Toshihiro Horiguchi *Editor*

Biological Effects by Organotins

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Preface

Organotin compounds are known as methyl-, ethyl-, propyl-, butyl-, pentyl-, hexyl-, octyl-, phenyl-tin compounds, and so on. Except for methyltins, organotin compounds are artificially synthesized chemical substances. Among these organotin compounds, especially tri-organotins, such as trimethyltin (TMT), triethyltin (TET), and tributyltin (TBT), have strong toxicities to various kinds of organisms, both vertebrates and invertebrates. That is why tri-organotin compounds, such as TBT and triphenyltin (TPhT), have been used as biocides, for example, as agricultural chemicals and boosters in antifouling paints.

Although TBT and TPhT are known to be persistent, accumulative, and toxic chemicals, their use in antifouling paints for ships and fishing nets had rapidly increased worldwide since the mid-1960s, due to their low expense and long-term continuing strong efficiency to prevent sessile organisms (i.e., barnacles and mussels) from adhering to ship hulls and fishing nets. The spread of using organotins in antifouling paints worldwide resulted in extensive marine and freshwater pollutions by TBT and TPhT all over the world, which indicated to occur contamination in fish and shellfish as food for humans and also adverse effects to aquatic organisms of ecological significance. Imposex phenomenon is one of typical adverse effects by TBT and TPhT in gastropod mollusks.

Legislation on the use of organotin compounds, such as TBT, in antifouling paints has started in European countries (i.e., France and the UK) and the USA since the 1980s, but it was not total/entire but partial legislation, because only ships and boats smaller than 25 m in length were prohibited to use organotins in antifouling paints. Although it was necessary to establish a new treaty for the worldwide total ban of organotin compounds used in antifouling paints, it took a lot of time for the new international treaty. Finally, the International Maritime Organization (IMO) decided to phase out TBT in antifouling paints over the period from 2003 to 2008, at its assembly in November 1999. An International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention: 21 Articles) was then adopted by the IMO on 5 October 2001. However, it finally came into force on

17 September 2008, because it had taken more time than expected for the AFS Convention to be ratified by member states.

On the other hand, scientific researches on gastropod imposex as well as contamination by organotin compounds in the aquatic environment have been continued more than 40 years. Several books, which focus on organotins and their adverse effects to organisms, have been already issued.

This book provides an overview of the induction mechanism of imposex caused by organotin compounds in gastropods, as well as fundamental information on the physiology and biochemistry of reproduction in mollusks. There have been several questions about basic biology of gastropod mollusks: Are the sex hormones of gastropod mollusks vertebrate-type steroids or neuropeptides? What about lipid disturbance and membrane toxicity due to organotin compounds? The book also discusses the latest findings on the role of nuclear receptors, such as retinoid X receptor (RXR), retinoic acid receptor (RAR), and peroxisome proliferator-activated receptor (PPAR), in the development of imposex in gastropods.

Further, it describes the current state of contamination by organotins in the marine environment and gastropod imposex, especially focusing on Europe and Asia, introduces readers to analytical techniques for organotin compounds, and assesses the contamination and adverse effects of alternatives to organotin-based antifouling paints.

Imposex, a superimposition of male genital tracts, such as the penis and vas deferens, on female gastropod mollusks, is known as a typical phenomenon or consequence of endocrine disruption in wildlife. Imposex is typically induced by very low concentrations of organotin compounds, such as TBT and TPhT from antifouling paints on ships and fishing nets. Reproductive failure may be brought about in severely affected stages of imposex, resulting in population decline and/or mass extinction. Thus, gastropod imposex has been recognized as a critical environmental pollution issue. Although gastropod imposex is also highly interesting for the biological sciences because of its acquired pseudohermaphroditism and/or sex change by certain chemicals, such as TBT and TPhT, the mechanism that induces the development of imposex remains unclear, possibly due to our limited understanding of the endocrinology of gastropod mollusks. This book offers a useful guide for professionals and students interested in the fields of aquatic biology, invertebrate physiology, ecotoxicology, and environmental science.

We strongly hope that this book will contribute to both the ultimate solution of issues on environmental pollution by organotin compounds and development of scientific researches on basic biology (i.e., reproductive physiology and endocrinology) of gastropod mollusks.

On behalf of all authors
Toshihiro Horiguchi

Tsukuba, Japan
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Contents

Part I
Analytical Techniques for Trace Levels of Organotin Compounds and Contamination by Organotin and Alternative Antifouling Paints in the Marine Environment

Chapter 1
Analytical Techniques for Trace Levels of Organotin Compounds in the Marine Environment

Babu Rajendran Ramaswamy

Abstract Organotins still remain a major concern for the safety of the marine environment, and their determination is covered under legislation in quite a number of nations. Because their usage is totally banned, the demand for determining organotins at sub-nanogram concentrations is ever increasing, which is achieved by elimination of matrix interferences, reduction of sample volume, and analyte enrichment. Organotin speciation is a complex technique involving a long and laborious sample treatment procedure that is prone to various uncertainties. To overcome the shortfalls in extraction and pre-treatment, newer microextraction techniques were developed with reduction in sample and solvent volume, extraction time, and enrichment procedures. Moreover, the recent techniques are developed with a major focus on green analytical chemistry to reduce the impact of anthropogenic (laboratory) activities on the environment. Decreasing the detection limit of methods without greatly compromising their sensitivity was a profound topic of environmental research for organotin analysis. In the case of analytical technique, from the late 1970s, the usage of titrometric and spectrophotometric methods were substituted with more sensitive and lower-cost detectors at nanogram level. Furthermore, detection at femtogram levels was achieved by a mass spectrometer coupled to either gas chromatography (GC) or liquid chromatography (LC) systems. One of the significant developments in instrumentation is the application of the isotope dilution technique to detect the transformation/degradation of organotin species during extraction analysis steps. This chapter discusses the methods available for measuring organotins and their metabolites in seawater, sediment, and biota such as fish and oysters and compares the performance of the various analytical methods available.

Keywords Organotin speciation • Analytical methods • Gas chromatography • Liquid chromatography • Mass spectrometry • Seawater • Sediment • Biota

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1.1 Introduction

Since the 1940s–1950s, industrial development in the United States and Europe paved the way for the widespread use of organotins (OTs) use in the plastic industry as additives (for thermal and light stability) and catalysts (polyurethane foams and silicones) (Fent 1996a). Later, in the 1960s, inclusion of organotins in antifouling paints for ships and offshore structures brought about its rampant usage (Oliveira and Santelli 2010). In addition, organotin compounds were used in disinfectants, preservatives, and pesticides (algicides, fungicides, bactericides) (Hoch 2001).

In the past 50 years we have seen tremendous usage of organotin compounds in various sectors of industry. Especially, paints used in ship hull coatings to prevent fouling organisms contributed mainly to their occurrence in the marine environment (Dubalska et al. 2013). Even though the usage of antifoulants has been banned globally, their persistence (>30 years) and toxic behaviour still pose a threat to the marine environment (Costa et al. 2013). A wide range of adverse effects of organotins on aquatic biota are well documented even at concentration of a few nanograms per liter (ng/l) in water (Fent 1996b; Binato et al. 1998), especially larval mortality, reduced growth, imposex, etc. in gastropods (Rainbow 1995; Arambarri et al. 2003; Neal et al. 2011).

The occurrence of Sn (IV) is predominant over the Sn (II) form in the environment, and its hydrophobicity is governed by the degree of alkylation/arylation of R groups (and its chain length) attached to the central tin atom. In the aquatic environment they may exist as cationic compounds (tributyltin, TBT^{+}; dibutyltin, DBT^{2+}; monobutyltin, MBT^{3+}) or neutral compounds (TBTOH, DBTOH, MBTOH, etc.) (Oliveira and Santelli 2010). Apart from their antifouling applications, they elicited considerable toxicity in wild organisms and showed sufficient persistence to be transferred along the trophic food chain (Oliveira and Santelli 2010). Pertaining to the effects, regulatory actions were taken to ban or restrict the usage of organotins, and one such was the total ban imposed by the International Maritime Organization since 2008. However, because of their persistence, possible effects of organotins on gastropods are still observed (Costa et al. 2013).

In pursuit of the foregoing, monitoring of organotins is always an important perspective in matrices such as seawater, sediment, and biota (fish, gastropods, plants, etc.). Pertaining to the need of environmental monitoring, determination of organotins is considered a prerequisite for understanding the fate of organotins at this post-ban era. Although many analytical methods have been developed, in particular techniques based on gas chromatography are widely applied with coupled instruments for its versatility, whereas liquid chromatography also seems to be equally good and an alternatively used technique. In this review, analytical methods are compared in such terms as limit of detection, simplicity, and environmental application.

1.2 Sampling and Extraction of Organotins

Sampling is the preliminary step in chemical speciation, which requires extreme caution because of their existence at lower concentration (nanogram levels in water, sediment, biota). In water, the organotin concentrations are influenced by many factors, including organic matter (dissolved and suspended), temperature, salinity, pH, and solubility (Oliveira and Santelli 2010). The distribution of organotins in sediment varies with respect to the composition (i.e., organic matter, humic substances, etc.) of the sediment (Pinochet et al. 2009).

Generally, environmental samples require intensive processing (i.e., changing their physical and chemical nature) for identification or quantification of organotins, and extreme care is warranted to avoid loss or transformation of organotins and to reduce artefacts. Various extraction techniques exist to isolate and concentrate the target analytes from environmental matrices. The most preferred are liquid–liquid extraction (LLE), solid-phase extraction (SPE), soxhlet extraction, supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), and microextractions (Abalos et al. 1997; Oliveira and Santelli 2010; Rutkowska et al. 2014) Furthermore, extracts need to be derivatized for gas chromatography (GC) analysis. The sequential procedure for determination of organotins is shown in Fig. 1.1.

To determine the efficacy of the analytical method (recovery %), certified reference materials (CRMs) including PACS-1 (harbour sediment), CRM-462 (coastal sediment), CRM-77 (mussels), and NIES-11 (sea bass) are used. In the absence of CRMs, spiking the known amount of analytes before extraction is practiced to determine the recovery percentage (Oliveira and Santelli 2010). Biological samples are spiked in wet condition (dry biota is seldom spiked), whereas sediment samples are spiked in dry condition. The limit of detection (LOD) and limit of quantification (LOQ) of the method are based on both the extraction technique and the instrument used.

1.3 Analytical Methods for Organotins Analysis

The determination of organotin compounds requires instruments with selectivity and sufficient sensitivity. In earlier times (1950s), the determination of tin was performed by photometric or titrimetric methods. In subsequent years, spectrophotometric methods based on reagents (cacotheline, dithiol, hematoxylin, etc.) were used to analyse tins (trialkyltin, dialkyltin, diaryltin). Inorganic tin was also determined by the spectrofluorimetric method using 3-hydroxyflavone as a complexing agent. All these methods relied on the conversion of the organotins into inorganic form through digestion with mineral acid and ignition. However, they suffered by sensitivity with LOD in the ppm range (0.02–10 mg/l) and also poor selectivity (Attar 1996). Even electrochemical techniques developed during the 1970s, based

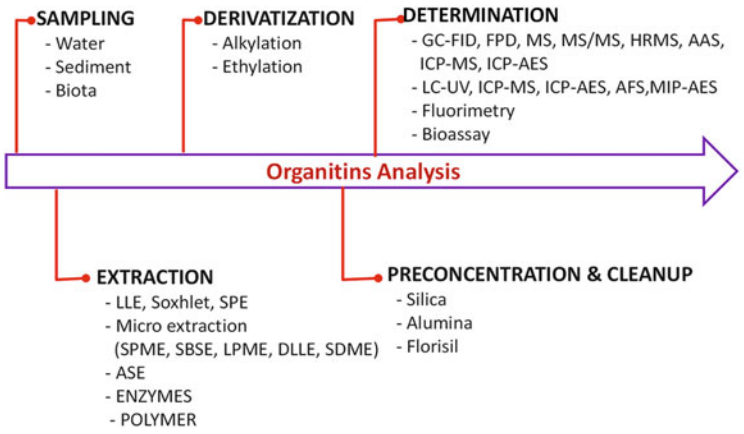

Fig. 1.1 Stages of organotin analysis based on available techniques

on measuring redox potentials of organotins, suffered severely from sample matrix (Nwata 1994).

In the late 1970s, the robust gas chromatograph (GC) or liquid chromatograph (LC) coupled to element-specific detection systems were used to increase sensitivity and performance. Moreover, GC constitutes about 64 % of the determined organotin methods, and capillary electrophoresis (CE) and LC detection account for 13 % (Abalos et al. 1997). The GC detection techniques such as flame ionization detection (FID) and electron-capture detection (ECD) were not useful for determinations at nanogram levels; therefore, they were seldom used after the 1990s (Abalos et al. 1997). Fom the late 1970s on, more sensitive and specific detectors capable of detecting micro- to nanogram levels were employed, including the atomic emission detector (AED), flame photometric detector (FPD), and pulsed flame photometric detector (PFPD). In the past two decades, detection at femtogram levels was achieved by mass spectrometry [MS, MS/MS, inductively coupled plasma (ICP)-MS, high-resolution mass spectrometry (HRMS)] techniques coupled to either GC or LC systems.

1.3.1 Analysis of Organotins in Seawater

1.3.1.1 GC-Based Analysis

Gas chromatography is a highly preferred technique for organotin analysis in seawater. The well-known method for extraction of organotins was LLE. Generally, LLE uses a complexing agent (tropolone or sodium diethyldithiocarbamate) along with volatile organic solvent(s) for effective extraction. Before analysis, extracts need to be derivatized (during/after extraction) to improve the volatilization of the organotins. Alkylation (using Grignard reagents) and ethylation (using $NaBEt_4$)

were widely used derivatization methods, whereas generation of hydrides by $NaBH_4$ is especially performed for GC-atomic absorption spectroscopy (AAS). The ethylation method with single-step extraction to produce alkyl borates showed more reproducibility than alkylation (Takeuchi et al. 2000).

Atomic spectroscopy was one of the earlier techniques used to quantify tins; however, it lacks specificity to distinguish different organotins. Therefore, a hyphenated technique was developed consisting of GC-AAS and GC-atomic emission detector (AED) for the purpose of separation as well as for detection. Dirkx et al. (1992) developed a method based on citric acid extraction and pentylmagnesium bromide derivatization for organotin analysis in GC-AAS. A packed glass column (1.8 m × 2 mm i.d., packed with 3 % OV-101 on Chromosorb WHP; 100–120 mesh) was used for the separation, and the relative LOD obtained using GC-AAS was in the range of 4–10 ng/l. Later, Narasaki and Wang (1998) reported an LOD one to two orders of magnitude lower (0.1 ng/l) than the earlier study by using a capillary column (DB-1 fused silica Megabore; 30 m × 0.53 mm i.d., 1.5 μm thick). Further, the capillary column provided sharp peaks and was devoid of ghost peaks.

Ceulemans et al. (1993) analyzed butyl- and phenyltin compounds in harbour water using GC-microwave-induced plasma (MIP)-AED with detection limit of 0.1 ng/l (as Sn) obtained through injecting 25 μl of sample with programmed temperature vaporization (PTV) technique. This method also facilitated online preconcentration of analytes before separation in GC. When considering the derivatizing agents for AED, sodium tetra (*n*-propyl) borate was found to be better than sodium tetraethylborate because of its capacity to derivatize butyl-, phenyl-, and ethyltins with an acceptable LOD of 3–12 ng/l (Schubert et al. 2000). Apart from conventional techniques, Folsvik et al. (2000) validated a semipermeable membrane device (SPMD), a passive sampling technique, for monitoring butyltins in seawater using AED with lower LOD (0.5 ng/l) than other methods already described (Table 1.1). The SPMD offers time-bound onsite water sampling with reduced contaminants/interferences. Although it is a solvent-free extraction procedure using triolein, direct application in seawater depends on various parameters such as rate of uptake, water temperature, etc., which still need validation (Folsvik et al. 2000). Campillo et al. (2004) used the purge-and-trap extraction technique for determining dimethyltin (DMT), trimethyltin (TMT), MBT, DBT, TBT, and monophenyl tin (MPhT) in southeast Spain using AED. The method was performed in short duration (22 min for the whole procedure) and showed comparably lower LOD (11–50 ng/l) (Table 1.1). Beside its performance, GC-MIP-AED is not widely preferred because of its high cost and maintenance. Other than AAS and AED, the atomic fluorescence spectrometer (AFS) can also be coupled to GC for organotin analysis; however, the technique is least considered because of its elevated detection limit (100–10,000 ng/l), as reported by Shi and Jiang (2011) (Table 1.1).

