

Tributyltin (TBT) contamination and impacts on imposex in *Thalessa aculeata* (Mollusca: Neogastropoda: Muricidae) in Minahasa Peninsula coastal waters, North Sulawesi, Indonesia

^{1,2}Natalie D. Rumampuk, ²Inneke F. Rumengan, ¹Rizald M. Rompas, ³Suzanne L. Undap, ²Farnis B. Boneka, ⁴Kathe R. Jensen, ³Markus T. Lasut

¹ Postgraduate Program of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado, Indonesia; ² Department of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado, Indonesia; ³ Post-graduate Program of Aquatic Science, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado, Indonesia; ⁴ Natural History Museum of Denmark, Universitetsparken 15, 2100 København Ø, Denmark. Corresponding author: M. T. Lasut, lasut.markus@unsrat.ac.id

Abstract. Contamination and impact of tributyltin (TBT) in the marine environment have been studied at three localities in Minahasa Peninsula Coastal waters (MPCW) using sediment samples and tissues of the marine gastropod *Thalessa aculeata*. The study aimed to determine the TBT concentration in sediment and snail tissues, to evaluate the occurrence of imposex, and to identify histological condition of gonad tissues of imposex affected snails. The study areas and sampling sites were selected for differences in sources of TBT, mainly shipping activities. Manado Bay (A) is an area of relatively low shipping activities, Lembeh Strait (B) has very high level of shipping activities, and Maluku Sea (C) has very low level of such activities. TBT concentrations in the sediment and gastropod tissues reflected the levels of shipping activities, and were below detection limit at locality C. Imposex was detected in snails from all three localities, and in up to 40% of the females at locality B. Relative female penis size index was more than 50% in the most heavily affected snails. Imposex affected females had shrinking of oocytes and obstruction of meiosis. We conclude that TBT contamination is still present in MPCW and affects reproduction of gastropods, possibly causing decrease in populations of these animals.

Key Words: sediment, tissue, gastropod, locality, reproduction.

Introduction. Organotin compounds (OTCs), in particular tributyltin (TBT), have received considerable attention since the introduction of TBT as a biocide in antifouling ship paint in the 1960s. TBT has severe effects on non-target organisms, in particular molluscs, in the marine environment. In marine snails, especially neogastropods, TBT causes a permanent, irreversible condition known as imposex, in which female snails develop male reproductive structures such as penis and vas deferens, eventually leading to sterility and even death of the affected snails. The development of imposex is dose dependent and, although different species show different sensitivity and different sequences of developing imposex, this condition has been used as an indicator of TBT contamination in the environment, even at very low concentrations (Bryan et al 1987; Gibbs et al 1987; Ellis & Pattisina 1990; Alzieu 1991).

Due to its severe effects on aquatic life, national and international legislation have gradually imposed bans on the use of TBT in antifouling paints, and in 2001 the International Maritime Organization (IMO) adopted the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS), which went into force in September 2008. After this date there has been a global ban on the use of TBT as an anti-fouling agent. However, TBT has accumulated in sediments and biota, and with a half-life in sediment of 1-5 years (Wilson et al 2015), it is to be expected that effects on

biota will continue to be present for some time. In addition, it is likely that several countries will have difficulties enforcing the ban, and there may be effects from “historical” paint layers even in well managed marine areas, such as the Baltic Sea (Lagerström et al 2017).

Several recent studies have shown that TBT is still in use in Indonesia (Sudaryanto et al 2005; Harino et al 2012; Kusumastuti et al 2013), and specifically in Minahasa Peninsula coastal waters (MPCW) located in the northern region of Sulawesi Island (Noor et al 2013; Undap et al 2013a). Only one study exists on the concentration of butyltin in the sediment in this area (Razak 2004). Imposex has been studied in several gastropod species from different localities in the Indonesian Archipelago (Ellis & Patissina 1990; Pandey & Evans 1996; Kusumastuti et al 2013; Noor et al 2013). These studies concluded that TBT contamination was widespread and that the MPCW area was a TBT-polluted area. Indonesia signed the AFS convention in 2014, and it will be important to monitor the status of TBT pollution in Indonesian waters over the coming years.

