

## Bioaccumulation of tributyltin in gastropods Thalessa aculeata from Bitung waters, North Sulawesi

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**Abstract**. This study aims to describe the histopathology of the male reproductive organs of the imposex female gastropod *T. aculeata* contaminated with organotin compounds (TBT) in Bitung waters. Thirty individuals were collected from four sampling sites (Polytechnic Pier, Monitoring Port of the Ministry of Marine Affairs and Fisheries, Fisheries Port, and Bitung Port) and put into a seawater-containing bucket to keep them alive while transported to the laboratory for imposex observations and histopathological tests. The imposex symptom evaluation was estimated by relative penis length index (RPLI). This examination was done through microscopic observations of the reproductive organs of normal males, normal females, and the imposex females. Results showed the RPLI of 79.71-89.29% and caused changes in the cell structure, especially in the reproductive organs. The histopathological images of the male reproductive organs from all sampling sites showed an atrophy with shrunk seminiferous tubular cells, very low sperm production, and lysed septa.

**Key Words**: histology, imposex, lysis, reproductive organs, *T. aculeata*.

**Introduction**. Oceans are known as one of the largest, most complex, and most dynamic ecosystems in the world. The interaction of the physical, chemical, and biological factors in the waters occurs fast and continuously which determines the condition of the aquatic ecosystem. Benthos is one of the animal groups living on the bottom by attaching themselves to the substrate and marine structures or ships, and/or burying in the sediment (Noor et al 2013). Their settlement on marine structures or ships can cause functional deficiencies and accelerate corrosion on the ship keel which can increase the risk of leaks and reduce the service life of the ship.

Tributyltin (TBT) has been used as an antifouling since the mid-1960s (Goldberg 1986; Giulianelli et al 2020). The ship breaking industry has also generated a huge quantity of solid waste in the form of broken wood, rubber, insulation materials, paper, metals, glass and ceramics, plastics, leather, textiles, food waste, chemicals, paints, thermocol, sponge, ash, oil mixed sponges, organometallic contaminants, miscellaneous combustible and non-combustible hazardous toxicants (Rodríguez-Grimon et al 2020).

The investigation of Munawar & Afsar (2023) clearly indicated that the imposex individuals were present at Gadani ship breaking yards, where ship scrap arrived from all over the world to dismantle and these older ships still contain antifouling paint coatings on the ship hull composed of TBT. The TBT leaches out directly in water column and results in endocrine disruption and imposex phenomenon in marine gastropods which are normally dioecious. Since gastropods live in the subtidal and intertidal zones for a sufficiently long period at various environmental conditions, they are highly affected by physicochemical factors including pollutants entering the water.

TBT very effectively kills larvae or adult sessile organisms (Dobson & Cabridenc 1990). It is also very dangerous for aquatic organisms due to its negative impact. One of

the phenomenal and specific TBT pollutions is imposex symptom in gastropods (Evans et al 1995; Giulianelli et al 2020), namely the appearance of male sexual organ (penis and/or vas deferens) in female gastropods due to hormonal system disturbance (Bryan et al 1987; Gibbs et al 1987; Oehlmann et al 1998). On a population scale, the imposex occurrence in gastropods can result in their drastic population decline (Bryan et al 1986).

Monitoring marine pollution and toxicity of organotin compounds has received a serious attention in many developed countries in which TBT is responsible for the endocrine system disturbance in several female sea snails that experience the development of male sex organ characteristics. TBT also causes immune system disorders in the sea shells that form a shell anomaly after TBT disposals at a very low concentration (Sudaryanto 2001).

The present study was carried out in Bitung waters, especially the harbor area, as an important area to support transportation, economy, and trading. The presence of the harbor includes several activities, such as going in and out of large and small ships, ship berths and dockings (Rumampuk et al 2018). This study is aimed at describing the histopathology of the male reproductive organs in the imposex female gastropod *T. aculeata* contaminated with organotin compounds (TBT) in Bitung harbor as a TBT pollution indicator in the shipping activity areas.

**Material and Method**. This study was conducted from July to August 2023. Gastropods were collected in Bitung waters around the harbor region (Figure 1). Four sampling points were selected, namely Polytechnic Pier (B1), the Monitoring Port of the Ministry of Marine Affairs and Fisheries (B2), Fisheries port (B3), and Samudera Bitung port (B4). Thirty individuals were taken from each sampling site and put into a seawater-containing bucket to keep them alive while transported to the laboratory for imposex observations and histopathological tests. Species identification followed Dharma (1988) and Dharma (2005).



Figure 1. Sampling locations in Lembeh Strait (B1 - Polytechnic Pier, B2 - the Monitoring Port of the Ministry of Marine Affairs and Fisheries, B3 - Fisheries port, B4 - Samudera Bitung port).

**Imposex observations.** The gastropod shells were broken down using a hammer in order to observe the imposex characteristics. The soft part of the gastropod was removed from the shell, placed on a Petri dish. Due to its motility, the sample was then soaked in the mixture of 7%  $MgCl_2$  and seawater at 1:1 ratio for about 15 min for anesthesia in order to ease the observation (Fernandez et al 2002).