Among the common GC detectors, GC-FID showed poor performance with LOD of 900–1200 ng/l (Millan and Pawliszyn 2000). Cukrowska et al. (2004) also reported higher LOD (500–1500 ng/l) for MBT and TPhT analysis in seawater samples. Therefore, FPD was used in most of the studies in quantifying organotins

Table 1.1 Extraction and detection techniques frequently used for organotin analysis in water samples

Volume (ml)	Extraction	Derivatization	Instrument	LOD (ng/l)			References
				MBT	DBT	TBT	
10	Purge and trap	$NaBEt_4$	GC-MIP-AED	16	18	11	Campillo et al. (2004)
4 weeks[a]	SPMD	$NaBEt_4$	GC-AED	0.5	0.5	0.5	Folsvik et al. (2000)
50	SPME	KBH_4	GC-AFS	10,000	200	100	Shi and Jiang (2011)
18	HS-SPME	$NaBEt_4$	GC-FID	1000	1200	900	Millan and Pawliszyn (2000)
5	DLL-ME	$NaBEt_4$	GC-FPD	0.5	0.3	0.2	Birjandi et al. (2008)
450	LLE	$NaBEt_4$	GC-FPD	0.3–1.3			Tang and Wang (2009)
100	SPME	$NaBEt_4$	GC-FPD	0.031	0.007	0.006	Aguerre et al. (2000)
500	SPE	$C_5H_{11}MgBr$	GC-MS (LP)	9.6	1.5	3	Vidal et al. (2003)
500	LLE	$NaBEt_4$	GC-MS	2.2	2.9	3.7	Radke et al. (2013)
12.7	HS-SPME	$NaBEt_4$	GC-MS	–	–	0.025	Segovia-Martinez et al. (2010)
–	HS-SDME	$NaBEt_4$	GC-ICP-MS	1.4	1.8	0.8	Xiao et al. (2008)
1000	LLE	$NaBEt_4$	PTV-GC-ICP-MS	0.00005	0.00017	0.0000038	Tao et al. (1999)
250	LLE	$NaBEt_4$	GC-HRMS	0.0024	0.0018	0.0024	Ikonomou et al. (2002)
–	SPE	–	HPLC	–	–	4500	Compano et al. (1995)
250	SPE-C18	–	HPLC	–	1.5	30	Gonzalez and Ortuno (2002)
5	LLE	–	LC-APCI-MS	–	–	80	Bekri et al. (2006)
50	Ultrasonication	–	LC-MS/MS	–	–	50	Zhu et al. (2013a)
450	SPE	–	CE-TOF-MS	–	1800	3200	Malik et al. (2013)

[a]Exposure period: – not applicable/not performed

from seawater (Table 1.1). FPD is based on detecting the emission of tin species in a hydrogen-rich flame (610 nm). Generally, FPD showed superior sensitivity, selectivity, and reproducibility than ECD for trialkyl (butyl) and aryltins (phenyl) (Tolosa et al. 1991). Tolosa et al. (1991) employed simple LLE with methylmagnesium chloride as the derivatizing agent for the extraction and further analysis in the filter-less FPD provided relative detection limits of 0.5–6.5 ng/l for butyl and phenyltins with recovery >80 %. As a result of rapid developments in extraction methods, SPE and microextraction (e.g., SPME) techniques are often employed in organotin analysis. Especially, in solid-phase microextraction (SPME), reduction in matrix interferences was experienced with reduced sample volume (up to 5 ml). Based on SPME technique with optimal operating conditions (adsorption time, 60 min; sample volume, 100 ml; nature of the fibre, PDMS; injection temperature, 250 °C; and desorption time, 1 min), Aguerre et al. (2000) investigated butyl (MBT, DBT, TBT) and phenyl (MPhT, DPhT and TPhT) tins in seawater from Mimizan (Atlantic Coast), France, and reported the levels at 0.9–14 ng/l and LOD of 0.006–0.583 ng/l, which is 10–100 times lower than LLE and SFE. However, the usage of LLE is quite significant, as it has been widely used until the last decade. For instance, LLE (glacial acetic acid and potassium hydroxide–isooctane) used by Tang and Wang (2009) with GC-FPD for butyl and phenyltin estimation in waters of Luermen Stream Estuary, Taiwan (<0.4–96 ng/l), showed comparable LOD of 0.3–1.3 and 0.4–2.1 ng/l, respectively, with good recovery (72–119 %) (Table 1.1).

Because of the interference and sensitivity issues of conventional detectors, the mass selective detector (MSD) is widely adopted because of its selective ion monitoring analysis with higher sensitivity. Stab et al. (1993) inferred that LOD achieved by GC-MS [sub-picogram (-pg) level] was about 10 times lower than GC-FID. Using a gas chromatograph–inductively coupled plasma mass spectrometer (GC-ICP-MS), Smaele et al. (1996) reported butyltins (mo-, di-, tri-) in Belgian harbours with TBT as low as 1.65 ng/l with the instrument LOD of 15–35 fg/l, which is considerably lower than the GC-MIP-AED (18 pg/l) and GC-AAS (100 pg/l) techniques available at that time. Tao et al. (1999) used PTV in GC-ICP-MS to analyse organotins (butyl-, propyl-, pentyl-, phenyl-) in seawater at ultra-trace levels. Together with PTV (large volume injection up to 100 μl), the operation of a shield torch at normal plasma conditions in ICP-MS enabled them to reach the absolute instrumental detection limit at sub-femtogram level (0.7–1.8 fg as Sn), which is about 100 fold higher than other reported detection limits based on GC-ICP-MS, GC-MIP (microwave-induced plasma)-AES, and LC-ICP-MS. Based on the developed technique, they quantified organotins in open ocean water samples from Seto Inland Sea, Japan, at the level of 1–100 pg/l.

Following its rapid development and wide application, SPE was adopted for chemical speciation. Vidal et al. (2003) adopted SPE for analyzing butyl, phenyl, and pentyl tins in the Mediterranean Sea and obtained low LOD (0.1 and 9.6 ng/l) with good recovery (97–109 %) compared to the LLE method. According to Vidal et al. (2003), the use of GC-MS (low pressure-LP) allows large-volume injection and reduces the analysis time by a factor of two. Subsequently, the application of

the microextraction (SPME) technique also resulted in lower detection limit (<1 ng/l) with GC-MS. Segovia-Martinez et al. (2010) employed the extraction and analysis by headspace (HS)-SPME and GC-MS, respectively, for organotin analysis in seawater and found that the method detection limit (1 ng/l for TET; 0.025 ng/l for TBT and DPhT; 0.5 ng/l for TPhT) was at least 100 times lower than with GC-FID. Furthermore, the study facilitated DPhT determination at the sub-nanogram level (0.14 ng/l) in the Comunidad Valenciana coast of Spain. Because of the better performance of LLE, combined GC-MS is still used in organotin monitoring. For example, Radke (2013) investigated nine organotins consisting of butyltins (MBT, DBT, TBT), phenyltins (MPhT, TPhT, DPhT), ocytltins (MOT, DOT), and tricyclohexyltin (TChT) in Gdynia Harbor, Poland, using LLE-GC-MS and found higher levels of butyltin (3.2–60.7 ng/l).

Single-quadrupole GC-MS often lack sensitivity from interference in the low molecular mass region where most of the organotin diagnostic ions appear. Therefore, the triple-quadrupole technique was used to overcome the problem. Beceiro et al. (2009) used HS-SPME to compare the performance of GC/MS and GC/MS/MS. As expected, GC-MS/MS showed one order of magnitude lower LOD (MBT, 4 ng/l; TBT, 9 ng/l) than GC-MS (MBT, 28 ng/l; TBT, 27 ng/l). In addition to MS/MS quantification, high-resolution mass spectrometry (HRMS) was used for organotin analysis in water for the first time by Ikonomou et al. (2002). The resolution achieved was very high (up to 10,000 atomic mass units) by narrow mass channels, and the LOD achieved was among the lowest (0.0018–0.0024 ng/l) for organotins (butyl, phenyl, cyclohexyl) (Table 1.1). Nevertheless, HRMS analysis has not been widely exploited for environmental samples because of its higher operational cost.

1.3.1.2 LC-Based Analysis

In addition to GC, LC-based quantification methods are used. As suggested by Gonzalez et al. (2003), fluorimetry, MS, and ICP-MS are techniques well suited for organotin analysis in the environment. One of the prime advantages of the LC methods was that the samples do not require derivatization and extra cleanup, which ultimately reduces the uncertainty in results. However, the methods are limited by the poorer resolution and inefficient sensitivity of most of the detectors (UV-VIS, fluorescence, etc.). Mostly ion-exchange and reverse-phase columns are used for organotin determination by high-performance liquid chromatography (HPLC). The reverse-phase technique was first successfully applied by Kadokami et al. (1988) for organotins (methyl, butyl, phenyl) from Dokai Bay of Japan (TBT, 0.08 μg/l) based on LC-AAS with LOD of 30 ng/l as Sn; however, Compano et al. (1995) determined TBT and TPhT in harbour waters from Spain with LOD of 4500 ng/l and 150 ng/l, respectively, using SPE (C18; elution with methanol) and HPLC analysis in an ion-exchange column with an injection volume of 200 μl. Furthermore, Compano et al. (1995) used fisetin as the complexing agent for detection of organotins using the fluorescent technique. Later, the use of online

SPE-HPLC-fluorimetry by Gonzalez and Ortuno (2002) showed lower LOD for DPhT (0.5 ng/l), DBT (1.5 ng/l), TPhT (1 ng/l), and TBT (30 ng/l) than offline SPE.

Later, the sensitivity of LC detection was increased by combining with MSD, and the matrix effect was also considerably reduced. Using LC-MS, Bekri et al. (2006) obtained a detection limit of 80 ng/l and 100 ng/l for TBT and 4-hydroxybutyldibutyltin, respectively, for a small volume of sample (5 ml) using LLE. Moreover, the performance of LC-ICP-MS was far better than with LC-MS. Inoue et al. (1995) used the micellar mobile phase in LC-ICP-MS equipped with glass concentric nebulizer and tapered ICP torch for analyzing DMT, TMT, DBT, TBT, DPhT, and TPhT and obtained the LOD of 51, 35, 27, 29, 27, and 24 pg as Sn, respectively. Also, the method greatly reduced the matrix interference by using tris (hydroxymethyl)aminomethane dodecylsulfate (micellar type) instead of the sodium dodecyl sulfate mobile phase (clog at sampling orifice and ICP torch). Furthermore, the study demonstrated that the LOD achieved by the glass concentric nebulizer was quite lower than the common nebulizers such as cross-flow and Meinhard. Advance extraction methods are incorporated in LC techniques for lowering the LOD. Recently, Camino et al. (2012) validated stir-bar sorptive extraction (SBSE) for TBT analysis in seawater using LC-MS and achieved low LOD (0.8 ng/l) and LOQ (2.5 ng/l) with good recovery (92–102 %).

Apart from the GC and LC techniques, CE is rarely used. Malik et al. (2013) coupled CE with TOF-MS for the first time and attained an LOQ of 1800 ng/l and 3100 ng/l for DBT and TBT, respectively, with an acceptable recovery range of 67.2–81 % (Table 1.1). Based on various analytical methods listed in Fig. 1.2a, it is obvious that most of the GC-based methods are capable of quantifying TBT (as well as other organotins) at a sub-nanogram level compared to LC methods. Further, most of the GC-based methods can detect concentrations below the TBT guidelines of the UK Environmental Quality Target (EQT) (2 ng TBT/l), the US EPA chronic criterion (7 ng TBT/l), and the Dutch government environmental quality limit (11 ng TBT/l) (Kim et al. 2014).

Other than instrument-based analytical techniques, Kabiersch et al. (2013) reported a bioassay (bioluminescence yeast assay) for organotin analysis in sediment using *Saccharomyces cerevisiae*, BMAEREluc/hRXR-ER. The DNA-binding domain of the human retinoid X receptor (RXR) was embedded in the yeast genome, which is very sensitive towards TBT at nanomolar concentration. Although this assay cannot distinguish organotin species, it seems to be a useful tool for initial screening of the environmental matrix for organotins. This inexpensive small-scale high-throughput analysis requires only a day and is highly suitable for mobile laboratories and in situ analysis.

1.3.2 Analysis of Organotins in Sediment

Sediment is the repository of environmental contaminants and acts as a sink. Moreover, sediment concentration can reveal the temporal status of the organotins

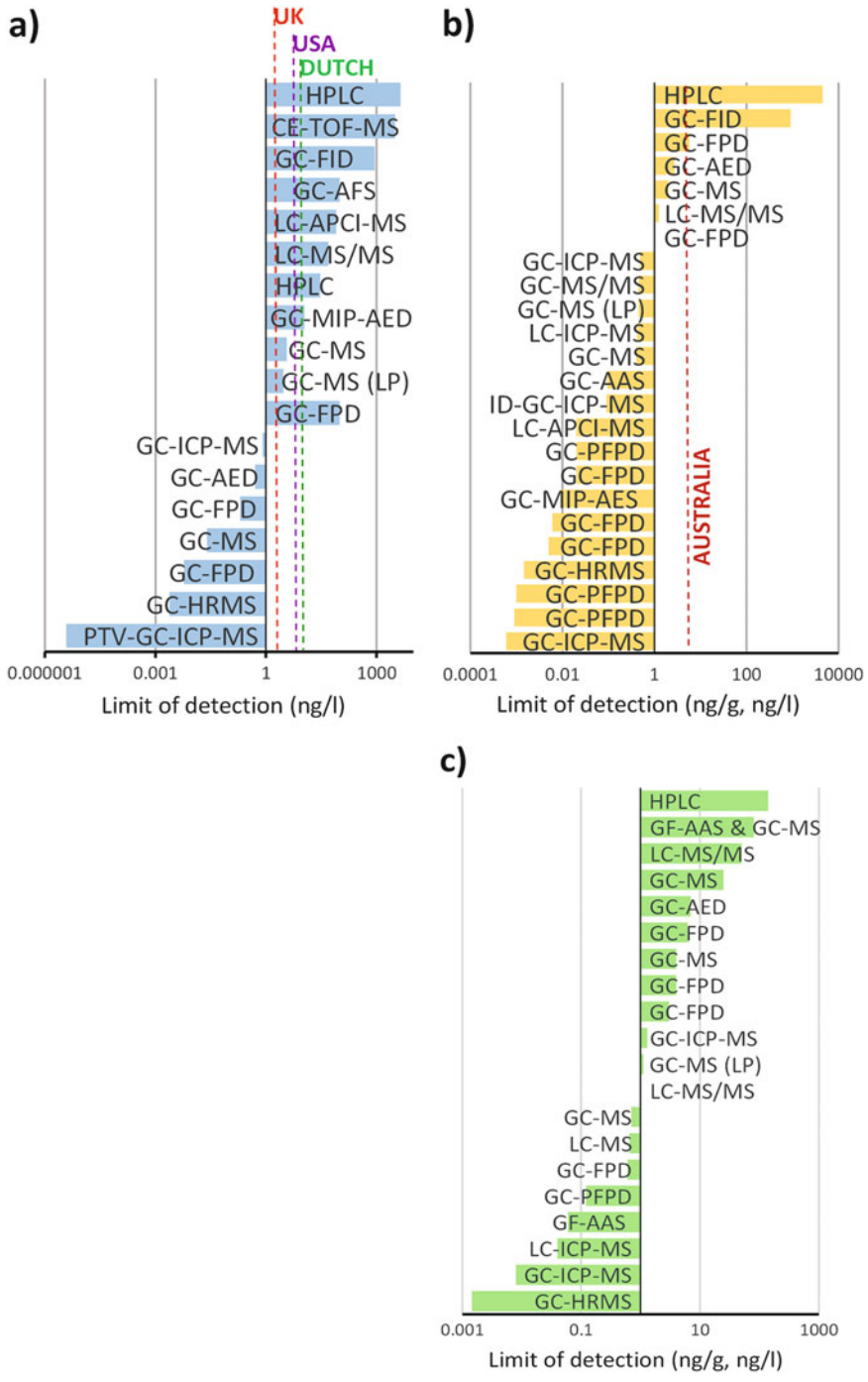

Fig. 1.2 Comparison of performance of tributyltin (TBT) analysis for water (**a**), sediment (**b**) and biota (**c**) by various analytical techniques (Values obtained from references provided in Tables 1.1, 1.2, and 1.3)

in the environment. Understanding the OT levels in sediment is important to know its exposure level for sediment dwellers. The commonly used method to extract organotins from sediment is leaching by using acid reagents aided by sonication, stirring, and shaking, or soxhlet extraction with an organic solvent (Stab et al. 1993). The acidic condition of the solution reduces the strength of the metal binding, and breaking the bonds from the matrix provides a positive charge to metal species. Further, low- to medium-polarity solvents such as toluene with complexing agents/acids (i.e., tropolone, diethylthiocarbamate, acetic acid) offers compromised efficacy and selectivity for organotins analysis from sediment. An advantage for sediment over a water matrix is the availability of CRM to validate the methods. Therefore, most of the methods reported were based on CRM.

1.3.2.1 GC-Based Analysis

Among the available reported methods, GC-ECD is rarely used for sediment. Tolosa et al. (1991) reported a method for butyl and phenyl tins analysis using acid digestion (HCl-tropolone-diethyl ether) and alumina cleanup before analyzing in GC-ECD and GC-FPD. Based upon the validation of spiked sediment (from Barcelona Harbour, Spain), the performance of FPD (LOD, 0.1–2 ng/g) with a 600-nm interference filter was found better than with ECD. Similar to ECD, because of poor performance, FID is also least used and is replaced by other GC detectors (FPD, PFPD, MS), which often resulted in a LOD two to three orders of magnitude lower than with FID. Millan and Pawliszyn (2000) investigated the performance of HS-SPME-GC-FID using PACS-2 and observed very high LOD such as 1000, 1200, and 900 ng/l (as Sn) for MBT, DBT, and TBT, respectively.

Earlier, Takahashi et al. (1999) reported butyltins in sediments of Otsuchi Bay, Japan in the range of 86–260 ng/g using GC-FPD with detection limits of 1–5 ng/g (wet wt.). Among the methods reported for GC-FID, microwave-assisted extraction developed by Donard et al. (1995) showed 1 ng/g as the detection limit even with minimal matrix (0.1 g PACS-1/CRM 462) (Table 1.2). Aguerre et al. (2000) optimized direct (DI)-SPME in GC-FPD and observed enhanced recovery and repeatability compared to SPE and LLE methods. The detection limit obtained for butyltins (0.02–0.07 ng/g dw) was rather lower than earlier reports, whereas phenyltins showed a detection limit ten times higher (0.13–28 ng/g) than butyltins.