The present study aimed to determine the level of TBT in sediment and tissues of the snail *Thalessa aculeata* (Deshayes, 1844) (Mollusca: Neogastropoda: Muricidae) in three localities in MPCW, to evaluate imposex incidence, severity, and histological condition of gonad tissues of this snail in areas subject to different levels of TBT pollution. This study is related to two prior studies published only in Indonesian language (Mnsen et al 2016; Mamonto et al 2017).

Material and Method

Study areas and sampling sites. Minahasa Peninsula is located at the northern end of the K-shaped island of Sulawesi, which is located in the central part of the Indonesian archipelago (Figure 1). Three coastal localities were selected with regard to sources of TBT contamination from maritime and shipping activities: locality A: Manado Bay, facing Manado City, and subject to relatively low levels of maritime activities; locality B: Lembeh Strait, facing Bitung City and Lembeh Island, and subject to high levels of maritime activities; locality C: Maluku Sea, facing Belang City, and subject to very low levels of maritime activities. Within each of these areas two sample sites were selected: Manado Port (A1), Manado Fishing Port (A2), Bitung Fishing Port (B1), Bitung Harbour (B2), Belang Fishing Port (C1) and Belang Beach (B2). These sites were selected because prior studies, identified by GPS coordinates, had confirmed the presence of *T. aculeata*.

Sample collection and preparation. Sediment samples and gastropod specimens were collected simultaneously from each locality in July-August 2015. Sediment samples were collected from the surface layer (0-5 cm) in shallow water (50 cm depth) using a polyethylene (PE) spoon (Lasut et al 2010). Gastropods were collected haphazardly by hand. For TBT determination two snail specimens from each sampling site were combined to yield sufficient material for analysis. All samples were placed in separate PE bags and stored in a Styrofoam box with ice pack during transportation to the laboratory. Species identification was confirmed in the laboratory by one of us (FBB) using the books by Dharma (1988) and Dharma et al (2005).

Determination of TBT compound. Sample analyses were carried out at the Laboratory of Marine Environmental Science (MES), Faculty of Agriculture, Kyushu University, Fukuoka, Japan. Concentrations of TBT were determined using the method described by Undap et al (2013a, b) with slight modifications. Briefly, each sediment sample (1 g wet weight) was spiked with 1 µg of TBTCI-*d*27 as internal standard and homogenized with a 1:1:1 mixture of 1 M hydrobromic acid in methanol, ethyl acetate, and deionized water. For gastropod tissues, after removal of the shell, the samples were homogenized in a polytron homogenizer. They were then shaken for 30 min and centrifuged at 3000 rpm for 5 min. The supernatant was separated, and then extracted twice with 10 mL of 3:2 ethyl acetate: hexane, shaken for 10 min and centrifuged again. The organic layers were concentrated to 1 mL under gentle nitrogen gas flow at 35°C. The concentrated extract was then ethylated by adding 0.5 mL of a 5% sodium tetraethylborate solution and

deionized water and shaken for 30 min, after which 10 mL 1 M KOH solution was added and the sample shaken for 60 min. After centrifugation, the supernatant was passed through a Florisil column. The eluate was evaporated to 1 mL and then analysed using an Agilent 6890 gas chromatograph (Avondale, PA, USA) equipped with an Agilent 5973 mass spectrometer operated in EI+ mode. The mass to charge ratios used for quantitation of TBT and TBTCl-*d*27 ions were monitored at *m/z* 263 and 318 respectively.

