

# Imposex of *Thais clavigera* and *T. luteostoma* (Muricidae) as an Evidence of Organotin Pollution in Chinhae Bay

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= 국문요약 =

진해만의 유기주석 오염을 나타내는 대수리와 뿔두드럭고둥의 임포섹스

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진해만 바위해안 조간대에 서식하는 뿔소라과 2종, 대수리(*Thais clavigera*)와 뿔두드럭고둥(*T. luteostoma*), 에서 수컷의 성징이 암컷에게서 발현되는 임포섹스 현상을 조사하였다. 임포섹스는 전 조사 정점에서 100% 나타나고 있었으므로 본 조사에서 수컷의 성징을 보이지 않은 암컷은 발견할 수 없었다. 임포섹스의 강도를 나타내는 상대 성기 길이 지수(relative penis length index: RPL)는 34.7%에서 81.1%의 범위에 있었다. 특히 마산만 안쪽의 조사 정점에서는 암컷의 구성비가 크게 감소하고 어린 개체를 거의 발견할 수 없는 등 개체군이 임포섹스의 영향을 받고 있음을 알 수 있었다. 두 종의 체내에 함유된 트리부틸주석(TBT)과 트리페닐주석(TPT)의 농도를 분석한 결과 각각 0.18-1.45 µg/g, 0.42-6.30 µg/g의 범위에 있었으며, 임포섹스의 정도는 트리부틸주석과 트리페닐주석의 체내 농도와 밀접한 관계를 보였다. 대수리와 뿔두드럭고둥의 임포섹스는 우리 나라에서 유기주석 화합물의 오염을 나타내는 좋은 지표로 사용될 수 있으며, TBT의 사용 규제이후 그 효과를 감시하기 위한 용도로도 유용하게 사용될 수 있다.

## INTRODUCTION

Organotin compounds, especially tributyltin (TBT), have been widely used as antifouling additives for paints applied to the hulls of ships and boats since mid-1960s (Anderson and Dalley, 1986). The increasing use of TBT-based antifoulants, however,

raised considerable concern about the deleterious effects on non-target marine organisms. TBT was suspected of having adverse effects on the culture of Pacific oyster *Crassostrea gigas* in the late 1970s (Alzieu, 1991). Field experiments and bioassays revealed that spatfall inhibition and shell thickening in oysters can be induced at TBT concentrations of only a few part per trillion (Waldock and Thain, 1983; Alzieu *et al.*, 1986).

Another notable sublethal effect of organotin

compounds is the induction of 'imposex', a phenomenon involving the superimposition of male sex characters onto female gastropods. The expression of imposex and its cause was first investigated in the intertidal mud snail, *Nassarius obsoletus* by Smith (1980), who found strong evidence that it was induced by exposure to organotin compounds leaching from antifouling paints. Occurrence and effects of imposex have been investigated intensively in the UK in the common dog-whelk, *Nucella lapillus* (Gibbs *et al.*, 1987). Females develop a small, sometimes malformed, penis close to and behind the right tentacle. A superficial vas deferens grows between genital papilla and the penis, eventually in the most intense imposex overgrowing and occluding the papilla, thus preventing egg liberation and breeding (Gibbs *et al.*, 1987).

Studies on *N. lapillus* in England indicated that populations have declined markedly over the past 10-15 years (Bryan *et al.*, 1986). Surviving animals were subject to a high degree of 'imposex' and they contained high concentrations of hexane-extractable organic tin (Bryan *et al.*, 1987). Imposex was readily induced by exposure to 1 ng/L of TBT leached from antifouling paint in the laboratory experiments (Bryan *et al.*, 1988). It was also suggested that alteration of testosterone levels might be related to imposex (Spooneer *et al.*, 1991). But detailed mechanism of imposex induction in female marine gastropods has not yet been elucidated.