Although the performance of GC-FPD is noticeable, it often suffers from inorganic contaminants (e.g., sulfur, phosphorus); therefore, FPD was replaced with PFPD to increase the sensitivity and reduce the matrix effect. Godoi and Words (2003) compared the performance of FPD and PFPD by using toluene-acetic acid-ammonium pyrrolidine dithiocarbamate (APDC) extraction, pentylmagnesium bromide (PeMgBr) derivatization, and aluminium oxide cleanup,. The LOD achieved for PFPD (0.3–2.2 pg/g) was at least one order of magnitude lower than with FPD (5–79.2 pg/g). Leermakers et al. (2005) also stated that the detection limit attained for PFPD is approximately 100 times lower than that of the FPD.

Table 1.2 Extraction and detection techniques frequently used for organotin analysis in sediment samples

Volume (g)	Extraction	Derivatization	Instrument	LOD (ng/g [a]ng/l)			Reference
				MBT	DBT	TBT	
2	Ultrasonication: purge and trap	$NaBEt_4$	GC-AED	4	4.5	2.7	Campillo et al. (2004)
0.5	Ultrasonication HS-SPME	$NaBEt_4$	GC-FID	1000	1200	900[a]	Millan and Pawliszyn (2000)
2	PLLE	$NaBEt_4$	GC-FPD	3.6	5.1	5.7	Radke et al. (2013)
0.1	MAE	$C_5H_{11}MgBr$	GC-FPD	–	–	1	Donard et al. (1995)
2	SPME	$NaBEt_4$	GC-FPD	0.07	0.02	0.02	Aguerre et al. (2000)
2	Ultrasonication	EtMgCl/PeMgBr	GC-FPD	8.9	12.2	0.005	Godoi and Words (2003)
2	Ultrasonication	EtMgCl/PeMgBr	GC-PFPD	0.6	0.5	0.0009	Godoi and Words (2003)
0.5	HS-SPME	$NaBEt_4$	GC-PFPD	0.02	0.05	0.02[a]	Bravo et al. (2005)
1	Acid digestion	$NaBH_4$	GC-AAS	–	–	0.095	Feng and Narasaki (2002)
0.5	Ultrasonication HS-SPME	$NaBEt_4$	GC-MS	0.3	0.3	0.4	Devos et al. (2005)
2.5	ASE	$NaBEt_4$	GC-MS	0.4–2			Berg et al. (2001)
2	Ultrasonication	$C_5H_{11}MgBr$	GC-MS (LP)	0.75	0.07	0.45	Vidal et al. (2003)
0.5	Acid digestion	$NaBEt_4$	GC-ICP-MS	0.23–0.48			Rajendran et al. (2000)
0.5	Microwave digestion-HS-SPME	$NaBEt_4$	ID-GC-ICP-MS	–	–	0.09	Yang et al. (2002)
2	Ultrasonication	$C_5H_{11}MgBr$	GC-MS/MS	0.75	0.07	0.45[a]	Vidal et al. (2003)
–	Ultrasonication	$NaBEt_4$	GC-HRMS	0.00035–0.00145			Ikonomou et al. (2002)
1	SPME	$NaBEt_4$	GC-FPD	0.031	0.007	0.006[a]	Aguerre et al. (2001)
1	SPME	$NaBEt_4$	GC-PFPD	0.004	0.001	0.001[a]	Aguerre et al. (2001)
1	SPME	$NaBEt_4$	GC-MIP-AES	0.042	0.011	0.009[a]	Aguerre et al. (2001)
1	SPME	$NaBEt_4$	GC-ICP-MS	0.002	0.0007	0.0006[a]	Aguerre et al. (2001)
1	Ultrasonication	–	HPLC	–	–	4500[a]	Compano et al. (1995)
10	ASE	–	LC-MS/MS	–	–	1.25	Nichols et al. (2014)
1	Ultrasonication	–	LC-ICP-MS	–	0.37	0.42	Yu et al. (2011)
1	Acid digestion	–	LC-APCI-MS	–	0.065	0.02	Rosenberg and Kmetov (2000)

– not applicable/performed

In case of AED, extraction by the purge-and-trap method (2 g sample derivatized in 5 ml acetate buffer; helium gas purging for a 10-min cycle at 35 °C; desorbing cycle for 8 min at 250 °C) yielded sufficient recovery of methyl, butyl, and phenyltins (93.4 %) with LOD in the range of 2.7–12.5 ng/g (Campillo et al. 2004). This purge-and-trap technique allows extraction of samples with high inorganic compounds to inject total extract without any purification procedure.

Mass selective detectors were also found suitable for organotin analysis in sediment, which gave detection limits lower than FPD and AED. When GC-ICP-MS was first used for TBT analysis in environmental samples (water and sediment) from the Dutch coast by Ritsema et al. (1998), the LOD obtained was 0.1 ng/g butyltin (as cation) based on classical acid-leaching extraction. Moreover, it is to be noted that analytical advancements have drastically reduced the detection range up to sub-picogram level, as discussed next. Rajendran et al. (2000) also used GC-ICP-MS for tributyl and triphenyltin analysis in Seto Inland Sea near Hiroshima, Japan and reported organotins (butyl and phenyl) based on the lowest LOD (0.23–0.48 ng/g) achieved compared to other available techniques (GC-AAS, GC-ICP-AED, GC-FPD). Further, the extraction method developed by Rajendran et al. (2000) showed good recovery (96–102 %) and LOD even with a large dilution factor.

Apart from acid leaching, Aguerre et al. (2001) used DI-SPME to investigate the performance of four different detectors in GC (FPD, PFPD, MIP-AES, ICP-MS) and reported ICP-MS as the best one with lowest LOD for butyltins (MBT, 0.002 ng/l; DBT, 0.0007 ng/l; TBT, 0.0006 ng/l), and for phenyltins as well. Although the LOD obtained through GC-ICP-MS was ten times lower than with GC-PFPD, Aguerre et al. (2001) suggested GC-PFPD for the purpose of routine monitoring for reasons of low consumption of gases and low operating cost.

ASE was also optimized for organotin extraction that is independent of analyte and matrix interferences and provides cleaner extracts. The whole process in ASE is based on application of increased temperatures and pressure for accelerating the extraction while keeping the solvent below its boiling point. The extract obtained by ASE can be analyzed without further cleanup and showed a detection limit in the range of 0.4–2 ng/g with high recovery (93–104 %) using GC-MS for butyltins (MBT, DBT, TBT) in harbour sediment cores from Wadenswil, Switzerland (Berg et al. 2001) (Table 1.2). Furthermore, Wahlen and Wolff-Briche (2003) performed ASE (0.2 g sample) for TBT analysis using the isotope dilution (ID) technique in GC-ICP-IDMS and achieved LOD as low as 0.03 pg/g, which is four orders of magnitude lower than LOD achieved by ordinary GC-MS. Additionally, Wahlen and Wolff-Briche (2003) concluded that the GC-ICP-IDMS method was far more sensitive (greater signal-to-noise ratio) and reliable than LC-ICP-IDMS technique (LOD, 3 pg/g). The use of isotope dilution method is gaining importance because it negates the dependence on CRM, and can identify and correct the loss of analytes during extraction, derivatization, instrument drift, species transformation, etc. (Van 2006). However, the high temperature used in the ASE method can initiate degradation of TBT to DBT and DBT to MBT, and the recovery may reach 140 % for MBT (Staniszewska et al. 2008 and references therein).

Tessier et al. (2002) effectively used a purge and cryogenic trapping device to quantify organotins in sediments from European estuaries (5–2000 fmol/l) using GC-ICP-MS with detection limit <1 fmol/l (1 ng/g). The method uses extraction of 10 g fresh sediment with 20 ml milli-Q water by purging with helium (300 ml/min for 10 min) and collection of gaseous species in cryotraps with total extraction time of 30 min. The LOD reported for purge-and-trap extraction with ICP-MS was comparable with LOD in the purge-and-trap method based on GC-AED (Campillo et al. 2004). Similar to ICP-MS studies, high-resolution mass spectrometry also showed robust detection at the sub-picogram level (LOD of 0.35–1.45 pg/g) for nine organotins in sediment from Botanical Beach, Canada (Ikonomou et al. 2002).

1.3.2.2 LC-Based Analysis

Among LC techniques for sediment analysis, UV, ICP-OES (inductively coupled plasma optical emission spectrometer), and AAS detectors are seldom used because of lack of sensitivity. Therefore, most of the earlier studies used fluorimetry-based detection whereas recent reports are based on ICP-MS (Gonzalez et al. 2003). Mainly, the LC-based methods eliminate possible sources of uncertainty and systematic errors that may arise from the derivatization reaction. Among the conventional methods, Compano et al. (1995) described a simple ultrasonication (i.e., extraction of 1 g sediment by 8 ml methanol) method for analysis of triphenyltin and tributyltin in HPLC using a fluorescent complexing agent (fistein) with emission maxima at 496 nm and 499 nm for TBT and TPhT, respectively. Furthermore, they obtained detection limits of 0.03 and 20.9 ng as Sn for TPhT and TBT, respectively, by injecting 200 µl of the extract (Table 1.2).

Fluorimetry has been phased out by mass spectrometry (MS and ICP-MS) with the LC system. Mass spectrometry-based techniques showed high sensitivity, wide linear range, and isotopic selectivity (Toledo et al. 2003). Rosenberg and Kmetov (2000) used the acid leaching extraction technique and quantified butyl- and phenytins in LC-APCI (atmospheric pressure chemical ionisation)-MS with LOD of 0.02 ng/g and 0.065 ng/g for TBT and DBT, respectively. Apart from individual studies, the reference material certification campaign of EU programme compared the sensitivity of the various analytical techniques based on the results from the laboratories. Quevauviller et al. (2000) analysed TBT from CRM-462 and found LC-ICP-MS as the most reliable instrument with less error. Apart from single quadrupole, tandem MS was also used with the LC system. Nichols et al. (2014) standardized ASE (MeOH/acetic acid with three cycles of extraction with purge time of 120 s, temperature of 100 °C, and pressure of 1500 psi) for LC-MS/MS using PACS-2. The recovery range observed was 72.4–80.5 % with low LOD (1.25 ng/g) (Table 1.2). Preferably, the ASE-based method allows direct injection of a 40-µl sample, thereby avoiding a preconcentration step without compensating the sensitivity.

Based on the LOD of TBT, the analytical techniques represented in Fig. 1.2b imply that methods presently available are sufficient to monitor TBTs in sediment

below the Australian sediment quality guideline (5 ng/g), with the MS technique outperforming other methods.

1.3.3 Analysis of Organotins in Biota

Investigating the organotin residues in biota provides an overview to understand the transport of organotins from environment to resident organisms and its biomagnification through the food chain. The quantification of OT residues also enables ecotoxicologists to estimate the toxic levels to organisms and their communities. Recent developments in analytical technique provide promising methods to quantify organotins, even at the femotogram level. Organotins in marine organisms are analysed by various methods, most of which showed good recovery and comparable LOD (Table 1.3). The most common methods of extraction for organotins speciation using GC analysis involve the process of adding tissue solubilizer (tetraethylammonium hydroxide, TEAH), acid-leaching (HCl/acetate buffer), derivatization ($NaBEt_4$), and cleanup by gel permeation chromatography (Folsvik et al. 1999).

1.3.3.1 GC-Based Analysis

Using classical GC-AED, Folsvik et al. (1999) obtained the LOD of 7 ng/g for organotins, and TBT in dog whelks of the Norwegian coast was found in the range of 48–1096 ng/g. Takahashi et al. (1999) collected biota samples (amphipods, mussels, ascidians, sea urchins, several fish species) at Otsuchi Bay, Japan for butyltin analysis using tropolone-acetone-HCl-grignard reagent-benzene/hexane extraction and quantification with GC-FPD. The method showed detection limits for MBT, DBT, and TBT as 9, 1, and 1 ng/g wet wt., respectively, with average recovery ($n = 5$) of 104 %, 117 %, and 108 %, respectively, and their concentration were observed in the range of <9–120, 1.8–600, and 5.1–310 ng/g, respectively, with plankton having the highest accumulation of butyltins (up to 1700 ng/g).

In another study, GC-FPD was used to determine butyltins (MBT, DBT, TBT) in fish, mussel, oyster, and barnacles (Cassi et al. 2002), where simultaneous extraction and derivatization greatly reduced the matrix effect caused by $NaBEt_4$, and the LOD reported was in the range of 3–4 ng/g. Ciesielski et al. (2004) analyzed MBT, DBT, and TBT in the liver of marine mammals based on ASE-GC-FPD (LOD, 6.2–10 ng/g), and the total butlytins were found in the range of 43.9–7698 ng/g dw. In contrast to conventional extraction methods, Gallego et al. (2006) used an ultrasonic probe (for effective leaching) and SPE [molecularly imprinted polymers (MImP) specially designed for TBT] to reduce the extraction and analysis time for about 40 min. Furthermore, the MImP has drastically reduced the interferences and helped to achieve lower LOD (3 ng/g) and good recovery (70–105 %) for TBT. Further, the use of potassium hydroxide-based digestion may increase the recovery

Table 1.3 Extraction and detection techniques frequently used for organotin analysis in biota samples

Sample type	Volume (g)	Extraction	Derivatization	Instrument	LOD (ng/g)			Reference
					MBT	DBT	TBT	
Mussel	1–2	Solubilizer (TEAH)	$NaBEt_4$	GC-AED	7	7	7	Folsvik et al. (2000)
Liver tissue: marine mammals	1	ASE	$NaBEt_4$	GC-FPD	10	7	6.2	Ciesielski et al. (2004)
Oyster and rock shells	0.2	KOH digestion	$NaBEt_4$	GC-FPD	0.2–0.6			Tang and Wang (2009)
Mussel	0.5	Ultrasonication – SPE	$NaBEt_4$	GC-FPD	3–4			Cassi et al. (2002)
Mussel, oyster, fish, barnacles	0.1–0.5	Ultrasonication – polymer extraction	$NaBEt_4$	GC-FPD	3			Gallego et al. (2006)
Algae (*Ulva lactuca*)	0.5	HS-SPME	$NaBEt_4$	GC-PFPD	0.21	0.49	0.12	El Hellal et al. (2006)
Mussel	20	Microwave digestion	$C_5H_{11}MgBr$	GF-AAS & GC-MS	80			Binato et al. (1998)
Mussel	0.5	Ultrasonication – LLE -SPE	$NaBEt_4$	GC-MS	19	13	25	Liscio et al. (2009)
Mussel	5	Sonication	$C_5H_{11}MgBr$	GC-MS (LP)	5	0.42	1.12	Vidal et al. (2003)
Barnacles, mussels, fish	0.5	Solubilizer TMAH – ultrasonication	$NaBEt_4$	GC-MS	0.57–5.5	0.56–5.1	0.8–4.0	Kucuksezgin et al. (2011)
Nucella lapillus	0.5	Digestion- TMAH	$NaBEt_4$	GC-MS	0.8	1.5	0.7	Guomundsdottir et al. (2011)
Mussel	0.5	Ultrasonication-SDME	$NaBEt_4$	GC-ICP-MS	0.7	1.2	1.3	Xiao et al. (2008)
Biota	1	Microwave–ultrasonication	$NaBEt_4$	GC-ICP-MS	0.006	0.001	0.008	Point et al. (2007)
Mussel	–	Ultrasonication	$NaBEt_4$	GC-HRMS	0.0035–0.00145			Ikonomou et al. (2002)
Shellfish	0.2	Sonication	–	HPLC	–	8	140	Gonzalez et al. (2001)
Shellfish	1–2	Sonication	–	LC-MS	–	0.95	0.65	Magi and Ianni (1998)
Mussel tissue	0.2	ASE	–	LC-ICP-MS	–	–	0.04	Wahlen and Wolff-Briche (2003)
Mussel	0.1	Ultrasonication – polymer	–	LC-MS/MS	–	–	50	Zhu et al. (2013a)
Mussel	0.1	MNP	–	LC-MS/MS	–	–	1	Zhu et al. (2013b)
Mussel, fish	Whole	CPE	–	GF-AAS	–	–	0.06	Louppis et al. (2010)

– not applicable/performed

values for about 151 % because of the probable degradation of tri- and di-substituted tins to mono form (Tang and Wang 2009).

The trend of using PFPD rather than FPD was also observed for biota. El Hellal et al. (2006) monitored methyl, ethyl, butyl, and phenyl tins in aquatic plants (*Elodea*) and algae from Bizerte Lagoon (Tunisia) using HS-SPME-GC-PFPD. Also, the method seems to perform better for all the organotins (LOD, 0.12–6.7 ng/g) (except TPhT, 40 ng/g) than GC-MS [large-volume injection (LVI), low-pressure (LP)] methods as given in Table 1.3. The SPME method showed an LOD 10–100 times higher than LLE (even for a complex matrix such as algae), and also extracted methyltins, which are not extracted by LLE.

Less frequently, GC-AAS is also used for organotins monitoring because its performance is poorer than GC-FPD/PFPD. Binato et al. (1998) reported a higher detection limit (80 ng/g Sn) by using the microwave digestion-GC-AAS technique. However, Louppis et al. (2010) used cloud point extraction (CPE) to investigate TBT contamination in 77 seafood samples including sardines, anchovies, and mussels collected from the Aegean and the Ionian Sea by using GC-AAS. The LOD achieved for CPE (0.06 ng/g) is comparable with other sensitive GC techniques.