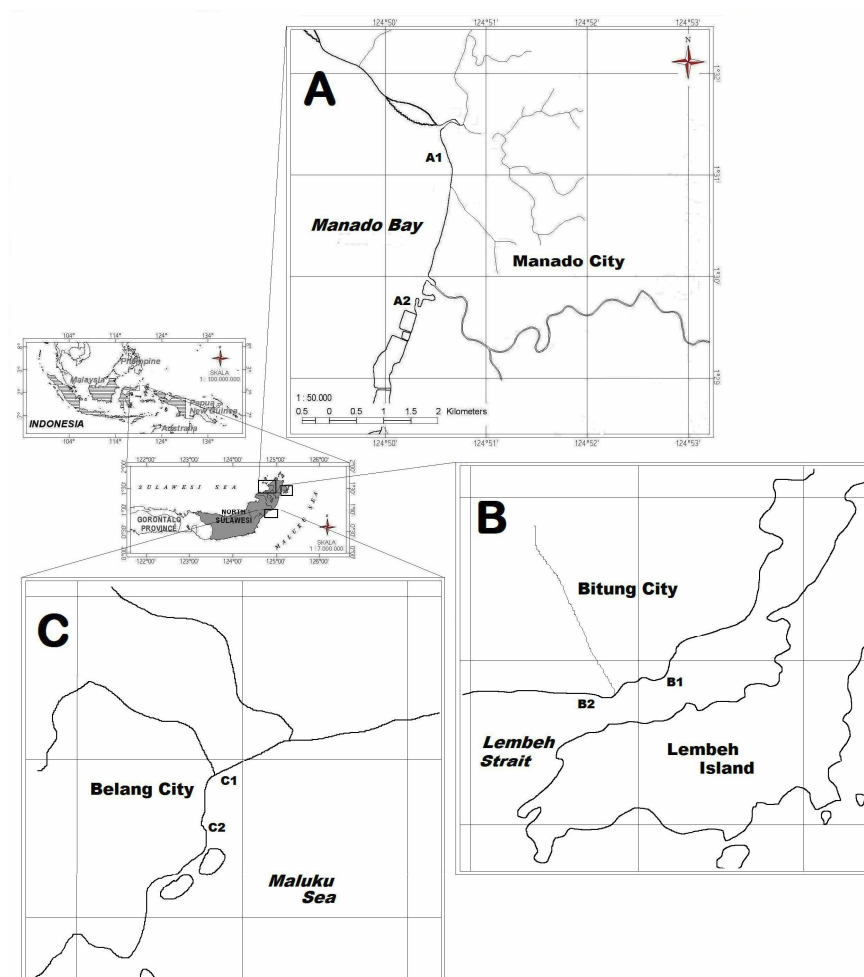


Figure 1. Study area (A: Manado Bay; B: Lembeh Strait; C: Maluku Sea) and sampling sites (A1: Manado Fishing Port; A2: Manado Port; B1: Bitung Fishing Port; B2: Bitung Harbour; C1: Belang Fishing Port; C2: Belang Beach).

Quantification of imposex. Shell length of gastropods was measured using calipers (accuracy 0.1 mm). Snails were then anesthetized in 7% MgCl₂ for 15 min before the soft parts were removed from the shell. Snails were sexed using colour of the gonads. In *T. aculeata* ovaries are light yellow and testes are dark yellow or orange. The length of the penis, which is located behind the right cephalic tentacle, was measured. Two indices were used to quantify imposex: percentage of imposexed females (*I*%) and relative penis size index (*RPSI*). The first is an index that calculates the percentage of imposexed females: (number of females with imposex/total number of females) X 100. *RPSI* is one of the indices that quantify the degree of imposex by measuring the size of the penis in females with imposex relative to the size of the penis in normal males: (mean length of female penis³/mean length of male penis³) X 100 (Gibbs et al 1987).

Histological observation. Changes caused by TBT to reproductive organs were studied in serial sections of imposex-affected females as well as normal females and males. Specimens were fixed in 10% formaldehyde for 72 h, rinsed in tap water, dehydrated and

embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin-eosin and mounted with DPX resin (Prophet et al 1992; Lopes-dos-Santos et al 2014).