The imposex observations were conducted under a microscope. Female sex organs were identified from the presence of a cream or white capsule gland, dark brown or black-colored albumen gland, soft-yellow-colored sperm ingesting gland, and soft yellow-colored ovary. Male sexual organs were identified based on the presence of yellow and brownish orange-colored prostate gland (pg) and dark yellow or orange-colored testis. In males, seminalis vesicle can also be found as a white-colored winding tube (Tan 1997). The imposed female individuals were identified through the appearance of penis on the seminalis vesicle (Mohamat-Yusuff et al 2014). Penis length was measured using a 0.01 mm digital caliper. The imposex symptom was estimated by relative penis length index (RPLI) as follows:

RPLI (%) =  $\frac{\text{Mean penis size of female}}{\text{Mean penis size of male}} \times 100$ 

**Histopathological tests**. The examination was done through microscopic observations on the reproductive organs of normal males, normal females, and the imposex penis of females. These reproductive organs were fixed in 10% formaldehyde for 3 days, washed in running water, dehydrated in 70%, 80%, and 95% alcohol, cleared using xylol-alcohol, xylol I, xylol II, infiltrated with paraffin, and embedded. The paraffin block was cut at 5 µm thick, developed in a water bath at 45°C, placed on the object glass, deparaffinated, rehydrated in 95%, 80%, 70% alcohol, and stained with hematoxylin-eosin (HE). The image of histological changes of the male reproductive organ of the imposed females was compared with that of the normal male as a control individual (Horiguchi et al 2006).

**Data analysis**. Data counts were calculated to obtain the mean RPLI and the standard deviation for all ranges of the study sites.

**Results**. As many as 120 gastropod specimens (30 from each sampling site) were measured for sex and imposex ratio. The gastropods (*T. aculeata*) collected from Polytechnique Pier, the Monitoring port of the Ministry of Marine Affairs and Fisheries, Fisheries port, and Samudera Bitung port had mean penis size of 3.2-3.83 mm in normal males and 2.66-3.42 mm in imposed females, indicating that the imposed females averagely have smaller penis than the normal males. The RPLI of the gastropods ranged from 79.71 to 89.29% (Table 1) with the highest in the gastropods from the Fisheries Port.

Table 1

<i>Geographic</i> position	Sampling point	<i>Men penis size of normal male (mm)</i>	<i>Mean penis size of imposed female (mm)</i>	% RPLI	SD
01°27′27.13″ N	Polvtechnique Pier	3.2	2.66	83.13	
125°12′51.19″ E	.,				
01°27′8.19″ N	Monitoring port of	3.5	2.79	79.71	
125°12′49.60″ E	the Ministry of				
	Marine Affairs and				4 16
	Fisheries				4.10
01°26′96.08″ N	Fisheries port	3.83	3.42	89.29	
125°12′26.40″ E					
01°26′33.49″ N	Samudera Bitung	3.52	2.87	81.53	
125°11′51.33″ E	port				

Mean penis size in normal males and imposed females of *T. aculeata*. Males and females show the imposex character with the locality in Bitung waters

SD = standard deviation.

The histological examination of the reproductive organs showed that the histology of the normal male reproductive organ had the structure of seminiferous tubules (smt) with full of sperms containing round-shaped spermatids (S), headed fiber-like spermatozoa flagella, and the presence of septa as the boundary of tubule (Figure 2). The male reproductive organ in the imposed female exhibited an atrophy with frowned seminiferous tubule cells, and despite sperm presence, its production is very few, whereas septa are lysed (Figures 2A, B, and E). The male reproductive organs of the imposed females collected in the Fisheries port showed that the sperms-emptiedseminiferous tubule had no spermatids or spermatozoid flagella (Figure 2D).



Figure 2. The reproductive cells of normal males (A) and imposed females from Bitung waters: B. Polytechnique pier; C. Monitoring port of the Ministry of Marine Affairs and Fisheries; D. Fisheries port; E. Samudera Bitung port. SMT: tubulus seminiferi, S: spermatid, F: flagella spermatozoa, Sp: septa. Magnification: 20X.

## Discussion

**Imposex**. Based on the RPLI of the male reproductive organ in imposed female *T. aculeata*, the highest value reached  $89.29 \pm 4.16\%$  around the Fisheries port and the

lowest in the Monitoring port of the Ministry of Marine Affairs and Fisheries, 79.71±4.16%. The imposex symptom seems to be higher than that in the previous finding in Bitung waters (Rumampuk et al 2018) for the same species, in Fisheries port, 43.48%. Noor et al (2013) found the highest imposex of 60% in *Thais tuberosa* and 44% in *Monodonta labio* in Manado waters. The comparison between these two findings reflects that the imposex symptom in Bitung waters is increasing with higher shipping activities. Other imposex phenomena were recorded in *Stramonita haemastoma* in Pecém port, Brazil, with an RPLI of 0-28.37% (Castro et al 2005), *Thaisella chocolata* in Callao port, Peru, with an RPLI of 8.4-28.4% (Castro et al 2018), and along the Atlantic coast, Morocco, 5.07 to 66.66% (Mortaji et al 2020).