Application of mass spectrometry in extraction method development is obvious in many studies. In one such study, the feasibility of different extraction procedures (mechanical shaking, ultrasonication, and closed vessel microwave-assisted extraction) for the simultaneous determination of organotins (propyl, butyl, phenyl, and octyl) in reference material CE477 was investigated by Nemanic et al. (2002) using GC-MS. Ultrasonic extraction at 50 °C for 1 h with hydrochloric acid in methanol, followed by derivatization, showed the highest sensitivity for organotins without any interfering peaks in GC chromatograms and LOD in the range of 3–20 ng Sn/g. Based on this method, organotin compounds were quantified in mussels (*Mytilus galloprovincialis*) collected from the Slovenian costal area with elevated concentrations of butyl tins (1090, 565, and 102 ng Sn/g for TBT, DBT, and MBT, respectively). In another study, Liscio et al. (2009) investigated the efficacy of $NaBEt_4$ and *n*-pentylmagnesium bromide derivatization for organotins (MBT, DBT, and TBT) from *M. galloprovincialis*, and the LOD achieved by using $NaBEt_4$ (13–25 ng/g) was one order of magnitude lower than *n*-pentylmagnesium bromide (99–377 ng/g) in GC-MS. The performance of $NaBEt_4$ was better because of less noise and nondetection of butyltins in the blank in contrast to *n*-pentylmagnesium bromide.

The GC-ICP-MS performance is often enhanced by adopting microextraction. Xiao et al. (2008) used headspace–single-drop microextraction (HS-SDME) for MBT, DBT, and TBT in mussels (0.5 g sample) and obtained LOD in the range of 0.7–1.3 ng/g; the method is found to be simple, quick (faster than DI-SPME), inexpensive, and effective (without matrix interference). Point et al. (2007) used microwave-assisted ultrasonic extraction with GC-ICP-MS and achieved an LOD two orders of magnitude lower (6–8 pg/g) than with HS-SDME (Table 1.3). Similar to GC-ICP-MS, HRMS also tends to perform much better with LOD at the pg level

(1.45–3.5 pg/g) for mussel tissue (CRM-QSP001BT) by classical extraction methods (acid digestion, derivatization, cleanup) (Ikonomou et al. 2002).

1.3.3.2 LC-Based Analysis

LC fluorimetry shows acceptable performance for biota samples (LOD, 8–140 ng/g) with good recovery, 96–102 % (Toledo et al. 2003). Other than fluorimetry, Rivaro et al. (1995) used HPLC-hydride generation-ICP-AES for MBT, DBT, TBT, and TPhT analysis in oyster with a detection limit of 7 ng Sn. Later, with the help of LC–particle beam (PB)-MS, Magi and Ianni (1998) analyzed organotins in shellfish with LOD of 0.65–0.95 ng/g. Furthermore, based on LC-ICP-MS, Wahlen and Catterick (2003) developed an analytical method for MBT, DBT, TBT, MPhT, DPhT, and TPhT using ASE, and the LOD achieved (40 pg/g) was several times lower than with fluorimetry (Toledo et al. 2003) and ICP-AES (Rivaro et al. 1995) analyses. Furthermore, to speed up the extraction process, Zhu et al. (2013a, b) used a novel SPE cartridge packed with either imprinted polymer or magnetic molecularly imprinted polymer particles (Fe_3O_4@MIPs), and these extraction techniques showed LOD of 1 ng/g and 50 ng/g, respectively, for mussel sample (0.1 g) in LC-MS/MS (Table 1.3). The imprinted polymer exhibits quick adsorptive capacity and allows selective retention of organotins (e.g., TBT). Therefore, the foregoing analytical method is considered to be useful to investigate the bioaccumulation of OTs in biota.

While looking into the various extraction techniques available for biota, GC seems to provide a wider choice than LC techniques because the lowest LOD for TBT was achieved using GC-HRMS (Fig. 1.2c). Next to GC-HRMS, ICP-MS performed better. The detection limit of TBT for widely used GC-FPD and GC-PFPD varied with respect to extraction methods.

1.4 Conclusion

Compared to earlier days, the choice of extraction and detection techniques available for OTs is ample. The use of advanced extraction methods (e.g., SPME, SDME, MIP) rather than classical LLE made possible rapid improvements in sample treatment in terms of reduction of processing time, sample volume, and reagent consumption. Moreover, online and in situ derivatization approaches have greatly reduced the matrix effect during GC analysis. Although GC provides good resolution and separates most of the species in a single run compared to LC methods, sample preparation seems laborious. Few instruments offer cost-effectiveness (e.g., GC-FPD, GC-AAS), although mass spectrometry (MS, MS/MS) offers detailed information on the types of organotins. The application of isotope dilution has offered a solution for tracing the analyte transformation/degradation reactions during analysis. Among the available detection techniques,

some of them outperform with lowest detection limits at the picogram level, which is sensitive enough to report concentration below safety/toxic levels in the environment.

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Chapter 2
Continuing Issues of Contamination by Organotins in the Marine Environment After Domestic and International Legislation

Yuji Takao

Abstract Organotin compounds, which are used for painting on bottoms of ship's hull, have high residual property in the environment, and examples of their detection in the environment have been reported even after the introduction of international regulations on their use. To better understand the residual concentrations of organotins in marine environments, studies have been conducted on regional and small-scale fishing harbors, rather than in areas with integrated, large-scale commercial harbors and dockyards and areas where industrial facilities unrelated to the fishing industry exist. This approach of using small fishing harbors has advantages because there are many sites available for analysis, and the analytical noise is low. It is found that the organotin compound concentrations in sediments of fishing harbors with repainting facilities are clearly higher than those of harbors without such facilities. The organotin contamination depends on the presence of the repainting facilities and not on the scale of the fishing harbor. It is proposed that paint flakes produced during the repainting of hull bottoms are discharged into the sea, and these paint flakes are a high concentration source of contamination. Since the difference in organotin values between harbors with and without repainting facilities is less than ten times at the median values, it is considered that organotin compounds are released intermittently into the environment during periods that a ship is in harbor and that the quantities of organotins in the environment are not as low as expected.

Keywords Tributyltin • Sediment • Hull bottom paint • Fishing harbor • Repaint facility • Paint flakes

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2.1 Contamination that Originates Mainly from Hull Bottom Paint

To consider the effects of chemical substances contained in hull bottom paint on the marine environment, it is necessary to understand the purpose of this paint and its characteristics during and after use. Shells and seaweeds adhere and grow over time on the surfaces of sunken artifacts irrespective of the material of artifacts, especially those at depths reached by the sunlight. Adhesion of such matter to the underside of a ship decreases, for example, its speed and fuel efficiency. Also, adhesion of such matter to fishing nets impairs the utility of the nets. Because of their strong antifouling effects and high durability, organotin compounds were used widely around the world from the 1960s. It became clear, however, that organotin compounds cause reproductive abnormalities in shellfish at extremely low concentrations; thus, most countries, including Japan, regulate the use of these compounds strictly (Dafforn et al. 2011; de Castro et al. 2012). However, organotin compounds have high residual properties in the environment, and their detection in sediments and in marine wildlife is widely reported up until quite recently (Díez et al. 2002; Takeuchi et al. 2004; de Castro et al. 2012; Smith et al. 2006). The specific organotins used as antifouling agents are tributyltin (TBT) and triphenyltin (TPT): in particular, the quantities of TBT in use have been large. The half-life of TBT is reported to be about 1–2 weeks in water (Seligman et al. 1986; Lee et al. 1989; Watanabe et al. 1992) and about 1–4 years in sediments (de Mora et al. 1989, 1995; Dowson et al. 1993; Anderson et al. 2002).

Because the main intended use of organotins is in hull bottom paint, their analyses in dockyards, commercial ports, marinas, bay, ship courses, etc. have been reported by researchers worldwide (Birchenough et al. 2002; Rato et al. 2006; Wang and Tam 2012). According to reported values, concentrations near dockyards and ship-repair yards are higher than in other areas (Ko et al. 1995; Antizar-Ladislao 2008; Choi et al. 2009; Undap et al. 2013; Pougnet et al. 2014). TBT has several other uses, in addition to hull bottom paint. Figure 2.1 schematically shows how much TBT has been released into the environment. The size of each arrow shows the released quantity ideationally, not a ratio of exact released quantities. Although this figure also shows dibutyltin (DBT), a decomposition product of TBT, the toxicity of DBT to organisms is lower than that of TBT. As shown in Fig. 2.1, an overwhelmingly large quantity of TBT has been released in the environment from ships. Except for special small racing crafts, the undersides of most boats and ships are submerged in seawater during operation, as well as while they are in harbor, and there are always organisms trying to adhere to these surfaces. Antifouling chemicals contained in paint applied to hull bottom prevent these marine organisms from adhering by dissolving gradually in the seawater. Chemical substances that initially had a high concentration on the painted surface dissolve in seawater over time, and the concentration decreases gradually. Therefore, organisms are able to adhere to the painted surface. Hence, hull bottom paint that has self-polishing properties is widely used (Kiil et al. 2001, 2002, Dafforn et al. 2011). New

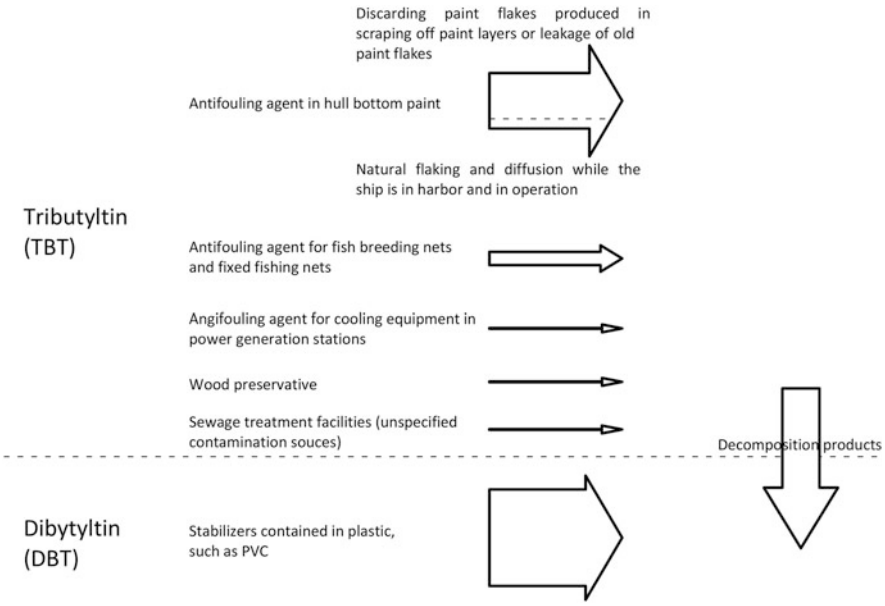

Fig. 2.1 Summary of quantities of tributyltin released to the environment

paint surfaces are constantly exposed to the seawater, thereby preventing organisms from adhering to the surface. Since the paint thickness decreases gradually, repainting becomes necessary about every 2–5 years, even if there is no damage or adhesion of organisms on the paint surface. These two effects, diffusion of antifouling agent from the paint surface into the seawater and natural micro-flaking of the surface, increase the concentration of antifouling chemicals in seawater, sediments, and organisms at places where many ships moor and pass: typically large-scale commercial harbors and sea-lanes.

The underside of the ships is repainted once a year or more. Surface preparation is well known to be important to ensure quality of painting. New paint is not simply applied directly on the old paint surface during a repainting process. Usually, the old paint is scraped off with organisms such as barnacles, and surface is prepared uniformly by sanding and smoothing. An uneven surface decreases fuel efficiency and induces adhesion of organisms. Contamination by paint flakes produced during this process has been identified as a contamination source (Ko et al. 1995; Barakat et al. 2001; Birchenough et al. 2002; Song et al. 2005; Eklund et al. 2008; Turner 2010; Jones and Turner 2010; Du et al. 2014). We also believe that discarding these flakes and diffusion of unrecovered flakes into the sea are important contamination sources. We represent this by the far left arrow in Fig. 2.1. Figure 2.2 summarizes the analyte concentrations (on a logarithmic scale) reported by researchers (Page et al. 1996; Ko et al. 1995; Thomas et al. 2000; Barakat et al. 2001; Díez et al. 2002; Harino et al. 2007; Berto et al. 2007; Eklund et al. 2008; Choi et al. 2009; Undap et al. 2013; Pougnet et al. 2014; Filipkowska et al. 2014) around the world from the

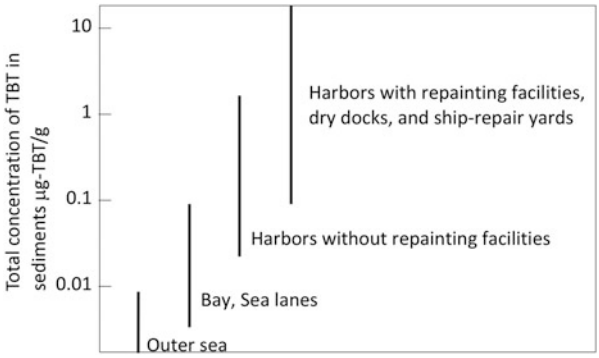

Fig. 2.2 Rough relationships between sampling locations and TBT concentrations in sediment

standpoint of the existence of repainting facilities. The concentration of TBT is high in places where there are repainting facilities, where many ships gather, and where ships pass. As described above, researchers have reported high concentrations near dockyards. A dockyard has two important functions: building of new ships and repair and maintenance. The cause of high organotin concentrations near dockyards is not considered to be the ship-building work but rather the repainting of ships in maintenance.

2.2 Classification of Fishing Harbors and State of Contamination

We measured the concentration of organotin compounds in sediments taken at various locations in Japan. Similar to the reports of other researchers, high concentrations of organotins were detected in sediments near dockyards and ship-repair yards. However, we found extremely high concentrations of organotin compounds accumulated in the sediments of regional and small-scale fishing harbors. We classified these harbors and found that the levels depend largely on the presence of repainting facilities. These results are described here.

Sediments were sampled at 82 points in five prefectures of northern Kyushu in Japan between 2003 and 2007. These were classified into five categories: suburban areas, urban areas, fishing harbors without repainting facilities, fishing harbors with repainting facilities, and dockyards or ship-repair yards. River mouths and coasts that unrelated to the ship were classified in the suburban or urban area categories.

A fishing harbor has a characteristic structure depending on its surrounding area. Figure 2.3 shows the typical shape of a fishing harbor in northern Kyushu. There are following characteristics in this area: About half the fishing harbors have a concrete slope, shown as *s* in the figure, and facilities to enable fishing boats to land. In most cases, there is a hut housing a wheeled cart to hoist boats, associated wire ropes, an electric winch, work tools, and other items. Figure 2.4 shows a photograph of a typical fishing harbor slope. These facilities are used by fishermen to land their

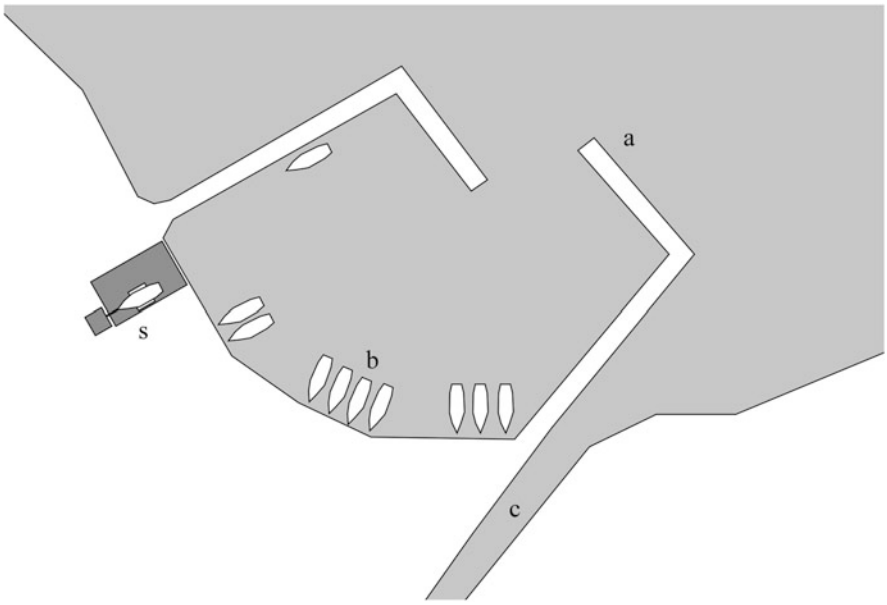

Fig. 2.3 Typical image of fishing harbor in northern Kyushu. *s* repainting facility; *a* breakwater; *b* fishing boats; *c* small river

boats once a year or so; scrape off organisms, such as shells that adhere to the hull bottom, and old paint; and apply new paint. At comparatively large-scale fishing harbors with more than 100 boats, these facilities become sophisticated with more several carts, but the basic function is the same. In most cases, these facilities are grouped together in one place. These facilities are shared, so hull bottom work by a fisherman can be regularly seen. These facilities are unattended when they are not in use. In rare cases, these facilities are covered with a roof (similar to that of a ship-repair yard), several workers are stationed, and repair work, as well as repainting, can be conducted. In this section, such large-scale attended facilities are classified as ship-repair docks for the sampling site classification. As shown by *b* in Fig. 2.3, in the Kyushu area, returning fishing boats moor in harbors. This is often observed in areas having rocky coasts, such as the eastern Tohoku coast, the main island area of Okinawa, and the south-west area of Hokkaido in Japan, and at fishing harbors in the south of Korea. In the eastern Chiba prefecture in Japan, many fishing harbors have more than half of the land-sea boundary within the harbor developed from sloping sandy beach. Fishing boats that return to such harbors are pulled up onto their own slopes on the beach to wait for their next fishing. In rare cases, boats are pulled up by a cart dedicated for ships, but in many cases, about ten wooden or plastic logs are buried perpendicular to the boat on a concrete slope, and the boat is pulled up to the land and moored by sliding over these logs. Even in a harbor of this type, repainting facilities *s* are easily distinguished as carts, which make work on the hull bottom easy, and are used to pull the boats on land for repainting. A breakwater, shown as *a* in Fig. 2.3, absorbs waves, so exchange of the seawater and

Fig. 2.4 Example of repainting facilities in a fishing harbor. There are three sets of carts and rails to haul boats up a concrete slope. The *red* paint color adheres to the slope and carts

sediment is comparatively low inside the harbor, and diffusion of contaminants and aerobic degradation are slow. *c* in Fig. 2.3 shows a small-scale river. In many cases, its flow rate is low and its width narrow. In this figure, it is shown outside the fishing harbor; in some cases, however, it flows into the fishing harbor. In case of a large-scale river, part of the river mouth is used as a harbor. As shown in Fig. 2.3, examples of an integrated small-scale river mouth and fishing harbor are often observed in northern Kyushu. Two types of fishing harbors, shown in Fig. 2.4, are classified, depending on the presence of *s* facilities.