Results

Concentration of TBT. The level of TBT in sediments samples varied between 'not detected' (nd) and 0.313 μg per gram wet weight (Table 1). The highest concentration was found at B1 (Bitung Fishing Port), and the levels at B1 and B2 were 31 and 16 times higher than at A2. TBT was found in gastropod tissues at A and B but not detected at C. Concentration at B was 9 times higher than at A. At locality A concentration in gastropod tissues was higher than that of the sediment, but at B the tissue concentration was lower than in the sediment, indicating that there is no direct correlation between sediment and tissue levels.

Incidence of imposex in *T. aculeata*. A total of 180 specimens of *T. aculeata* were collected, measured for shell length, sex ratio and imposex (Table 2). Only sexually mature specimens were included (mean shell length varied from 30.67 to 40.38 mm). Females affected by imposex were found at all sites, but incidence was very low at C (Maluku Sea). Again, incidence was highest (45%) at B (Table 2).

The same pattern was found when calculating the *RPSI* to quantify the degree of imposex (Table 3 and Figure 2). The sites that had high incidence also had high values of *RPSI*, up to 70% of the male penis length at B1.

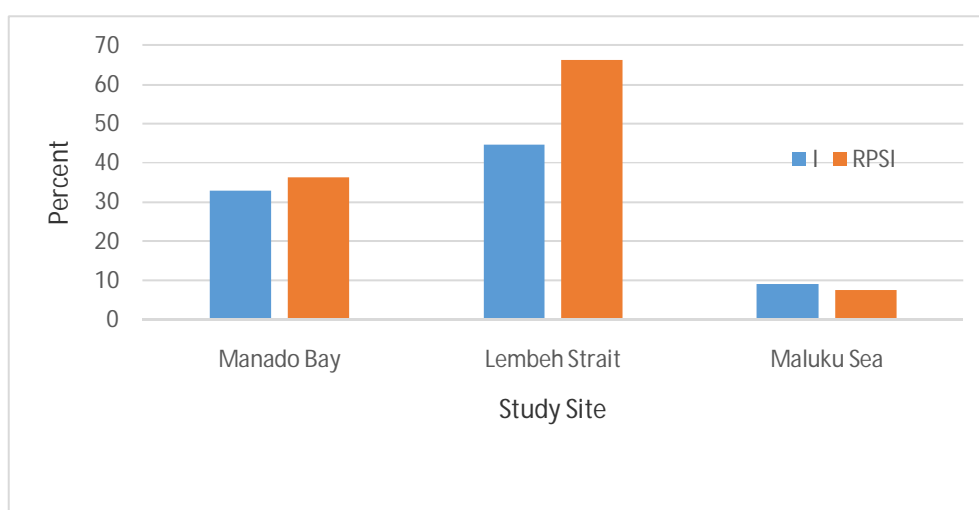


Figure 2. Average of imposex (*I*) and relative penis size index (*RPSI*) of *T. aculeata* at the study areas.

Table 1

Tributyltin concentration in sample of sediment and gastropod at the study areas and sampling sites

Study area	Sampling sites	Geographical positions	TBT concentrations ($\mu\text{g g}^{-1}$ ww)	
			Sediments	Gastropods
(A) Manado Bay	(A1) Manado Fishing Port	01°31' 24,76" N; 124° 50' 38,43" E	nd	0.017
	(A2) Manado Port	01°29' 71,13" N; 124° 50' 38,43" E	0.010	
(B) Lembah Straits	(B1) Bitung Fishing Port	01°26' 96,08" N; 125°12' 26, 40" E	0.313	0.163
	(B2) Bitung Harbour	01° 26' 33,49" N; 125°11' 51, 33" E	0.167	
(C) Maluku Sea	(C1) Belang Fishing Port	00°56' 77,02" N; 124° 47' 30,98" E	nd	nd

Note: nd: not detected; ww: wet weight).