Furthermore, imposex observations in Southeast Asia in 1988 and 1989 have shown a variety of imposex conditions in several gastropod species like Thais luteostoma in Ambon Bay, Indonesia, 30-100% (Ellis & Pattisina 1990) and 49.58% in Takale ship dock, Ambon Bay (Islami et al 2019), Babylonia spirata in Tanjung Mas port, Semarang, 39.47%, and 40.27% in Delta Wulan waters, Demak, Indonesia (Nuraini et al 2020). Pandey & Evans (1996) also reported that the imposex proportion in adult gastropods Morula granulata from Manado port was 85% and in Thais sp. from Bitung port it was 75%, whereas in Dickson port, Malaysia, the highest imposex frequency (100%) occured in T. luteostoma (Wagiman 2004) and in all individuals of Thais tuberosa in Peninsular Malaysia (Mohamat-Yusuff et al 2014). These values are relatively not different from the percent imposex in several ports in Bitung waters. Nevertheless, Bech (2002) who evaluated various impacts of the cruise ship activities in Phangan Bay, Thailand, using Thais distinguenda as a TBT contamination indicator found the imposex up to 100%. According to Castro et al (2008), the TBT-induced phenomenon generally occurs in regions where the sea transportation and industrial activity rate are high, such as seaports and ship docks; low imposex frequencies occur in protected coastal areas with low transportation activity.

The present study in Bitung waters clearly indicated that the TBT-induced imposex symptom seems to be similar to that in Europe, the United States, or other countries in Southeast Asia. It is in agreement with Goldberg (1986) that in the 1970s, TBT was evident to be the most toxic substance released into the ocean. However, there are still research spaces to be done to indicate other factors that possibly cause the imposex in the gastropod population.

A body of water with high shipping activities indicates a high imposex symptom. The imposex phenomenon in the present study has proved that organotin (TBT) compounds are toxic to gastropods because they trigger the development of male reproductive organs in females which could result in reproductive failures and organism extinction. Therefore, limitation or inhibition of TBT utilization as an antifouling paint should be applied.

**Reproductive organ histology in gastropods**.TBT accumulation in living organisms causes various disturbances in reproductive systems so gastropods do not produce gonads and cannot breed. Imposex in the reproductive system is the most specific response of the gastropod to TBT contamination (Rumampuk et al 2018). The TBT can also sink into the bottom sediment. Rumampuk et al (2019) recorded the presence of TBT in the sediment as much as 0.313 ppm in Bitung waters with an RPLI below 50%, whereas Harino et al (2012) and Undap et al (2013) detected TBT concentration in the sediment as much as 160  $\mu$ g kg<sup>-1</sup> and 4.25  $\mu$ g kg<sup>-1</sup>, respectively. Rumampuk et al (2018) also found an amount of TBT of 0.163 ppm in the gastropod tissue from Bitung waters. The imposex occurrence could result from TBT absorption of the gastropod living in a soft bottom substrate.

According to Rompas et al (2016), various conditions can influence P-450 system and alter the pollutant toxicity into more or less toxic metabolites. However, the inhibition of P450 cytochrome supporting aromatase (CYP19) activity (responsible for changing androgen to estrogen) by organotin compounds could function as a trigger of imposex mechanisms. This inhibition causes an increase in androgen hormone concentration in the body (Meirelles et al 2008). P-450 cytochrome is an enzymatic system importantly functioning in xenobiotic and endogenous metabolisms, such as hormones and fatty acids. Several types of P450 cytochrome enzymes are involved in xenobiotic metabolism for sex hormone synthesis, including aromatase inhibition, namely changes in androgen and estrogen. As a result, testosterone concentration rises and the imposex development in female gastropods occurs (Morcillo & Porte 1998).

Iguchi et al (2007) described the interrelationship between P405 cytochrome activity and the failure of the testosterone change to 17  $\beta$ -estradiol. This condition makes the androgen-estrogen ratio unbalanced in the gastropod body. The increased testosterone does make the penis develop in females. According to Horiguchi (2006), reproduction-related steroid hormones, such as testosterone and 17 $\beta$ -estradiol, are physiologically important compounds in the reproductive organ formation and gonad maturation (spermatogenesis and oogenesis) in invertebrates including gastropods. Spermatogenesis of the ovary in females exposed to tributyltin or triphenyltin could become one of the reasons for the declined breeding capacity of the female *Babylonia japonica* from Japan. Failures in reproduction will ecologically reduce the reproduction rate and eventually threaten the sustainability of the gastropod population.

**Conclusions**. Gastropods *Thalessa aculeata* collected from several ports in Bitung waters have been contaminated with tributyltin. The damages to the reproductive organ cell structure in gastropods *Thalessa aculeata*, such as the absence of spermatids and spermatozoa flagella in seminiferous tubules and lysed septae, cause the organisms unable to breed. This incidence requires monitoring activities to avoid a worse marine environmental condition.

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**Conflict of interest**. The authors declare that there is no conflict of interest.

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