Figure 2.5 classifies organotin concentrations in sediments, depending on where the sample was taken. The height of a bar shows the total organotin compound concentration, representing six organotin compounds. The five classified sampling areas are sorted in decreasing order of total concentration. The black part of a bar shows the TBT concentration, a major component of hull bottom paint. In 44 % of the places sampled, the concentration of TBT was the highest of the organotin compounds. Figure 2.5 shows that the highest sediment concentrations are found for dockyards and ship-repair yards and fishing harbors with repainting facilities that are in the left two groups, compared to the other three groups. To clarify the comparison of these five groups, Table 2.1 shows the number of samples taken, the median and average concentrations of TBT, and the median and average of total organotin compound concentrations (ΣOTs). Table 2.1 confirms that the highest

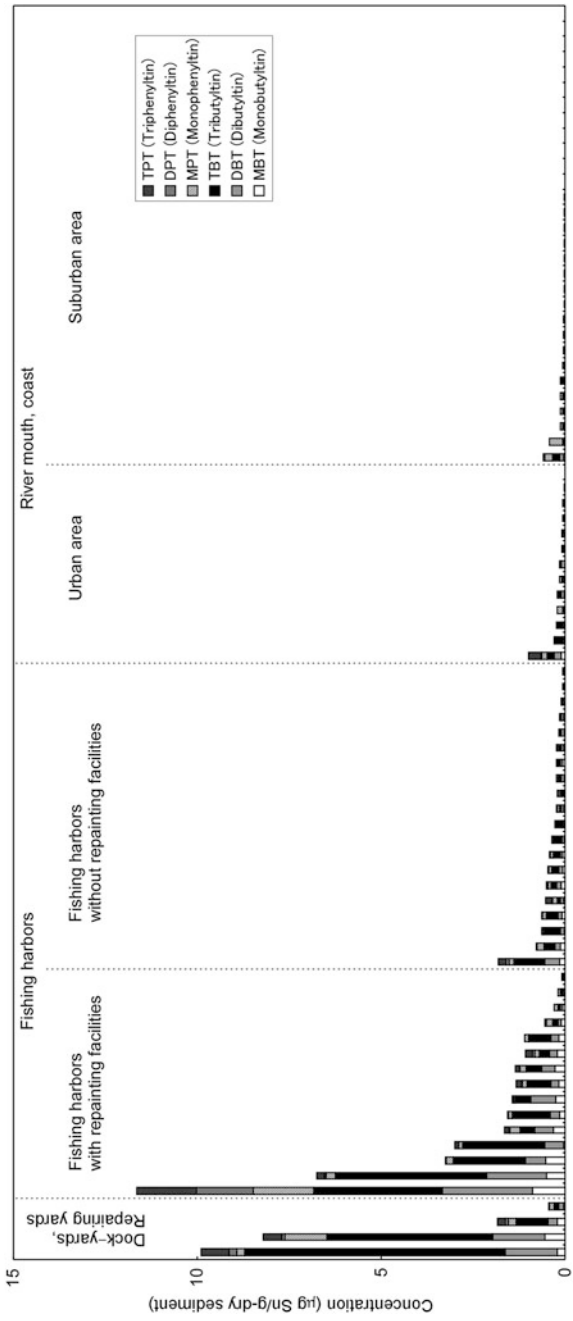

Fig. 2.5 Concentration of organotin (mg as Sn g^{-1} dry wt.) in northern Kyushu sediments sampled between 2003 and 2007 and classified by location

Table 2.1 Median and average concentrations of tributyltin (TBT) and total concentrations of organotin compounds (ΣOTs; tributyltin, dibutyltin, monobutyltin, triphenyltin, diphenyltin, and monophenyltin) in classified sediments

	Location	*n*	Concentration (μg Sn/g-dry)			
			Tributyltin(TBT)		ΣOTs	
			Median	Average	Median	Average
Dockyard or repairing yard		4	2.681	3.144	5.014	5.094
Fishing harbor	With repainting facility	15	0.434	1.064	1.341	2.350
	Without repainting facility	20	0.099	0.173	0.255	0.406
River mouth	City area	13	0.026	0.062	0.140	0.193
	Country side	30	<0.01	0.013	0.022	0.022

n is the number of samples taken for each classification

concentrations are found in sediments of dockyards and ship-repair yards among five groups as previously reported. Comparing the two fishing harbor groups, those with repainting facilities show concentrations 4.2–6.2 times higher: i.e., the concentration of organotin compounds in the sediment depends strongly on the presence of repainting facilities. Figure 2.5 shows low and similar organotin concentrations at river mouths in both areas; however, Table 2.1 shows that sediments of river mouths in urban areas have values of 4.7–8.7 times higher. The reason is thought that organotin compounds are also used as wood preservatives and as catalysts and stabilizers for polymers.

2.3 Relationship Between Organotin Compound Concentrations in Fishing Harbors and Number of Registered Ships

In general, to be able to define the charges for the expenses involved in improvement and maintenance of harbors and state of use of these facilities, fishing boats moored in fishing harbors in Japan are registered. Since we were able to obtain the number of registered boats in harbors of Kyushu, we created Fig. 2.6. This figure shows the number of boats registered in each fishing harbor in Kyushu on the horizontal axis and total concentration of organotin compounds in that harbor on the vertical axis. Black and white circles show fishing harbors with and without repainting facilities, respectively. Figure 2.6 shows that even where the number of registered boats is large, for example, 350–550, the sediment organotin concentration is low in fishing harbors without repainting facilities. On the contrary, in fishing harbors with repainting facilities, even if the number of registered boats is small, for example, 20–30, the organotin compound concentrations tend to be high. This demonstrates that the presence of repainting facilities is an important factor in determining the level of organotin compound concentration, irrespective of number of registered boats and scale of a fishing harbor.

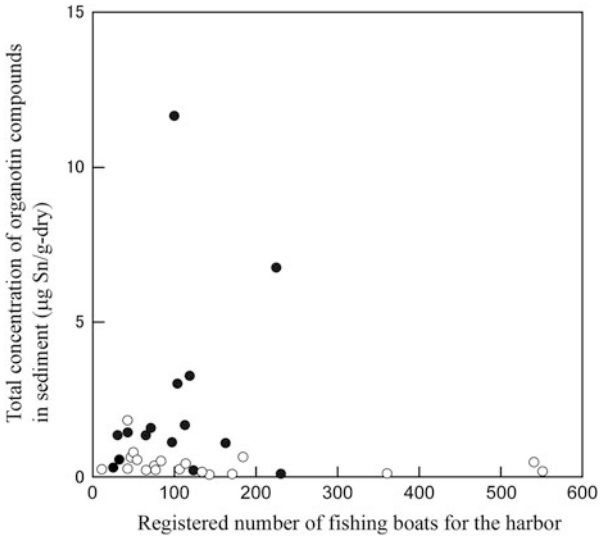

Fig. 2.6 Relationship between total concentration of organotin compounds in sediments and registered number of fishing boat for that harbor. *Open* and *solid circle* indicates fishing harbor without and with repainting facilities, respectively

2.4 Relationship Between Distance from Repainting Facilities and Concentration of Organotin Compounds

Figures 2.7a and 2.7b show analytical results for sediment organotin concentrations in fishing harbors A and B, respectively. Both harbors have repainting facilities at the sites denoted *s*, which include features such as slopes, carts mounted on rails, wires, and winches. Small-scale rivers flow in both fishing harbors. There are approximately double the number of boats registered at A as at B (when the sediments were actually sampled, a larger number of boats were moored in A), and the scale of other facilities, such as the fishery cooperative, was larger at A. Black circles in the figures show sites where the sediments were sampled. In A, shown in Fig. 2.7a, the concentration of organotin compounds in the sediment was high at site 1 (near repainting facilities s) and low at sites 2–4, which are further away from s. At site 5, on the opposite side of the harbor to the repainting facilities s, the concentration was slightly high. In B, shown in Fig. 2.7b, high concentrations of organotin compounds were detected at site 1 near the repainting facilities s, and the concentration decreased on moving away from s to sites 2 and 3. Although the same numbers of fishing boats were moored at sites 2 and 3 and at sites 4 and 5, the organotin contamination at the latter was lower due to these sites being further away from s.

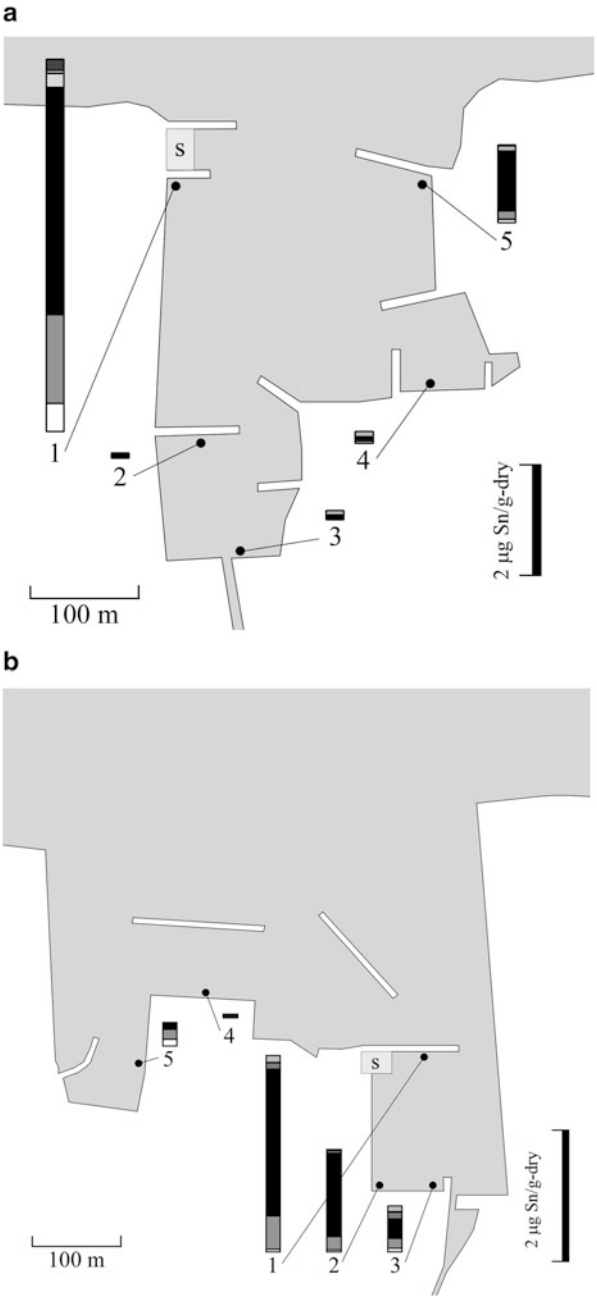

Fig. 2.7 (**a**, **b**) Concentration of organotin compounds in sediment at a fishing harbor. *Bar* marks are the same as for Fig. 2.5. The symbol "s" indicates the slope where fishing boats are pulled out of the sea for repainting

Table 2.2 Organotin concentrations in paint scrapings collected from the fishing harbor slope in Fig. 2.7b

	TBT	DBT	MBT	TPT	DPT	MPT	Total
Paint scrapings	4.82	0.47	0.17	0.12	0.10	1.10	6.78

2.5 Organotin Compound Concentrations in Paint Flakes Taken from Repainting Facilities

Table 2.2 shows the concentrations of organotins in paint flakes sampled from the slope at the repainting facilities *s* shown in Fig. 2.7b. These "paint flakes" comprise a sandy mixture of shells that were attached to the underside of boats and old rough paint flakes that are scraped off before new paint is applied when a boat is pulled up on a slope. These flakes were collected from gaps in the rails for a cart that was used to haul boats up the slope. The samples were physically similar to sand, in which fragments of shells of smaller than several mm and paint flakes smaller than 1 mm were mixed. The analytical results show that TBT is the main component (70.3 %) and that total concentration of organotin compounds was high at 6.78 μg Sn/g-dry. The comparison of these values with the results for the sediment at site 1 in Fig. 2.7b shows that the total concentration of organotins is about three times higher and that the proportion of organotins is very similar. This supports the conclusion that discarding of hull bottom paint flakes is a major factor in organotin compound contamination of sediments in fishing harbors.

Although these paint flakes are fundamentally industrial waste and must be recovered and disposed of properly, it is thought that this is not carried out thoroughly in many regional and small-scale harbors. The reason for this is considered to depend largely on the lack of awareness in the workers, i.e., fishermen. They know that the quantity of sandy old paint flakes scraped off from one boat is small, the flakes disappear as they are washed away by seawater, and the slope is washed by waves during rainy weather and high seas. There is also low awareness that this hull bottom paint contains hazardous components, so recovery of paint scraps and adherence to work procedure are insufficient.

2.6 Two Classifications of Fishing Harbors in Five Areas in Japan and Organotin Compound Concentrations

Figure 2.8 summarizes the survey results for total organotin compound concentrations in sediments collected from fishing harbors in five areas of Japan, including the above areas in northern Kyushu ($n = 49$, additional data in Fig. 2.5). The other sites surveyed are the Kanto area (Boso Peninsula and Miura Peninsula) ($n = 44$), eastern Tohoku ($n = 33$), southwestern Hokkaido ($n = 50$), and the main island area of Okinawa ($n = 27$). Japan has a long land distance, measured north to south: the

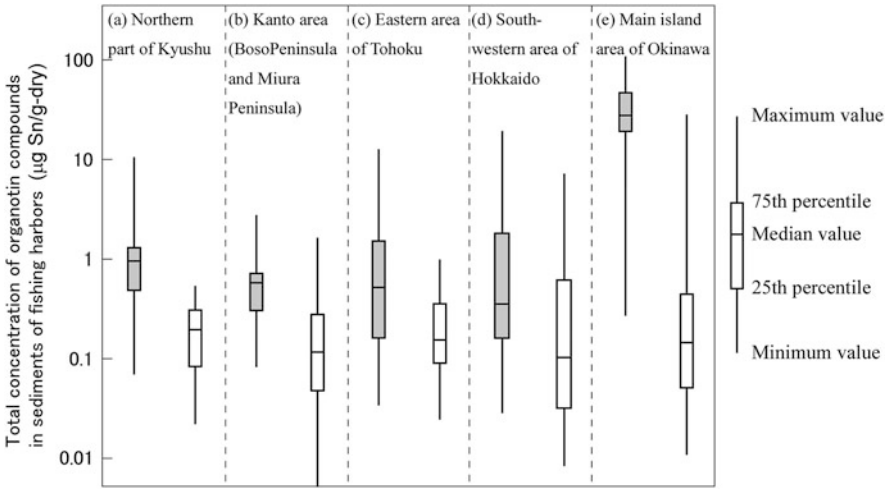

Fig. 2.8 Total concentrations of organotin compounds in sediments of fishing harbors from five areas in Japan. Results are classified based on the presence (*gray boxes* and *lines*) and absence (*white boxes* and *lines*) of repainting facilities

distance between Okinawa and Hokkaido is 2200 km. In this figure as well, results were totaled with classifications based on the presence/absence of repainting facilities in harbors. Figure 2.8 clearly shows that organotin concentrations in harbors with repainting facilities are higher in all the surveyed areas. This confirms that paint flakes produced during repainting of the hull bottom are a main source of contamination in fishing harbors, irrespective of location. Excluding Okinawa, the median value of difference in concentration between both groups is smaller than single digit. The reason that Okinawa fishing harbors with facilities are more heavily contaminated than other areas is unknown.

Surveys of regional and small-scale fishing harbors, such as those reported here, have the following merits. Firstly, in contrast to surveys of commercial harbors and dockyards, the number of fishing harbors is large and statistical reliability is higher. Secondly, activities carried out in most fishing harbors are similar; there are no large-scale chemical plants in the neighborhood and therefore no external factors to distort the analyses.