Table 2

Imposex quantification and biometric data of *T. aculeata* at the sampling sites

Study areas	Sampling sites	SL (mm) (mean)	T	M	NF	IF	F (NF + IF)	SR (M:F)	I%
(A) Manado Bay	(A1) Manado Fishing Port	30.67	30	15	11	4	15	1.00	26.67
	(A2) Manado Port	40.38	30	7	14	9	23	0.30	39.13
(B) Lembah Strait	(B1) Bitung Fishing Port	32.18	30	7	13	10	23	0.30	43.48
	(B2) Bitung Harbour	35.73	30	6	13	11	24	0.25	45.83
(C) Maluku Sea	(C1) Belang Fishing Port	32.07	30	8	20	2	22	0.36	9.09
	(C2) Belang Beach	31.69	30	8	20	2	22	0.36	9.09

Note: SL: shell length; T: total snails collected; M: male; NF: normal females; IF: imposexed females; F: total females; SR: sex ratio; I%: percentage of imposex.

Table 3

Penis size index of *T. aculeata* at the sampling sites

Study areas	Sampling sites	MP (mm)	IFP (mm)	MP ³ (mm ³)	IFP ³ (mm ³)	RPSI%
(A) Manado Bay	(A1) Manado Fishing Port	3.65	2.15	48.63	9.94	20.44
	(A2) Manado Port	3.41	2.74	39.65	20.57	51.88
(B) Lembah Strait	(B1) Bitung Fishing Port	3.89	3.47	58.86	41.78	70.98
	(B2) Bitung Harbour	3.20	2.72	32.77	20.12	61.41
(C) Maluku Sea	(C1) Belang Fishing Port	4.86	1.85	114.79	6.33	5.52
	(C2) Belang Beach	4.60	2.10	97.34	9.26	9.51

Note: MP: male penis; IFP: imposexed female penis; RPSI%: percentage of relative penis size index.

Histological observation of reproductive organs. For histological analysis gonad tissues of imposexed females were compared to normal females (Figure 3) as well as normal males. Comparisons of normal and imposexed females from A and B showed shrinking of oocytes in the imposexed females (Figure 3 AII and BII). This resulted in absence of meiosis and inability to form normal ova. Also, dead cells were observed in ovarian lobes, and pyknosis (condensation of chromatin) occurred in the nuclei. These changes were not observed in the imposex affected female from C (Figure 3 CII). Imposexed females did not show any tissues that could be unequivocally identified as sperm-producing when compared to testicular lobes of normal males.