The occurrence of imposex in marine neogastropods has been considered as one of the most distinct detrimental effects of organotin compounds. Imposex phenomenon has been described in 72 species belonging to 49 genera (Oehlmann *et al.*, 1991). *Thais clavigera* and *T. luteostoma* for evidence of imposex, which might be correlated with the probable degree of exposure to organotin compounds were examined in Chinhae Bay. Since these two intertidal neogastropods are common on the rocky shores in Korea (Choi, 1992), their im-

posex has great potential as indicators of the extent of TBT contamination. This study is an attempt to report the prevailing imposex phenomenon as well as to address the evidence of organotin contamination in Chinhae Bay, Korea.

## MATERIALS AND METHODS

Two species of the family Muricidae, *Thais clavigera* and *T. luteostoma*, were collected in June, 1994 at 13 sites in Chinhae Bay, Korea (Fig. 1). Over 50 adults with the shell height of 17 - 32 mm were frozen in the field and transferred to the laboratory. Specimens enough for analysis could not be collected at St. 10 due to high-tidal area.

After extracting by breaking the shell, sex was distinguished by the appearance of a prominent female sperm-ingesting gland. In both males and females, the length of the penis from its tip to its junction with the body wall behind the right tentacle was measured to the nearest 0.1 mm under a binocular microscope (Gibbs *et al.*, 1987; Ellis and Pattisina, 1990). Relative penis length (RPL) index, the ratio of female length/male length x 100, and relative penis size (RPS) index, the ratio of female length<sup>3</sup>/male length<sup>3</sup> x 100, were used as a measure of the degree of imposex at each site (Gibbs *et al.*, 1987).

Tissues for TBT analysis were placed in pre-combusted glass bottles after the measurement of imposex and kept frozen until analysis. The analytical procedures used were based on Stallard *et al.* (1989). Wet tissues were weighed to 5.00 ± 0.01 g in the 50 ml polypropylene centrifuge tubes and homogenized by Tekmar tissumizer. Dry/wet ratios were determined separately. Ten ml of 50% HCl were added to each sample and vortexed enough to mix adequately. Samples were allowed to sit 30 minutes before adding 20 ml of glass-distilled dichloromethane (HPLC grade). Samples were mixed with reciprocating shaker for 3 hours and centrifuged at 4000 rpm for 10 minutes. Two ml aliquots