With respect to fishing harbors without repainting facilities, it is interesting to note that the median, 25th, and 75th percentile values for the five areas are very similar. In addition, concentration differences between locations with and without repainting facilities are not as large as expected (except for the Okinawa main island): the difference between the median values is less than one order of magnitude, and the 25th percentile value with facilities and the 75th percentile value without facilities are similar or are reversed. Any moored ship deposits chemical substances into the sediment. It is estimated that the degree of supply is equivalent to the effects of a small fraction of paint flakes from repainting of hull bottom discarded into the sea. This represents that hull bottom paints have the

self-polishing property and a ship in a harbor continuously polishes its own bottom and continues to release chemical substances. Though it is a rough estimate, we can say that the contamination of chemical substances in the fishing harbors can be considered based on the following six assumptions: (1) the thickness of newly applied paint decreases to zero in 5 years by self-polishing; (2) boats are repainted once a year; (3) all old paint films are scraped off in repainting work; (4) old paint flakes produced in the work are discarded in the sea; (5) half of fishing harbors have repainting facilities, boats belonging to fishing harbors without repainting facilities move to harbors with facilities when repainting work is needed, and the number of boats belonging to these fishing harbors is the same; and (6) chemical substances have high residual effects in the environment. In fishing harbors without repainting facilities, one-fifth of the chemical substances contained in ship paint accumulates in 1 year (due to diffusion in the harbor); in fishing harbors with repainting facilities, in addition to this 1/5 accumulation in the harbor, twice the remaining 4/5 of chemical substances are discarded, giving a total of 9/5 accumulation of chemicals. Thus, the ratio between two types of harbors is nine. When the value of assumption (1) above decreases to 3 years, this ratio becomes five. When assumption (1) is 5 years and assumption (2) increases to 2 years, this ratio becomes four. These are close to the ratio of the median values shown in Fig. 2.8 (with the exception of Okinawa).

2.7 The Future

Many countries began to independently regulate organotin compounds, such as TBT, from about the 1980s. Manufacture and the use of organotins have been regulated in Japan since 1990, and international regulations were initiated by the AFS convention (The International Convention on the Control of Harmful Anti-Fouling System on Ships, 2001) in 2008. The harbor sediment samples shown in Fig. 2.8 were taken from northern Kyushu from 2003 to 2007, from the Kanto area in 2007, from Tohoku in 2008, from Hokkaido in 2010, and from Okinawa in 2009. Although more than 10 years had passed since the introduction of the usage regulations in Japan in 1990, organotin compounds were still detected in the sediments in many fishing harbors. Since 2010, many researchers in other parts of the world have also reported organotins in the sediments (Undap et al. 2013; Arp et al. 2014; Suzdalev et al. 2014), indicating that organotin compounds have high residual effects in the environment. For this reason, it is necessary to continue monitoring the concentrations of these chemicals in the environment and observe their decay.

Copper pyrithione, zinc pyrithione, Irgarol 1051, diuron, and other chemicals are now used as alternatives to organotins in hull bottom paint (Ranke and Jastorff 2000; Bao et al. 2011; Dafforn et al. 2011). It is expected that these have lower adverse effects on the ecosystem than organotin compounds. However, these chemicals have properties that prevent marine organisms from adhering to hull

bottoms. It is therefore important for the future maritime environment to use the valuable experience gained from organotin compounds. Firstly, paint flakes produced in repainting work of hull bottom paint should not be discarded in the sea but recovered and disposed properly. It is considered necessary to involve this educational campaign for owners of small crafts and for workers at comparatively large docks. Secondly, paint developers should continue to develop safer antifouling agents, bearing in mind that although self-polishing properties are useful and essential in hull bottom paints, the paint containing chemical substances is continuously discarded into the sea, and the quantities are not small.

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Chapter 3
Emerging Issues on Contamination and Adverse Effects by Alternative Antifouling Paints in the Marine Environments

Hiroya Harino

Abstract The current status of antifouling biocides contaminations was reviewed in water, sediment, and biological samples, and the effect of antifouling biocides for aquatic organisms was evaluated.

Irgarol 1051 (3-methylthio-4-tetrabutylamino-6-cyclopropylamino-*s*-triazine), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), sea nine 211 (2-*n*-octyl-4,5-dichloro-2-methyl-4-isothiazolin-3-one), chlorothalonil (2,4,5,6-tetrachloro-isophthalonitrile), dichrofluanid (*N*′-dimethyl-*N*-phenylsulphamide), metal pyrithions (metal complex of 2-mercaptopyridine-1-oxide), and PTPB (pyridine triphenylborane) in water were in the range of 0.5–2,430 ng/l, <0.7–6,742 ng/l, <0.3–3,700 ng/l, <1–1,380 ng/l, <1–55 ng/l, <80 ng/l, and 0.0036–0.021 ng/l, respectively. The concentrations of Irgarol 1051, diuron, sea nine 211, chlorothalonil, dichrofluanid, and pythiones in sediment were in the range of <0.02–816 μg/kg dry, <0.02–1,350 μg/kg dry, <0.04–150 μg/kg dry, <0.01–46.5 μg/kg dry, <0.1–688.2 μg/kg dry, <8–420 μg/kg dry, respectively. Irgarol 1051 was detected in the range of <0.1–35 μg/kg in clam, mussel, and oyster from Vietnam, Thailand, and Japan. The concentrations of diuron and sea nine 211 in bivalves were <0.1–9.6 μg/kg and <0.1–0.3 μg/kg, respectively.

EC_{50} and LC_{50} of Irgarol 1051 were in the range of 0.09–50,800 μg/l and 0.38 to >40,000 μg/l, respectively. EC_{50} of sea nine 211 were in the range of 0.42–12 μg/l. EC_{50} and LC_{50} of diuron were in the range of 4.3–43,000 μg/l and 5.9 to >127,000 μg/l, respectively. EC_{50} and LC_{50} of chlorothalonile were in the range of 4.4–390 μg/l and 12–110 μg/l, respectively. EC_{50} of dichlofluanid was in the range of 87–1,050 μg/l. EC_{50} of tolylfluanid was in the range of 9.9–405 μg/l. EC_{50} and LC_{50} of PTPB were in the range of 2.2–140 μg/l and 54 μg/l, respectively.

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EC_{50} of TCMTB ((2-thiocyanomethylthio) benzothiazole) was in the range of 46–433 μg/l.

Judging from toxicity data, most of these alternative biocides concentrations which were detected in the aquatic environment were below the level that causes an adverse effect in aquatic organisms.

Keywords Alternative biocide • Water • Sediment • Biota • Concentration • Toxicity

3.1 Introduction

Organotin (OT) compounds leaching from antifouling paints have caused many deleterious effects, including imposex and abnormal shell morphology, on nontarget aquatic organisms (Gibbs et al. 1988; Waldock and Thain 1983). As a result, OTs have led to a decrease in aquatic products (Bryan et al. 1986). OT application to large vessels has been banned or restricted in some countries since the 1980s. Even after the enforcement of the regulation, OTs have been detected at higher concentrations in water, sediment, and biota from harbours, marinas, and estuaries, particularly where boat activity is high and the water flushes poorly (Harino et al. 1998a, b). Furthermore, comparatively high concentrations of butyitin and phenyltin compounds have been detected in sediments, mussels, fish, and marine mammals (Tanabe et al. 1998; Harino et al. 1999, 2002, 2003, 2007a; Midorikawa et al. 2004). In October 2001, the International Maritime Organization (IMO) adopted the International Convention on the Control of Harmful Antifouling Systems (AFS Convention), which prohibited the use of OTs as active ingredients in antifouling systems for ships. Following the international restrictions on the use of OT-based antifoulants, paint manufacturers have developed many products as alternatives to the use of OTs. More than 20 chemical substances have been used or proposed as alternative compounds. When these antifouling biocides from the hulls of ships, fishing nets, etc. are released into the aquatic environment, these chemicals are distributed to water, sediment, and aquatic organisms. Therefore, it is important to identify the level of the antifouling biocides in the aquatic environment. Furthermore, the effects of alternative biocides on aquatic organisms are of concern, because most alternative biocides have been used as pesticides.

The research on contamination of alternative compounds in the aquatic environment started in early 1990. Since then many papers have been published. The distributions of representative antifouling biocides such as Irgarol 1051 (3-methylthio-4-tetrabutylamino-6-cyclopropylamino-*s*-triazine), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), sea nine 211 (2-*n*-octyl-4,5-dichloro-2-methyl-4-isothiazolin-3-one), chlorothalonil (2,4,5,6-tetrachloro-isophthalonitrile), dichlofluanid (*N*′-dimethyl-*N*-phenylsulphamide), zinc pyrithiones (zinc complex of 2-mercaptopyridine-1-oxide), copper pyrithiones (copper complex of 2-mercaptopyridine-1-oxide), and PTPB (pyridine triphenylborane)

in water, sediment, and biological samples and the effects of antifouling biocides on aquatic organisms are reviewed in this chapter.

3.2 Occurrences of Antifouling Biocide

3.2.1 Concentration in Aquatic Environment

There are many papers concerning the concentrations of Irgarol 1051. The concentrations of Irgarol 1051, which were reported between 1993 and 2012, are shown in Table 3.1. The presence of Irgarol 1051 in the surface waters of marinas on the Cote d'Azur, Monaco, was first reported at concentrations of up to 1,700 ng/l in 1993 by Readman et al. (1993). Since then, the occurrence of Irgarol 1051 has been reported in various European countries. The concentrations of Irgarol 1051 in coastal waters of England, German, Greece, Spain. Monaco, and Bermuda showed ranges of <0.5–1,424, 2.2–900, 11–440, 3–665, 5–1,700, and 10–590 ng/l, respectively (Readman et al. 1993; Gough et al. 1994; Toth et al. 1996; Tolosa et al. 1996; Connelly et al. 2001; Scarlett et al. 1999; Biselli et al. 2000; Ferrer and Barcelo 2001; Thomas et al. 2001, 2002; Boxall et al. 2000; Voulvoulis et al. 2000; Sargent et al. 2000; Sakkas et al. 2002; Martinez et al. 2001; Bowman et al. 2003; Lambert et al. 2006; Zhou 2008). Irgarol 1051 was detected in the range of 1–254 ng/l in water from the USA (Sapozhnikova et al. 2013). It was reported that Irgarol 1051 was also detected in Asian countries. The concentrations of Irgarol 1051 in Japan, Korea, and Malaysia were ranged <0.8–267, <1–14, and <1–2,012 ng/l, respectively (Okamura et al. 2000a; Harino et al. 2004; Eguchi et al. 2010; Liu et al. 1999; Ali et al. 2013). These levels found in the estuary may cause photosynthetic inhibition for nontarget algae (Sargent et al. 2000). GS26575 (2-methylthio-4-tert-buthylamino-6-amino-*s*-triazine) was detected in the area where Irgarol 1051 was detected. Okamura et al. (2000a) reported that the maximum concentration of GC26575 was 1,870 ng/l in the Seto Inland Sea of Japan (Tables 3.1 and 3.2).

The concentrations of diuron and its degradation compounds are shown in Table 3.2. Several studies have measured diuron in the coastal waters of England. The concentrations of diuron in water varied in each area, and its maximum concentration was 6,742 ng/l in Crouch Estuary, England (Thomas et al. 2001). The levels of diuron reported in England, Spain, Japan, USA, and Korea were in the range of <1–6,742, 2–1,030, <0.7–1,540, 2–68, and 35–1,360 ng/l, respectively. Concentrations of diuron reported in these countries were similar levels to those in England.

As the degradation products of diuron, DCPMU (1-(3,4-dichlorophenyl)-3-methylurea), DCPU (1-(3,4-dichlorophenyl)urea), and CPMU (1-(3-chlorophenyl)-3,1-dimethylurea) were confirmed to have been produced in the aquatic environment, and the concentrations of DCPMU, DCPU and CPMU were in the range of <1–78, <1–6, and <1–5 ng/l, respectively (Thomas et al. 2002). Thomas et al. (2002) reported

Table 3.1 Concentrations of Irgarol 1051 and its degradation compounds in water samples (means in parentheses)

Chemical	Location	Year	Concentrations (ng/l)	References
Irgarol 1051	Mediterranean Sea (Cote d'Azur), Monaco	1993	5–1,700	Readman et al. (1993)
	Medway estuary, England	1993	4–18 (11)	Gough et al. (1994)
	Humble estuary, England	1993	12–190 (84)	Gough et al. (1994)
	The Solent and English Channel, England	1993	2–11	Gough et al. (1994)
	Lake Geneva, Switzerland	1994–1995	2.5–145	Toth et al. (1996)
	Mediterranean Sea (Cote d'Azur), Monaco	1995	1.5–640	Tolosa et al. (1996)
	Hamilton Harbour, Bermuda, England	1995	10–590 (112)	Connelly et al. (2001)
	Seto Inland Sea, Japan	1996–1997	<5–264 (19)	Liu et al. (1999a)
	Plymouth Sound, England	1997	<1–127 (40)	Scarlett et al. (1999)
	North Sea, Germany	1997	11–170 (51)	Bissilli et al. (2000)
	Baltic Sea, Germany	1997	60–440 (266)	Bissilli et al. (2000)
	Masnou marina, Spain	1997–1998	3–119 (37)	Ferrer and Barcelo (2001)
	Crouch estuary, England (yachting season)	1998	<1–49 (9.2)	Thomas et al. (2001)
	Crouch estuary, England (off season)	1998	<1–9.4 (8.4)	Thomas et al. (2001)
	Southampton, England (yachting season)	1998	<1–141 (23)	Thomas et al. (2001)
	Southampton, England (off season)	1998	<1–1,421 (405)	Thomas et al. (2001)
	Sutton Harbour, England (yachting season)	1998	<1–84 (8.5)	Thomas et al. (2001)
	Sutton Harbour, England (off season)	1998	11–80 (54)	Thomas et al. (2001)
	Seto Inland Sea, Japan	1998	<5–142 (29)	Okamura et al. (2000a)
	Hamble estuary, England	1998–1999	(25)	Boxall et al. (2000)
	Owell estuary, England	1998–1999	(48)	Boxall et al. (2000)
	Blackwater estuary, England	1998–1999	<50–680 (148)	Voulvoulis et al. (2000)
	Corwy marina, England	1999	7–543 (126)	Sargent et al. (2000)
	Coastal area, Greece	1999–2000	2.2–900 (16)	Sakkas et al. (2002)

(continued)

Table 3.1 (continued)

Chemical	Location	Year	Concentrations (ng/l)	References
	Mediterranean Sea, Spain	1999–2000	20–665	Martinez et al. (2001)
	Florida (Biscayne Bay), USA	1999–2000	<1–60.9 (5.2)	Gardinali et al. (2002)
	Brighton marina, England	1999–2001	<1–964 (32)	Bowman et al. (2003)
	Southampton, England	2000	<1–305 (36)	Thomas et al. (2002)
	River, East Anglia, UK	2001	1–1,310 (259)	Lambert et al. (2006)
	Broads, East Anglia, UK	2001	12–2,430 (430)	Lambert et al. (2006)
	Broads, East Anglia, UK	2001	3–231 (48)	Lambert et al. (2006)
	The Port of Osaka, Japan	2002–2003	<0.8–267 (13)	Harino et al. (2004)
	Southern England	2004–2005	<3.1–89 (10)	Zhou (2008)
	Maizuru Bay, Japan	2007	2–18 (7)	Harino et al. (2010)
	California, USA	2008	2–254 (67)	Sapozhnikova et al. (2013)
	Jinhae Bay, Korea	2009	<0.1–14 (2.6)	Kim et al. (2014)
	Peninsular Malaysia, Malaysia	2011–2012	<1–2,012 (186)	Ali et al. (2013)
GS26575	Seto Inland Sea, Japan	1997–1998	<1–1,870 (15)	Okamura et al. (2000a)
	Masnou marina, Spain	1997–1998	4–23 (10)	Ferrer and Barcelo (2001)
	Southampton, England	2000	<1–59 (8)	Thomas et al. (2002)
	River, East Anglia, UK	2001	1–139 (16)	Lambert et al. (2006)
	Southern England	2004–2005	<0.5–30 (5.5)	Zhou (2008)
	Maizuru Bay, Japan	2007	<1.9	Harino et al. (2010)
	California, USA	2008	1–62 (18)	Sapozhnikova et al. (2013)

that the concentrations of DCPMU were high, because DCPMU is the primary degradation product of diuron in aerobic conditions.

There are many papers concerning the concentrations of sea nine 211 in water (Table 3.3). Sea nine 211 was not detected in water from marinas on the south coast of the UK and Maizuru Bay, Japan (Thomas et al. 2002; Eguchi et al. 2010).

Table 3.2 Concentrations of diuron and its degradation compounds in water samples

Chemical	Location	Year	Concentrations (ng/l)	References
Diuron	Crouch estuary, England (yachting season)	1998	<5–305 (36)	Thomas et al. (2001)
	Crouch estuary, England (off season)	1998	0.6–117 (22)	Thomas et al. (2001)
	Southampton, England (yachting season)	1998	1–33 (2265)	Thomas et al. (2001)
	Southampton, England (off season)	1998	<1–101 (8.3)	Thomas et al. (2001)
	Sutton Harbour, England (yachting season)	1998	<1–6,742 (90)	Thomas et al. (2001)
	Sutton Harbour, England (off season)	1998	<1–8.7 (14)	Thomas et al. (2001)
	Hamble estuary, England	1998–1999	(123)	Boxall et al. (2000)
	Owall estuary, England	1998–1999	(208)	Boxall et al. (2000)
	Mediterranean Sea, Spain	1999–2000	2–1,030	Martinez et al. (2001)
	Southampton, England	2000	16–1,249 (310)	Thomas et al. (2002)
	River, East Anglia, UK	2001	8–1,169 (58)	Lambert et al. (2006)
	Broads, East Anglia, UK	2001	65–249 (112)	Lambert et al. (2006)
	The Port of Osaka, Japan	2002–2003	<0.7–1,540 (126)	Harino et al. (2004)
	Maizuru Bay, Japan	2007	0.01–0.26 (0.08)	Eguchi et al. (2010)
	California, USA	2008	2–68 (6)	Sapozhnikova et al. (2013)
	Jinhae Bay, Korea	2009	35–1,360 (172)	Kim et al. (2014)
DCPMU	Southampton, England	2000	<1–78 (19)	Thomas et al. (2002)
DCPU	Southampton, England	2000	<1–6 (<1)	Thomas et al. (2002)
CPMU	Southampton, England	2000	<1–5 (<1)	Thomas et al. (2002)

Martinez et al. (2001) reported that sea nine 211 was detected in water from the Mediterranean Sea of Spain in the range of 2,600–3,700 ng/l. Furthermore, sea nine 211 in coastal water of Greece and Japan was detected in the range of 6.3–49 and <1–4 ng/l, respectively (Sakkas et al. 2002; Harino et al. 2004).