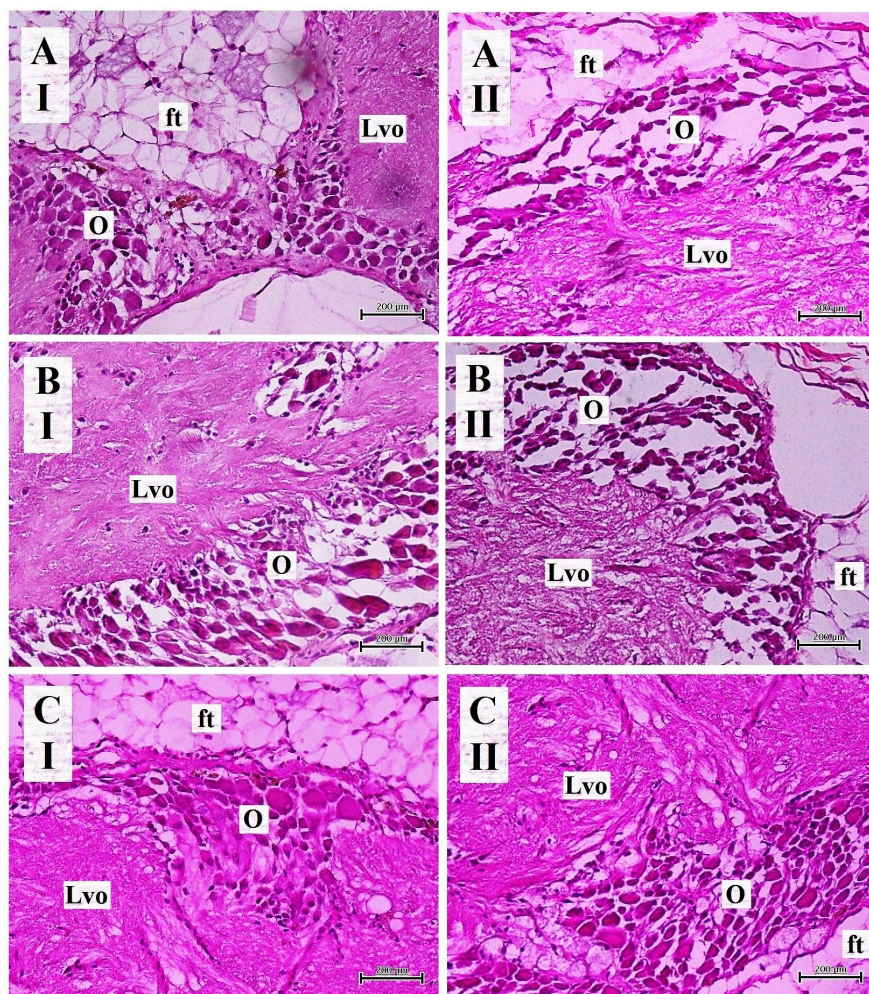


Figure 3. Reproductive cells of female of *T. aculeata*. A: Manado Bay; B: Lembeh Strait; C: Maluku Sea. I: normal female; II: imposexed female. O: oocyte; Lvo: ovarian lobe; ft: fat.

Discussion

Contamination of TBT. Concentrations of TBT in sediment samples as well as in gastropod tissues showed the highest levels of contamination at locality B (Lembeh Strait). Levels were lower at A and below detection limit at C (Table 1). Both sampling sites at locality B, Bitung Fishing Port (B1) and Bitung Harbour (B2), have high levels of shipping activities (maritime and industrial) and fisheries. TBT has been heavily used in antifouling paints on vessels of all sizes, and may still be discharged in port areas from ships with old paints leaching into the water, or from shipyards stripping off old paint layers (Anastasiou et al 2016; Batista et al 2016; Ytreberg et al 2016; Lagerström et al 2017). In addition, untreated wastewater is discharged from all urban areas into the marine environment (Lasut et al 2008). This could be an additional source of organotin compounds (Sudaryanto et al 2005). Low or undetected levels of TBT contamination have

also been found in other pristine waters of Indonesia, e.g. Gangga Island located in the northern part of MPCW where only $0.0007 \mu\text{g g}^{-1}$ dry weight (dw) was detected (Harino et al 2012).

Concentrations of TBT in sediments of Bitung Harbour (B2) appear to be highly variable, even over short time intervals. Our measurement of $0.17\text{--}0.31 \mu\text{g g}^{-1}$ wet weight (ww) is probably slightly higher than that found by Harino et al (2012), which was $0.16\text{--}0.35 \mu\text{g g}^{-1}$ dw, but lower than the $4.25 \mu\text{g g}^{-1}$ ww found by Undap et al (2013a). This indicates that TBT is still released to harbour sediments though not continuously. Similar results have been observed in international ports elsewhere, e.g. Cape Town, South Africa (Okoro et al 2016), and may indicate variable intensity in traffic in and out of port.

Compared to other localities in Indonesia, the concentrations found in the present study were higher or similar to the harbours of Jakarta, Surabaya, and Medan, which were $0.0023\text{--}0.19 \mu\text{g g}^{-1}$ dw (Sudaryanto et al 2005), in Jakarta Bay of $0.0004\text{--}0.052 \mu\text{g g}^{-1}$ dw (Harino et al 2012), and in Banten Bay of $0.012 \mu\text{g g}^{-1}$ ww. This also indicates high variability and persistence of TBT contamination of sediments.