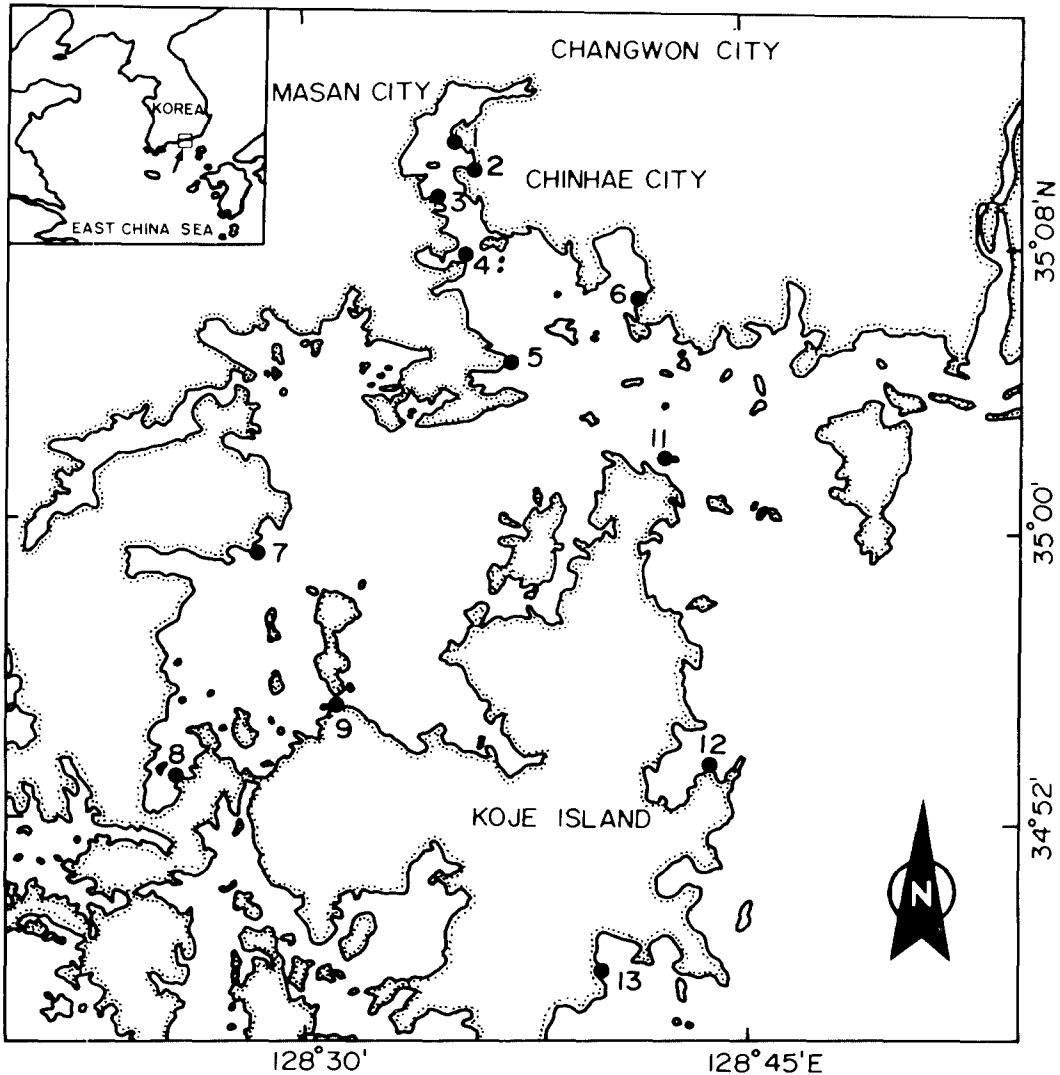


Fig. 1. Sites sampled for *Thais clavigera* and *T. luteostoma* in Chinhae Bay.

of the dichloromethane layer was removed for derivatization, and dried under nitrogen gas, leaving about 25  $\mu$ l of sample. Each extract was reconstituted in 2 ml of distilled hexane and 250  $\mu$ l of 2.0 M n-hexylmagnesium bromide was added. After 15 minutes, remaining Grignard reagent was destroyed by adding 4 ml of 0.4 N sulfuric acid. Organic layer was passed through Supelclean LC-florisil column and rinsed twice with 2 ml of hexane. Cleaned

extracts were concentrated to 200  $\mu$ l and analyzed by a gas chromatograph (Hewlett-Packard 5890 GC) equipped with a capillary column (SPB-1, 30 m x 0.25 mm i.d.) and a flame photometric detector (FPD). The filter (Dietrich Optical) had a range from 625 to 2000 nm. The column head pressure was 28 psi. Flow rates through the detector were 100  $\text{cm}^3/\text{min}$  for air and 170  $\text{cm}^3/\text{min}$  for hydrogen. Nitrogen make-up gas plus helium carrier gas had a

combined flow rate of 35 cm<sup>3</sup>/min. The GC temperature was programmed from 50°C for 1 minutes, at a rate of 30°C/min, to a final temperature of 200°C with a hold time of 8 minutes. The injector and detector temperatures were 225°C and 250°C, respectively. Triphenyltin is added just prior to florasil cleanup and therefore the recovery was calculated. Tributyltin (TBT) was analyzed for mussels and oysters, while dibutyltin (DBT), monobutyltin (MBT), triphenyltin (TPT) were additionally analyzed for gastropods.

The analytical data were confirmed, using the certified reference material (NIES CRM No. 11, sea bass, *Lateolabrax japonicus*). Determined values of TBT by this analytical procedure were within the certified value for 1.3 ± 0.1 µg TBT/g dry wt (Table 1).

