterone titers in imposexed females. Much of the research regarding TBT's mechanism of action for inducing imposex has assumed that testosterone is one of the major hormones involved in the development of this phenomenon (Matthiessen, 1995). This

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study demonstrated that the increase in immunodetectable testosterone is not caused by an increase in total testosterone rather; TBT increased the proportion of total testosterone that was available in the free, immunodetectable form (Addo, 1989).

Perhaps exposure of *T. fuscatus var radula* to the TBTO chemical for longer periods may produce a greater incidence of imposex. The fatty acid esterification of testosterone by *T. fuscatus var radula* species represents a minor process in the biotransformation of testosterone (Addo, 1989; Borg, 1995; LeBlanc and Bain, 1997). The reduced esterification of testosterone by TBT may be indicative of imposex without being directly responsible for the phenomenon. For example, high-affinity 17β-estradiol binding proteins have been measured in mussels Gooding (2003) and precedent exists for estrogens being functional in molluscs (Fernandez, 2005). The increase in free testosterone (the precursor to 17β-estradiol) resulting from TBT exposure increases synthesis of 17β-estradiol, with this hormone being the ultimate regulator of sex differentiation.

The precise mechanism by which TBT alters the free and esterified testosterone ratio in the mud snail was not established in this study. Additional experiments are imperative to elucidate the mechanism by which TBT affects the levels of free testosterone. The International Maritime Organization has called for a global prohibition on the use of organotin-based paints effective 1 January 2008 (Jenner, 1979). Undoubtedly, this compound will be replaced by other chemicals with antifouling properties. Elucidation of the mechanism by which TBT alters hormones responsible for sex differentiation in molluscs resulting in imposex would help ensure that future TBT replacements do not share this toxicological property.

Tissue analysis of *T. fuscatus var radula* for TBT concentration showed marked accumulation of TBT. Tissue concentration was dose dependent and exposure duration. The highest tissue concentration of TBT was observed in the highest concentration after 30 days of exposure.

TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) were not observed in *T. fuscatus var radula*. This could be as a result of low amount of cytochrome P-450 and limited action of the mono-oxygenase system (Livingstone and Farrar, 1985). This low detoxifying cytochrome P-450 and limited action of the mono-oxygenase system explain why *T. fuscatus var radula* is susceptible to TBT compound. TBT also binds to glutathione S- transferase and cytochrome P-450 results in the inhibition of these detoxifying enzymes systems (Rosenberg and Drummond, 1983; Fent and Stegeman, 1993). Further research is necessary to provide evidence of the linkage of TBT metabolizing enzyme systems to observed effects on testosterone elevation.

Conclusion

These findings suggest that elevation of testosterone in *T. fuscatus var radula* may lead to imposex expression. This endocrine alteration may have potentials to cause population decline of the periwinkle sails. Consequently, constant monitoring and assessment of TBT concentrations in harbours and marinas is necessary for protecting sensitive benthic invertebrates.

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