Concentrations of TBT in snail tissues followed the same trend as in sediments, i.e. highest concentrations in snails from Lembeh Strait (B), lower at A (Manado) and below detection level at C (Maluku Sea) (Table 1). The levels found in the present study were much higher than that from the same area found by Noor et al (2013), which was $0.0078 \mu\text{g g}^{-1}$ ww. However, different species of gastropods were analysed and methodology was somewhat different. In another study from Indonesian waters, Sudaryanto et al (2005) found concentrations of $0.0023\text{--}0.0037 \mu\text{g g}^{-1}$ dw of the mussel *Perna viridis* from three harbours. TBT has been found to be toxic to gastropods at concentrations as low as $0.001\text{--}0.002 \mu\text{g L}^{-1}$ (Gibbs et al 1987).

TBT impact on imposex and reproductive organs. TBT may be taken up by gastropods via sediment, water column or ingested prey organisms. TBT apparently accumulates in the tissues over time of exposure, and the occurrence and severity of imposex is related to the degree of exposure during the entire life span of an individual. Imposex is irreversible and affected individuals will remain affected even after exposure has diminished or ceased and tissue levels become lower. Snail populations severely affected by imposex will show sex ratios skewed towards males because female mortality will be high (Gibbs et al 1987). The low proportion of males and high number of normal females at almost all sites in the present study indicate that populations may be recovering from high exposure, and that the proportion of imposex affected females will decrease in the future.

Both imposex indices were correlated with sediment and tissue concentrations of TBT, i.e. the highest incidence as well as largest female penis size occurred in Lembeh Strait (B) (Table 1 and Figure 2). However, the imposex incidence in the present study was lower than for *Thais* sp. and *Thais tuberosa* from Malaysian coastal waters (Wagiman 2004; Mohamat-Yusuff et al 2014), for *Thais gradata* from Singapore (Tan 1999), and for *Thais distinguenda* from Thailand (Bech 2002a, b) for all of which imposex incidence was up to 100% in harbour areas. However, most of these studies were carried out before the AFS convention went into force and the use of TBT was still unregulated in Southeast Asia.

The present study showed impacts of TBT at the cellular level of the gonad of imposex affected females from localities A and B, but not from C (Figure 3). Similar cell death was observed by Mohamat-Yusuff et al (2014), and these authors also found testicular tissues in imposex affected female *Thais* sp. Our histological sections did not show tissues that could be unequivocally identified as testicular or sperm-producing in the imposex affected females.

The actual mechanism of TBT induction of imposex remains incompletely understood. It has been suggested that TBT interferes with steroid hormone metabolism (Morcillo & Porte 1998; Oberdörster et al 1998), and elevated levels of free testosterone have been found in mud snails (*Ilyanassa obsoleta*) exposed to TBT in the laboratory (Gooding et al 2003).

Conclusions. The main conclusions of this study are that harbour areas of MPCW are still contaminated by TBT and that gastropods, here *T. aculeata*, are also still affected. Incidence and severity of imposex was not as high as in other harbour areas in Southeast Asia, and snail populations may be in stages of recovery. However, it will be necessary to monitor snail populations over the coming years to see if the recovery is permanent or if intermittent TBT pollution may again produce imposex affected populations.

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Authors:

Natalie D. Rumampuk, Postgraduate Program of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: detynatalie@yahoo.com

Inneke F. Rumengan, Department of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: innekerumengan@unsrat.ac.id

Rizald M. Rompas, Postgraduate Program of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: rizald.rompas@gmail.com

Suzanne L. Undap, Post-graduate Program of Aquatic Science, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: suzanneundap@unsrat.ac.id

Farnis B. Boneka, Department of Marine Sciences, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: farnisb@gmail.com

Kathe R. Jensen, Natural History Museum of Denmark, Universitetsparken 15, 2100 København Ø, Denmark, e-mail: krjensen@snm.ku.dk

Markus T. Lasut, Post-graduate Program of Aquatic Science, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jln. Kampus Bahu, Manado 95115, North Sulawesi, Indonesia, e-mail: lasut.markus@unsrat.ac.id

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