## RESULTS AND DISCUSSION

The results of imposex survey and chemical analysis of the Muricidae, *T. clavigera* and *T. luteostoma*, collected from Chinhae Bay were summarized at Table 2. Imposex frequency has been expressed as the percentage of penis-bearing individuals in the female population (Gibbs *et al.*, 1987). The incidence of imposex in female from Chinhae Bay is virtually 100% at all sites; female lacking a penis could not be found in the survey.

Relative penis size (RPS) index and relative penis length (RPL) index were introduced to express the degree of imposex in terms of the relative sizes of the penis in male and female from each population. RPS and RPL values of *Thais* species ranged 4.9 - 53.4% and 34.7 - 81.1%, respectively. Bryan *et al.* (1986) demonstrated that when degrees of imposex (RPS) exceed 2% in *Nucella lapillus*, more than 80% of females were visibly affected, and at levels higher than 5% it was extremely rare to find a female lacking a penis.

In Chinhae Bay, RPS values were higher than 5% except St. 13. This result matches the finding that there was no female gastropod without a penis in this survey. TBT levels inducing imposex for *T. clavigera* were estimated to be 0.01 - 0.02 µg/g wet tissue (Horiguchi, 1994b). When changed to dry weight basis, the concentration range was about 0.03 - 0.06 µg/g dw. TBT tissue levels of Chinhae Bay gastropods exceeded this imposex induction threshold at all sites investigated.

Reference sites (St 11, Mangwoedo and St. 13, Haekumkang) in the east coast of Koje island, which were exposed directly to the clean waters of South Sea, showed relatively low intensities (RPL 52.2 and 34.7 %). But the degree of imposex was high at nearby sites, Okpo (St. 12, RPL 73.3%) and Sungpo (St. 9, RPL 90.0%), implying that large shipbuilding dockyards, Samsung and Daewoo ship-

**Table 1.** Precision and accuracy of tributyltin (TBT) and triphenyltin (TPT) analyses. The certified reference material (NIES CRM No. 11, sea bass, *Lateolabrax japonicus*) was analyzed by gas chromatograph with flame photometric detector.

Sample	No.*	TBT (µg/g dw)	TPT (µg/g dw)	Reference
NIES CRM No. 11	5	1.32 ± 0.10	7.20 ± 0.66	This study
Certified value		1.3 ± 0.1		NIES(1990)
Reference value**		6.3		NIES(1990)

\*No.: number of replicate samples, \*\* TPT value was not certified.

**Table 2.** Summary of Imposex survey and tissue organotin concentrations in *Thais clavigera* and *T. luteostoma* in Chinhae Bay

St. No.	Species	Numbers in sample		Imposex frequency	RPL (%)	RPS (%)	TBT	DBT	MBT	TPT
		♂	♀							
1	<i>T. clavigera</i>	22	4	100	81.1	53.4	0.68	0.62	0.10	2.62
1	<i>T. luteostoma</i>	16	10	100	76.7	45.1	0.60	0.44	0.13	2.44
2	<i>T. luteostoma</i>	20	9	100	80.6	52.3	0.41	0.52	0.34	0.58s
3	<i>T. clavigera</i>	11	10	100	58.8	20.4	0.30	0.29	0.10	1.34
4	<i>T. clavigera</i>	17	4	100	56.7	18.2	0.27	0.30	0.11	2.87
5	<i>T. clavigera</i>	25	9	100	72.7	38.4	0.47	0.33	0.26	1.78
6	<i>T. clavigera</i>	14	3	100	61.1	22.8	0.21	0.26	0.09	6.30
7	<i>T. luteostoma</i>	6	4	100	83.3	57.9	1.45	0.43	0.67	0.65
8	<i>T. luteostoma</i>	7	4	100	90.0	72.9	0.85	0.32	0.26	4.87
9	<i>T. clavigera</i>	9	9	100	63.3	25.4	0.57	2.75	0.63	1.73
11	<i>T. clavigera</i>	8	12	100	52.2	14.2	0.18	0.17	<0.05	0.42
12	<i>T. clavigera</i>	5	5	100	73.3	39.4	0.82	0.48	0.21	1.44
13	<i>T. clavigera</i>	22	22	100	34.7	4.9	0.19	0.07	0.15	0.87