There are a few papers concerning chlorothalonil, dichlofluanid pyrithions, and PTPB (Table 3.3). Chlorothalonil was not detected in water from Southampton,

Table 3.3 Concentrations of the other alternative biocides in water samples

Chemical	Location	Year	Concentrations (ng/l)	References
Sea nine 211	Mediterranean Sea, Spain	1999–2000	2,600–3,700	Martinez et al. (2001)
	Coastal area, Greece	1999–2000	6.3–49	Sakkas et al. (2002)
	Southampton, England	2000	<1	Thomas et al. (2002)
	The Port of Osaka, Japan	2002–2003	<0.3–4 (2)	Harino et al. (2004)
	Maizuru Bay, Japan	2007	<1	Harino et al. (2010)
Chlorothalonil	Blackwater estuary, England	1998–1999	<200–1,380 (252)	Voulvoulis et al. (2000)
	Southampton, England	2000	<1	Thomas et al. (2002)
Dichlofluanid	Blackwater estuary, England	1998–1999	<24	Voulvoulis et al. (2000)
	Coastal area, Greece	1999–2000	5.2–55 (21)	Sakkas et al. (2002)
	Mediterranean sea, Spain	1999–2000	<20	Martinez et al. (2001)
	Southampton, England	2000	<1	Thomas et al. (2002)
Pyrithion	Maizuru Bay, Japan	2007	<80	Harino et al. (2010)
PTPB (pyridine triphenylborane)	Hiroshima Bay, Japan	2011	0.0048–0.021 (0.01)	Mochida et al. (2012)

England and the Mediterranean Sea, Spain. However, the average concentration of chlorothalonil from Blackwater Estuary, England, was 252 ng/l (Voulvoulis et al. 2000). Dichlofluanid was not detected in water samples from most coastal areas. However, dichlofluanid was detected at 52–55 ng/l in the coastal waters of Greece (Sakkas et al. 2002). Pyrithiones (zinc pyrithione and copper pyrithione) were not detected in water samples of Maizuru Bay, Japan (Harino et al. 2010). PTPB, however, was detected in water in the range 4.8–21 pg/l (Mochida et al. 2012).

The concentrations of Irgarol 1051 in sediment are summarized in Table 3.4. Irgarol 1051 was detected in the range of <0.1–45 μg/kg dry in England (Thomas et al. 2002; Zhou 2008). In sediment from ASEAN countries, Irgarol 1051 was detected as 0.05–4.0 μg/kg dry, <0.1–4.9 μg/kg dry, and <0.02–14 μg/kg dry for Vietnam, Thailand, and Malaysia, respectively (Harino et al. 2004, 2007b, 2009b). The concentrations of Irgarol 1051 in sediment of the Port of Osaka, Otsuchi Bay, and Maizuru Bay, Japan were 7–816, <0.05–100, and <0.08–9.8 μg/kg dry, respectively (Harino et al. 2004, 2007b; Eguchi et al. 2010). Irgarol 1051 was

Table 3.4 Concentrations of Irgarol 1051 and GS26575 in sediment samples

Chemical	Location	Year	Concentrations (μg/kg dry)	References
Irgarol 1051	Southampton, England	2000	<0.1–0.3 (0.2)	Thomas et al. (2002)
	Southampton, England	2000	0.3–3.5 (0.8)	Thomas et al. (2002)
	Coastal area, Vietnam	2002	0.05–4.0	Harino et al. (2006a)
	The Port of Osaka, Japan	2002–2003	7–816 (641)	Harino et al. (2004)
	Gulf of Thailand, Thailand	2004	<0.1–4.9 (0.62)	Harino et al. (2006b)
	Southern England	2004–2005	<1.7–45 (16)	Zhou (2008)
	Otsuchi Bay, Japan	2005	<0.05–100 (4.7)	Harino et al. (2007b)
	Nankai Trough, Japan	2006	<0.1–0.2 (0.08)	Harino et al. (2009a)
	Peninsular Malaysia, Malaysia	2006	<0.02–14 (1.7)	Harino et al. (2009b)
	Melaka, Malaysia	2006	<0.02–0.21 (0.09)	Harino et al. (2009b)
	Johor, Malaysia	2006	<0.02–0.9 (0.25)	Harino et al. (2009b)
	Suruga Bay, Japan	2006–2007	0.1 (0.1)	Harino et al. (2009a)
	Tosa Bay, Japan	2007	<0.1–0.2 (0.08)	Harino et al. (2009a)
	Maizuru Bay, Japan	2007	<0.08–9.8 (3.9)	Harino et al. (2010)
GS26575	Coastal area, Vietnam	2002	<0.1–0.43	Harino et al. (2006a)
	Gulf of Thailand, Thailand	2004	0.03–3.2 (0.50)	Harino et al. (2007a)
	Southern England	2004–2005	<0.9–14 (4.3)	Zhou (2008)
	Otsuchi Bay, Japan	2005	<0.18–0.47 (0.04)	Harino et al. (2007b)
	Peninsular Malaysia, Malaysia	2006	<0.09	Harino et al. (2009b)
	Melaka, Malaysia	2006	<0.09–0.49 (0.04)	Harino et al. (2009b)
	Johor, Malaysia	2006	<0.09	Harino et al. (2009b)
	Maizuru Bay, Japan	2007	<0.18	Harino et al. (2010)

also detected even in sediment collected in the deep sea (water depth, 4000 m) of Nankai Trough, Suruga Bay, and Tosa Bay, Japan, although the concentrations were in the range of <0.1–0.2 μg/kg dry, which were lower than those detected in the coastal areas (Harino et al. 2009a).

The concentrations of diuron in sediment are shown in Table 3.5. Diuron was detected in various areas of England at concentrations in the range of <0.31–3,500 μg/kg dry (Gough et al. 1994; Thomas et al. 2000, 2002; Boxall et al. 2000; Voulvoulis et al. 2000). Furthermore, diuron was detected in sediments of Switzerland and Germany in the range of <0.2–8 and 3–220 μg/kg dry, respectively (Toth et al. 1996; Biselli et al. 2000). The concentrations of diuron in ASEAN countries such as Vietnam, Thailand, and Malaysia were <0.02–9.9 μg/kg dry (Harino et al. 2004, 2006, 2009b). Diuron was also detected in sediment of the coastal area and deep sea such as Nankai Trough, Suruga Bay, and Tosa Bay, Japan with concentrations of <0.2–1,350 μg/kg dry and <0.02–12 μg/kg dry, respectively (Harino et al. 2004, 2009a). The degradation product of diuron was not detected in sediment from England (Thomas et al. 2002).

The concentrations of sea nine 211 detected in sediment are summarized in Table 3.6. Harino et al. (2004) reported that concentrations of sea nine 211 in sediment from Japan ranged 0.04–150 μg/kg dry. It was reported that sea nine 211 was detected in sediment of ASEAN countries in the range of <0.02–4.2 μg/kg dry (Harino et al. 2006a, b; 2009b).

Chlorothalonil is known to have been detected in sediments of England at concentrations of <0.1–688 μg/kg dry (Voulvoulis et al. 2000; Thomas et al. 2002). On the other hand, no detection of dichlofluanid in sediment has been reported. Pyrithions were detected in sediment from Vietnam and Japan at concentrations of 422 μg/kg dry and 22 μg/kg dry, respectively (Harino et al. 2006a, 2007b).

There are only a few papers concerning the detection of alternative biocides in biological samples in benthic animals (Table 3.7). Irgarol 1051 was detected in the range of <0.1–35 μg/kg in benthos such as clam, mussels and oysters from Vietnam, Thailand, and Japan (Harino et al. 2006a, b, 2007b, 2010). The concentrations of diuron and sea nine 211 detected in biota were 0.1–9.6 μg/kg and <0.1–0.3 μg/kg, respectively.

3.2.2 Geological Distribution of Antifouling Biocides

Sargent et al. (2000) pointed out that the concentrations of Irgarol 1051 in water were not influenced by salinity, pH, or temperature and that there was a strong positive correlation between average concentrations of Irgarol 1051 and the density of boating activity. Concentrations of Irgarol 1051 in the lock marina were high and its concentrations in the open marina were low (Boxall et al. 2000). The lock marina was directly adjacent to the slipway where paint particles from pressure-washing and paint scraping may have entered the marina. Higher concentrations of Irgarol

Table 3.5 Concentrations of diuron and its degradation compounds in sediment samples

Chemical	Location	Year	Concentrations (μg/kg dry)	References
Diuron	Hamble estuary, England	1993	<10–132	Gough et al. (1994)
	Lake Geneva, Switzerland	1994–1995	<0.2–8	Toth et al. (1996)
	North Sea, Germany	1997	3–25 (12.5)	Bisslli et al. (2000)
	Baltic Sea, Germany	1997	4–220 (67.8)	Bisslli et al. (2000)
	Southampton, England	1998	<1–11 (8.2)	Thomas et al. (2000)
	Hamble estuary, England	1998–1999	6.3	Boxall et al. (2000)
	Orwell estuary, England	1998–1999	257	Boxall et al. (2000)
	Blackwater estuary, England	1998–1999	<0.31–222.3 (145)	Vouvoulis et al. (2000)
	Southampton, England	2000	0.4–6.2 (1.7)	Thomas et al. (2002)
	Southampton, England	2000	300–3,500 (880)	Thomas et al. (2002)
	Coastal area, Vietnam	2002	0.11–3.0	Harino et al. (2006a)
	The Port of Osaka, Japan	2002–2003	0.637–1,350 (39.4)	Harino et al. (2004)
	Gulf of Thailand, Thailand	2004	<0.08–5.7 (3.5)	Harino et al. (2006b)
	Otsuchi Bay, Japan	2005	0.06–530 (25.1)	Harino et al. (2007b)
	Nankai Trough, Japan	2006	<0.2–0.5 (0.2)	Harino et al. (2009a)
	Peninsular Malaysia, Malaysia	2006	<0.02–4.8 (0.84)	Harino et al. (2009b)
	Melaka, Malaysia	2006	<0.02–4.1 (0.51)	Harino et al. (2009b)
	Johor, Malaysia	2006	<0.02–9.9 (2.1)	Harino et al. (2009b)
	Suruga Bay, Japan	2006–2007	0.4–1.5 (0.95)	Harino et al. (2009a)
	Tosa Bay, Japan	2007	<0.2	Harino et al. (2009a)
	Maizuru Bay, Japan	2007	<0.08–12 (5.4)	Harino et al. (2010)

(continued)

Table 3.5 (continued)

Chemical	Location	Year	Concentrations (μg/kg dry)	References
DCPMU (1-(3,4-dichlorophenyl)-3-methylurea)	Southampton, England	2000	<0.1	Thomas et al. (2002)
DCPU (1-(3,4-dichlorophenyl) urea)	Southampton, England	2000	<0.1	Thomas et al. (2002)
CPMU (1-(3-chlorophenyl)-3,1-dimethylurea)	Southampton, England	2000	<0.1	Thomas et al. (2002)

1051 were determined in areas of both high yachting activity such as mooring areas and the marina (Thomas et al. 2001). Diuron shows a similar geological distribution to Irgarol 1051 (Boxall et al. 2000; Thomas et al. 2001). Harino et al. (2004) reported that drastically higher concentrations of alternative biocides were observed at certain locations, where small- and medium-hull vessels were moored in poorly flushed zones. These papers imply that the concentrations of alternative biocides tend to be higher in areas of high shipping activity with poor flushing zones.

Interestingly, it is reported that in marinas of England, the concentration of diuron were higher than those of Irgarol 1051 (Boxall et al. 2000). Harino et al. (2004) reported that the Port of Osaka, Japan showed a similar pattern to the marinas of England. Thus, higher concentrations of diuron were observed in marinas and trading ports. On the other hand, Liu et al. (1999) reported that Irgarol 1051 was found more frequently in fishery harbors than in marinas. These findings indicate that the levels of Irgarol 1051 and diuron seem to depend on the utilization form of ports, harbors, or marinas.

3.2.3 Seasonal Variation of Alternative Biocides

Comber et al. (2002) surveyed the concentrations of diuron and Irgarol 1051 in water from the Hamble and Orwell estuaries during the summer and winter seasons. Concentrations of diuron and Irgarol 1051 in water were significantly higher in summer compared with winter. The variation between summer and winter can be attributed to the decreased density of boats in winter. Biselli et al. (2000) reported that a seasonal dependence of Irgarol 1051 concentrations was found in both water and sediment samples, with maxima during the periods of March–May/July–September, whereas during the winter period of December–January low values were encountered, although measurable amounts remained in sediment. Albanis et al. (2002) monitored Irgarol 1051, chlorothalonil, and dichlofluanid in sediment. Maximum and minimum values were observed during the period June–September

Table 3.6 Concentrations of the other alternative biocides in sediment samples

Chemical	Location	Year	Concentrations (μg/kg dry)	References
Sea nine 211	Southampton, England	2000	<0.1	Thomas et al. (2002)
	Coastal area, Vietnam	2002	0.09–1.3	Harino et al. (2006a)
	The Port of Osaka, Japan	2002-2003	<0.2–2.35 (0.516)	Harino et al. (2004)
	Gulf of Thailand, Thailand	2004	<0.04–0.09 (0.01)	Harino et al. (2006b)
	Otsuchi Bay, Japan	2005	<0.04–150 (6.5)	Harino et al. (2007b)
	Peninsular Malaysia, Malaysia	2006	<0.04–1.7 (013)	Harino et al. (2009b)
	Melaka, Malaysia	2006	<0.02–4.2 (0.42)	Harino et al. (2009b)
	Johor, Malaysia	2006	<0.04–0.92 (0.19)	Harino et al. (2009b)
	Nankai Trough, Japan	2006	0.1–1.0 (0.44)	Harino et al. (2009a)
	Suruga Bay, Japan	2006–2007	0.2–1.2 (0.55)	Harino et al. (2009a)
	Tosa Bay, Japan	2007	<0.2	Harino et al. (2009a)
	Maizuru Bay, Japan	2007	<0.04–7.2 (1.1)	Harino et al. (2010)
Chlorothalonil	Blackwater estuary, England	1998–1999	<4.1–46.5 (14.6)	Vouvoulis et al. (2000)
	Southampton, England	2000	<0.1	Thomas et al. (2002)
Dichlofluanid	Blackwater estuary, England	1998–1999	<4.9–688.2 (567)	Vouvoulis et al. (2000)
	Southampton, England	2000	<0.1	Thomas et al. (2002)
	Coastal area, Vietnam	2002	<0.10–13	Harino et al. (2006a)
	Otsuchi Bay, Japan	2005	<0.4–14 (0.44)	Harino et al. (2007b)
	Peninsular Malaysia, Malaysia	2006	<0.1	Harino et al. (2009b)
	Melaka, Malaysia	2006	<0.1	Harino et al. (2009b)
	Johor, Malaysia	2006	<0.1	Harino et al. (2009b)

(continued)

Table 3.6 (continued)

Chemical	Location	Year	Concentrations (μg/kg dry)	References
Pyrithione	Coastal area, Vietnam	2002	<2–420	Harino et al. (2006a)
	Otsuchi Bay, Japan	2005	<8–8.8 (1.2)	Harino et al. (2007b)
	Peninsular Malaysia, Malaysia	2006	<20	Harino et al. (2009b)
	Melaka, Malaysia	2006	<20	Harino et al. (2009b)
	Johor, Malaysia	2006	<20	Harino et al. (2009b)
	Maizuru Bay, Japan	2007	<8	Harino et al. (2010)

and during the winter period (December–February), respectively. Thomas et al. (2001) reported that the concentrations of Irgarol 1051 were low in the yachting season in comparison of those in the off season; however, diuron was contrastingly high.

3.3 Adverse Effect of Alternative Biocides

3.3.1 Toxicity of Each Antifouling Biocide

It is found that high concentrations of alternative biocides were detected in various coastal areas. It is important to clarify how these alternative compounds would impact marine organisms to consider the ecological risk involved by those compounds. Toxicity data of Irgarol 1051 and its degradation compounds (GS26575) are summarized in Tables 3.8 and 3.9.

EC_{50} values for cyanobacteria, microalgae, periphyton, and cnidarians were in the range of 0.01–23 μg/l (Mohr et al. 2008; Okamura et al. 2000b; Scarlett et al. 1997, 1999; Lambert et al. 2006). Estimated values of EC_{50} of crustaceans were higher than those of cyanobacteria, microalgae, periphyton, and cnidarians, which were in the range of 10.8–50,800 μg/l (Okamura et al. 2000b; Toth et al. 1996; Fernandez-Alba et al. 2002). Crustaceans seemed to be insensitive species for Irgarol 1051. LC_{50} and NOEC of the common stonewort *Chara vulgaris* for Irgarol 1051 were 0.0168 μg/l and 0.0005 μg/l, respectively, which seems very sensitive to Irgarol 1051 (Lambert et al. 2006). It is well known that Irgarol 1051 effectively functions by blocking a pivotal step in the electron transport of photosystem II (PS II). The lower EC_{50} values of microalgae are considered the result of photosynthesis inhibition by Irgarol 1051.