Note. RPL: relative penis length index, RPS: relative penis size index, TBT: tributyltin, DBT: dibutyltin, MBT: monobutyltin, TPT: triphenyltin

building companies, near the sites might affect the local gastropod populations. RPL and RPS values were also high at St. 5 located in the vicinity of another shipbuilding dockyard, Hyungje shipbuilding company, and Chinhae Naval Base. It is well known that hull cleaning and painting operations are a major point source of TBT to the marine environment. A British survey of the inputs of TBT to the marine environment concluded that unregulated dry docking practices clearly result in the release of large quantities of TBT (Waldock and Thain, 1988). These results show that imposex measurements may present very specific and easily recognizable trends of TBT contamination around the TBT sources. Horiguchi (1994b) reported that the symptoms of imposex in the specimens were different, even at relatively close sites if there are local pollution sources.

The ratio of male and female gastropods has been suggested as an index of imposex intensity, because female mortality markedly increased at sites showing high imposex stage (Ellis and Pattisina, 1990). The proportion of females in *Thais* populations ranged from 50 - 60% at sites in Koje Island (St. 9, 11, 12, 13) to 15 - 38% at sites in inner Masan Bay (St. 1, 2). The numbers of females and males were nearly the same at St. 13, where the degree of imposex was the lowest. Populations having an excess of 50% females were found particularly at sites in Koje Island. Decline of female gastropods was obvious in inner Masan Bay. Site 1 had only 4 females of *T. clavigera* among 26 specimens collected. Sperm ingesting gland could not be easily discernible in some females collected from inner Masan Bay sites, while distended capsule glands were also observed in other females.

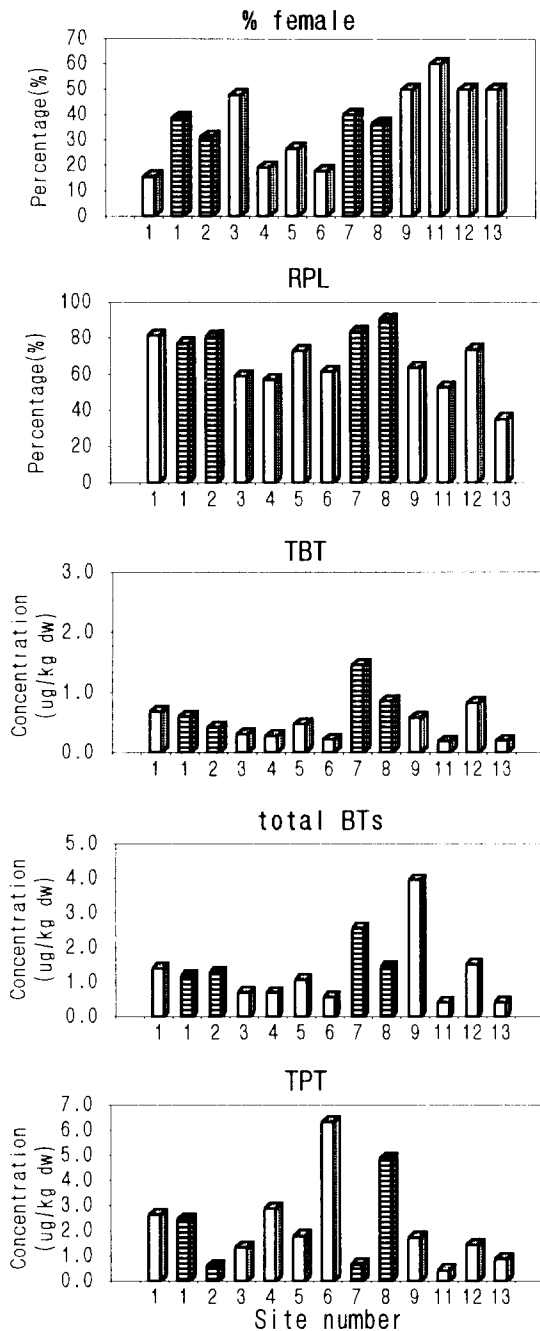


Fig. 2. Percentage females, relative female length (RPL), and tissue concentrations of tributyltin (TBT), total butyltins (BTs), and triphenyltin (TPT) in *Thais clavigera* (blank) and *T. luteostoma* (shaded) collected in Chinhae Bay.

Gibbs and Bryan (1986) demonstrated that in population having moderate to high degrees of imposex, the development of vas deferens tissue in females can occlude the oviduct, thus preventing the release of egg capsules. The build-up of unlaidd egg capsules in these sterile females may also lead to their premature death. They noted that declining populations usually contained an abnormally low percentage of females, a large proportion of which were sterile.

Although the numbers of individuals were abundant in inner Masan Bay, the populations of St. 1 and 2 consisted almost of old gastropods with no immature or juvenile specimens; most of these gastropods seemed to be at least 3 years old or more. Population decline is likely to be a slow process and remain undetected until well advanced in case of long-lived species such as *T. clavigera* and *T. luteostoma*. It suggested that there had been no recruitment for several years and *Thais* populations in inner Masan Bay might be declining.

Bryan *et al.* (1986) provided a convincing case that TBT was responsible for the decline of *N. lapillus* population in south-west England in the 1980s. There is no planktonic stage in the life history of *N. lapillus*, the young snails hatching directly from the attached egg capsules. Many populations of *N. lapillus*, which were sampled in 1980s, were characterized by small numbers, or the total absence, of juveniles (Evans *et al.*, 1991). However, proportions of juveniles have significantly increased and reproductive performances have improved since the introduction of TBT regulations in 1987 (Evans *et al.*, 1995).

The effects of imposex in *Thais* populations could not be easily forecasted in this study, because reduced recruitment might not necessarily mean the failure of reproduction. *T. clavigera* have a planktonic stage as a veliger larvae for about two months after hatching (Horiguchi, 1994b). Juveniles were found at nearby Gapo (St. 3), where tissue organotin concentrations were lower than inner bay

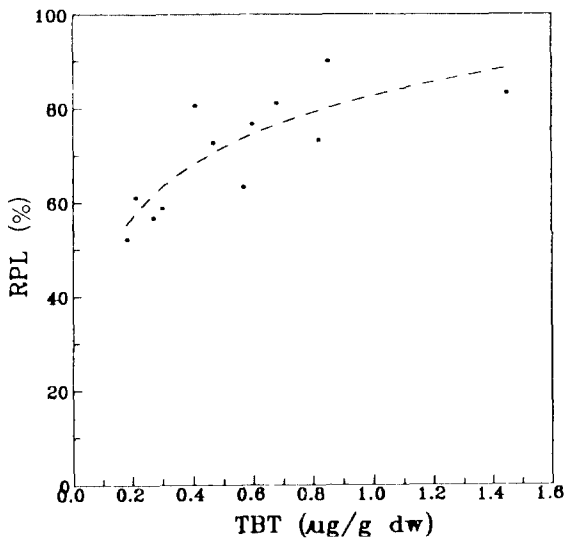


Fig. 3. Relationship between relative penis length (RPL) and TBT tissue concentrations in *Thais clavigera* and *T. luteostoma* collected in Chinhae Bay ( $r^2 = 0.80$ ,  $Y = 19.614 \times \ln(X) + 83.9471$ ).

sites (St. 1 and 2). Significant numbers of young animals were also found at some apparently affected site, St. 7. The absence of young gastropods in inner Masan Bay resulted perhaps from a failure to recruit young gastropods because juveniles might not survive due to other environmental stress such as diseases, effects of phytoplankton blooms and other pollution. In order to ascertain the successful recruitment of larvae in apparently TBT-affected sites of Masan Bay, there needs a long-term monitoring on population dynamics of *Thais* species. Further studies such as toxicity tests and transplanting experiments will confirm the effects of TBT on reproduction as well as the species specific differences in vulnerability to TBT.

Distributions of imposex in *T. clavigera* and *T. luteostoma* were closely associated with the TBT tissue concentrations in the study area. Figure 3 shows the correlations between TBT tissue concentrations and relative penis length index (RPL). The data demonstrated the high sensitivity of *T.*

*clavigera* and *T. luteostoma* to TBT in that the intensity of imposex increases rapidly at low concentrations in tissues.

TBT concentrations in gastropod tissues showed progressive decrease from inner to outer bay, representing the existence of major input source in Masan Bay. TBT input source may include various input originated from vessels in harbor as well as from sewage and wastewater discharged from Masan and Changwon City. Untreated wastewater is likely to contain various organotin compounds including DBT and TPT (Fent and Müller, 1991). Although DBT is mainly regarded as degradation product of TBT, DBT has been widely used as a heat stabilizer (Thompson *et al.*, 1985). Elevated DBT levels may be related with land based input sources. The increase in the TBT tissue levels at Wonmoon (St. 7) and Tongyoung (St. 8) may be due to small fishing boats or mariculture equipments. High TBT levels at the western parts of Chinhae Bay was also reported in mussels, oysters and sediment (Kahng, 1995; Shim, 1996).

The concentrations of triphenyltin (TPT) in gastropod samples ranged from 0.42 to 6.30 µg/g. The spatial variation of TPT concentrations was different from that of TBT. High TPT concentrations were detected in the tissues collected from St. 6 and St. 8. TPT has also been used as an antifouling agent in ship hulls, fishing nets and mariculture equipments. Furthermore, agricultural biocide use of TPT is regarded as an important source (Stab *et al.*, 1994). Recently it was reported that phenyltin compounds such as triphenyltin and monophenyltin could also induce imposex (Hawkins and Hutchinson, 1990; Horiguchi, 1994a), although deleterious effects of phenyltin compounds were not confirmed extensively (Fent and Hunn, 1991). As fate and effects of TPT in marine environment are fairly unknown, there is no evidence how much TPT increased imposex intensity of *Thais* species in Chinhae Bay.

This study indicates the widespread organotin

contamination in Chinhae Bay, implying that there needs regulations on the use of TBT for antifouling agent in Korea. Reduction of TBT levels in coastal waters has been already reported in most countries where regulations have been in force for several years (Cleary, 1991; Valkirs *et al.*, 1991; Waite *et al.*, 1991; Evans *et al.*, 1995). Imposex measurement in *Thais* species might be an excellent biomarker of exposure and effect of TBT contamination as well as a screening tool for monitoring the effectiveness of TBT regulations. It was estimated that the imposex of *T. clavigera* could be readily induced by exposure to extremely low concentrations as 1 ng/L (Horiguchi, 1994a). As the detection limit of seawater analysis of TBT by GC FPD is about 1 ng/L, imposex measurement is supposed to be more sensitive indicator of TBT pollution. Furthermore, *Thais* species will be very useful, because they are so widespread in Korean coasts, relatively large and visible, and can be easily sexed with a distinctive sperm-ingesting gland.

#### SUMMARY

A survey on intertidal neogastropods *Thais clavigera* and *T. luteostoma* in Chinhae Bay has revealed widespread imposex, the imposition of male characters on female snails. The incidence of imposex in female is virtually 100% at all sites; female lacking a penis could not be found in the survey. Relative penis length (RPL) index values of *Thais* species ranged 34.7 to 81.1%. The percentages of females declined significantly and populations consisted almost of old animals with no immature or juvenile specimens at the sites located in inner Masan Bay. The concentrations of TBT and TPT in gastropod samples ranged 0.18-1.45 µg/g, 0.42-6.30 µg/g, respectively. Distributions of imposex in *T. clavigera* and *T. luteostoma* were closely associated with the TBT and TPT tissue concentrations in the study area. Imposex measurement in *Thais clavigera* and *T. luteostoma* will be an

excellent biomarker of exposure and effect of organotin contamination as well as a screening tool for monitoring the effectiveness of TBT regulations after legislative controls.

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