Table 3.7 Concentrations of alternative biocides in biological samples

Chemical	Location	Year	Biota	Concentrations (μg/kg)	References
Irgarol 1051	Coastal area, Vietnam	2002	Clam	<0.10	Harino et al. (2006b)
	Awaji Island, Japan	2003	Mussel	1.1–4.7 (23.1)	Harino et al. (2010)
	Awaji Island, Japan	2003	Oyster	5–35 (11.9)	Harino et al. (2010)
	Gulf of Thailand, Thailand	2004	Mussel	<1	Harino et al. (2006b)
GS26575	Coastal area, Vietnam	2002	Clam	<0.20	Harino et al. (2006a)
	Awaji Island, Japan	2003	Mussel	<0.1	Harino et al. (2010)
	Awaji Island, Japan	2003	Oyster	<0.1–0.2 (0.1)	Harino et al. (2010)
	Gulf of Thailand, Thailand	2004	Mussel	<0.76	Harino et al. (2006b)
Diuron	Coastal area, Vietnam	2002	Clam	<0.20	Harino et al. (2006a)
	Awaji Island, Japan	2003	Mussel	<0.1–0.6 (0.1)	Harino et al. (2010)
	Awaji Island, Japan	2003	Oyster	0.1–0.2 (0.18)	Harino et al. (2010)
	Gulf of Thailand, Thailand	2004	Mussel	<0.64–9.6 (2.8)	Harino et al. (2006b)
Sea nine 211	Coastal area, Vietnam	2002	Clam	<0.10	Harino et al. (2006a)
	Awaji Island, Japan	2003	Mussel	<0.1	Harino et al. (2010)
	Awaji Island, Japan	2003	Oyster	0.1–0.3 (0.14)	Harino et al. (2010)
	Gulf of Thailand, Thailand	2004	Mussel	<0.24–0.24 (0.04)	Harino et al. (2004)
Dichlofluanid	Coastal area, Vietnam	2002	Clam	<0.20	Harino et al. (2006a)

LC_{50} values of GS26575, which was a degradation product of Irgarol 1051 for microalgae, were in the range of 73–83 μg/l (Gatidou and Thomaidis 2007), and NOEC of GS26575 for the microalgae *Chara vulgaris*, *Myriophyllum spicatum*, and *Apium modiflorum* were ranged from <0.00005 to 0.5 μg/l (Lambert et al. 2006). Thus, the toxicity of GS26575 to microalgae might be greater than that of Irgarol 1051.

Diuron has been used as a herbicide. Observed values of EC_{50} and LC_{50} for diuron and its degradation compounds are shown in Table 3.10. EC_{50} values of diuron for various organisms were 4.3–8600 μg/l (Lambert et al. 2006; Fernandez-

Table 3.8 EC_{50} and LC_{50} of Irgarol 1051

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LDC_{50} (μg/l)	References
Irgarol 1051	Periphyton	Chlorophytes	Mortality	0.34 (135 days)		Mohr et al. (2008)
		Epithemia adnata	Mortality	0.09 (58 days)		Mohr et al. (2008)
	Macrophytes	*Closterium ehrenbergii*	Sporophyte growth	2.5 (5 days)		Okamura et al. (2000b)
			Embryogenesis	3.6 (5 days)		Okamura et al. (2000b)
		Eisenia bicyclis	Gametophyte growth	5.9 (4 days)		Okamura et al. (2000b)
		Enteromorpha intestinalis	Zoospore germination	<2.5 (72 h)		Scarlett et al. (1997)
		Lemna gibba	Frond growth	11 (7 days)		Okamura et al. (2000b)
		Lemna minor	Frond growth	8.1 (7 days)		Okamura et al. (2000b)
		Porphyra yezoensis	Concospore growth	4.1 (4 days)		Okamura et al. (2000b)
			Concospore growth	0.6 (4 days)		Okamura et al. (2000b)
		Chara vulgaris	Photosynthesis	0.0168 (14 days)		Lambert et al. (2006)
		Myriophyllum spicatum	Photosynthesis	>2 (14 days)		Lambert et al. (2006)
		Apium nodiflorum	Photosynthesis	>2 (14 days)		Lambert et al. (2006)
		Zostera marina	Photosynthesis	0.2 (36 days)		Scarkett et al. (1999)
	Cnidarians	*Acropora formosa*	Photosynthesis	1.3 (10 h)		Jones et al. (2003)
		Galaxea fascicularis	Photosynthesis	5 (96 h)		Sheikn et al. (2009)
	Crustacea	*Megacyclops viridis*	Mortality	0.33 (92 days)		Mohr et al. (2008)
		Cyclopoid copepods	Mortality	0.09 (78 days)		Mohr et al. (2008)
		Cladocerans	Mortality	1.21 (148 days)		Mohr et al. (2008)
		Ostracods	Mortality	0.11 (148 days)		Mohr et al. (2008)
		Selenastrum capricornutum	Mortality	1.6 (72 h)		Fernandez-Alba et al. (2002)
		Artemia salina	Mortality		>40,000 (24 h)	Okamura et al. (2000b)
		Daphnia magna	Mortality	8,100 (48 h)	16,000 (24 h)	Toth et al. (1996)
			Mortality		8,300 (48 h)	Okamura et al. (2000b)
		Daphnia pulex	Mortality		5,700 (24 h)	Okamura et al. (2000b)
		Thamnocephalus platyurus	Mortality		12,000 (214 h)	Okamura et al. (2000b)

Table 3.9 EC_{50} and LC_{50} of Irgarol 1051 and GS26575

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
Irgarol 1051	Crustacea	*Selenastrum capricornutum*	Mortality	10.8 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	7,300 (48 h)		Fernandez-Alba et al. (2002)
		Vibrio fischeri	Mortality	50,800 (15 min)		Fernandez-Alba et al. (2002)
		Balanus amphitrite (larvae)	Mortality		2,200 (24 h)	Bao et al. (2011)
		Elasmopus rapax (juvenile)	Mortality		1,000 (96 h)	Bao et al. (2011)
		Tigriopus japonicus (adult)	Mortality		2,400 (96 h)	Bao et al. (2011)
		Oryzias merastigma (larvae)	Mortality		1,000 (96 h)	Bao et al. (2011)
	Cyanobacteria	*Chroococcus minor*	Growth	5.7 (7 days)		Bao et al. (2011)
		Synechococcus sp.	Growth	23 (96 h)		Bao et al. (2011)
	Microalgae	*Skeletonema costatum*	Growth	0.57 (96 h)		Bao et al. (2011)
		Thalassiosira pseudonana	Growth	0.38 (96 h)		Bao et al. (2011)
		Pyrocystis lunula	Growth	>15,000 (24 h)		Bao et al. (2011)
		Dunaliella tertiolecta	Growth		1.1 (96 h)	Gatidou and Thomaidis. (2007)
		Navicura forcipata	Growth		0.6 (96 h)	Gatidou and Thomaidis. (2007)
	Sea anemone	*Aiptasia* sp.	Growth			Bao et al. (2011)
	Polychaete	*Hydroides elegans* (larvae)	Mortality		2,600 (48 h)	Bao et al. (2011)
	Fish	*Oncorhynchus mykiss* (juvenile)	Mortality		880 (28 days)	Okamura et al. (2002)
GS26575	Microphytes	*Chara vulgaris*	Photosynthesis	>0.5 (14 days)		Lambert et al. (2006)
		Myriophyllum spicatum	Photosynthesis	>0.5 (14 days)		Lambert et al. (2006)
		Apium nodiflorum	Photosynthesis	>0.5 (14 days)		Lambert et al. (2006)
	Microalgae	*Dunaliella tertiolecta*	Growth		83 (96 h)	Gatidou and Thomaidis (2007)
		Navicura forcipata	Growth		73 (96 h)	Gatidou and Thomaidis (2007)

Table 3.10 EC_{50} and LC_{50} of diuron and its degradation compounds

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
Diuron	Ascidians	*Myriophyllum spicatum*	Photosynthesis	>5 (14 days)		Lambert et al. (2006)
		Apium nodiflorum	Photosynthesis	>5 (14 days)		Lambert et al. (2006)
	Cyanobacteria	*Chroococcus minor*	Growth	4.7 (7 days)		Bao et al. (2011)
		Synechococcus sp.	Growth	110 (96 h)		Bao et al. (2011)
	Crustacea	*Selenastrum capricornutum*	Mortality	45 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	8600 (48 h)		Fernandez-Alba et al. (2002)
		Balanus amphitrite (larvae)	Mortality		21,000 (24 h)	Bao et al. (2011)
		Elasmopus rapax (juvenile)	Mortality		>3,000 (96 h)	Bao et al. (2011)
		Tigriopus japonicus (adult)	Mortality		11,000 (96 h)	Bao et al. (2011)
	Coral	*Acropora tumida* (larvae)	Mortality		4,800 (24 h)	Bao et al. (2011)
	Microalgae	*Skeletonema costatum*	Growth	5.9 (96 h)		Bao et al. (2011)
		Thalassiosira pseudonana	Growth	4.3 (96 h)		Bao et al. (2011)
		Pyrocystis lunula	Growth	43,000 (24 h)		Bao et al. (2011)
		Dunaliella tertiolecta	Growth		5.9 (96 h)	Gatidou and Thomaidis (2007)
		Navicura forcipata	Growth		27 (96 h)	Gatidou and Thomaidis (2007)
		Dunaliella tertiolecta	Growth		8,500 (96 h)	Gatidou and Thomaidis (2007)
		Hormosira banksii	Germination	6.82 (72 h)		Myers et al. (2006)
	Sea anemone	*Aiptasia* sp.	Acute toxicity		>19,000 (96 h)	Bao et al. (2011)
	Polychaete	*Hydroides elegans* (larvae)	Acute toxicity		16,000 (48 h)	Bao et al. (2011)
		Hormosira banksii	Rhizoid growth	7.33 (72 h)		Myers et al. (2006)
	Fish	*Oryzias merastigma* (larvae)	Mortality		7,800 (96 h)	Bao et al. (2011)
		Oncorhynchus mykiss (juvenile)	Mortality		230 (28 days)	Okamura et al. (2002)
DCPMU	Microalgae	*Dunaliella tertiolecta*	Growth		345 (96 h)	Gatidou and Thomaidis (2007)
DCA	Microalgae	*Dunaliella tertiolecta*	Growth		6,381 (96 h)	Gatidou and Thomaidis (2007)
DCA	Microalgae	*Navicura forcipata*	Growth		6,269 (96 h)	Gatidou and Thomaidis (2007)

alba et al. 2002; Bao et al. 2011). LC_{50} of diuron for microalgae was in the range 5.9 to >19,000 μg/l (Bao et al. 2011; Gatidou and Thomaidis 2007). Generally, toxicity of diuron for microalgae was rather strong, which was almost the same level as Irgarol 1051. Diuron is degraded to DCPMU and DCA in the aquatic environment. LC_{50} values of DCPMU and DCA were reported as 345–6,381 μg/l (Gatidou and Thomaidis 2007). Acute toxicities of degradation products of diuron, therefore, were recognized to be weaker than those of diuron.

Table 3.11 represents the observed values of EC_{50} and LC_{50} for sea nine 211 and chlorotharonil. EC_{50} values of chlorothalonil for cyanobacteria, microalgae, and crustaceans were 150–390 μg/l, 64.4–190 μg/l, and 0.8–28 μg/l, respectively (Fernandez-alba et al. 2002; Bao et al. 2011). LC_{50} in polychaetes was in the range of 12 μg/l in 48 h, and LC_{50} of crustaceans were 67–110 μg/l in 96 h (Bao et al. 2011). Estimated values of 48 h LC_{50} (48-h LC_{50}) and 96-h LC_{50} (96-h LC_{50}) of chlorothalonil for polychaetes and crustaceans were 12 μg/l and in the range of 67–110 μg/l, respectively (Bao et al. 2011). Observed values of EC_{50} and LC_{50} of sea nine 211 for crustaceans were in the range of 0.42–12 μg/l (Myers et al. 2006) and 14 μg/l, respectively (Okamura et al. 2002).

In addition to alternative biocides, which were reviewed in the section on occurrences of antifouling paint, the toxicity data of tolylfluanid (*N*′-dimethyl-*N*-*p*-tolylsulfamide), TCMTB (2-(thiocyanomethylthio)benxothazole), and zineb (zinc ethylene bis-(dithocarbamete)) were reported. Estimated values of EC_{50} and LC_{50} for dichlofluanid, tolylfluanid, PTPB, TCMTB, and Zineb are shown in Table 3.12. EC_{50} values of dichlofluanid for crustaceans ranged from 81 to 1050 μg/l (Fernandez-alba et al. 2002; Bellas 2006). Because tolyfluonid has a similar molecular structure to dichlofluanid, the toxicity of tolyfluonid was compared to that of dichlofluanid. EC_{50} values of dichlofluanid and tolyfluonid for embryonic development and larval growth of mussels, sea urchins, and chordata were in the range of 9.9–627 μg/l and 74–405 μg/l, respectively (Bellas 2006). On the basis of the literature, it was also found that tolyfluonid had a similar toxicity to dichlofluanid. On the other hand, EC_{50} values of PTPB for algae were 2.2–140 μg/l and EC_{50} for crustaceans were 6.6–100 μg/l, respectively (Mochida et al. 2012). LC_{50} values of PTPB for invertebrates and fishes were 54 μg/l and 42–420 μg/l, respectively (Mochida et al. 2012; Okamura et al. 2002, 2009). EC_{50} values of TCMTB ranged from 46 to 433 μg/l (Fernandez-alba et al. 2002) Toxicity of zineb for a species of brown algae, Neptune's necklace (*Houmosira banksii*), was high because of its EC_{50} in the range of 0.241–0.49 μg/l (Myers et al. 2006).

Observed values of EC_{50} and LC_{50} for ZnPT and CuPT are shown in Tables 3.13 and 3.14, respectively. Those of EC_{50} and LC_{50} of ZnPT were 0.19–280 μg/l and 4.6–410 μg/l, respectively (Bao et al. 2011; Myers et al. 2006; Onzuka et al. 2010); those of EC_{50} and LC_{50} of CuPT were 0.7–50 and 1.3–2000 μg/l, respectively (Bao et al. 2011; Okamura et al. 2002; Onzuka et al. 2010).

Table 3.11 EC_{50} and LC_{50} of chlorothalonil and sea nine 211

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
Chlorothalonil	Chordata	*Ciona intestinalis*	Embryonic development	33 (24 h)		Bellas (2006)
		Ciona intestinalis	Larval attachment	42 (24 h)		Bellas (2006)
	Cyanobacteria	*Chroococcus minor*	Growth	150 (7 days)		Bao et al. (2011)
		Synechococcus sp.	Growth	390 (96 h)		Bao et al. (2011)
	Crustacea	*Elasmopus rapax* (juvenile)	Mortality		67 (96 h)	Bao et al. (2011)
		Tigriopus japonicus (adult)	Mortality		91 (96 h)	Bao et al. (2011)
		Oryzias merastigma (larvae)	Mortality		110 (96 h)	Bao et al. (2011)
		Selenastrum capricornutum	Mortality	6.8 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	28 (48 h)		Fernandez-Alba et al. (2002)
	Polychaete	*Hydroides elegans* (larvae)	Mortality		12 (48 h)	Bao et al. (2011)
	Microalgae	*Skeletonema costatum*	Growth	13 (96 h)		Bao et al. (2011)
		Thalassiosira pseudonana	Growth	4.4 (96 h)		Bao et al. (2011)
		Pyrocystis lunula	Growth	190 (24 h)		Bao et al. (2011)
	Mussel	*Mytilus edulis*	Embryonic development	8.7 (48 h)		Bellas (2006)
	Sea urchin	*Paracentrotus lividus*	Embryonic development	6.6 (48 h)		Bellas (2006)
Sea nine 211	Chordata	*Ciona intestinalis*	Embryonic development	104 (24 h)		Bellas (2006)
		Ciona intestinalis	Larval attachment	43 (24 h)		Bellas (2006)
	Crustacea	*Selenastrum capricornutum*	Mortality	3 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	4 (48 h)		Fernandez-Alba et al. (2002)
		Vibrio fischeri	Mortality	12 (15 min)		Fernandez-Alba et al. (2002)
		Hormosira banksii	Germination	0.42 (72 h)		Myers et al. (2006)
		Hormosira banksii	Rhizoid growth	0.46 (72 h)		Myers et al. (2006)
		Oncorhynchus mykiss (juvenile)	Mortality		14 (28 days)	Okamura et al. (2002)
	Sea urchin	*Paracentrotus lividus*	Embryonic development	12 (48 h)		Bellas (2006)
		Paracentrotus lividus	Larval growth	25 (48 h)		Bellas (2006)
	Mussel	*Mytilus edulis*	Embryonic development	11 (48 h)		Bellas (2006)

Table 3.12 EC_{50} and LC_{50} of dichlofluanid, tolylfluanid, PTPB, TCMTB, and Zineb

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
Dichlofluanid	Chordata	*Ciona intestinalis*	Embryonic development	282 (24 h)		Bellas (2006)
		Ciona intestinalis	Larval attachment	128 (24 h)		Bellas (2006)
	Crustacea	*Selenastrum capricornutum*	Mortality	133 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	1,050 (48 h)		Fernandez-Alba et al. (2002)
		Vibrio fischeri	Mortality	87 (min)		Fernandez-Alba et al. (2002)
	Sea urchin	*Paracentrotus lividus*	Embryonic development	627 (48 h)		Bellas (2006)
	Mussel	*Mytilus edulis*	Embryonic development	81 (48 h)		Bellas (2006)
Tolylfluanid	Chordata	*Ciona intestinalis*	Embryonic development	217 (24 h)		Bellas (2006)
		Ciona intestinalis	Larval attachment	95 (24 h)		Bellas (2006)
	Sea urchin	*Paracentrotus lividus*	Embryonic development	405 (48 h)		Bellas (2006)
		Paracentrotus lividus	Larval growth	9.9 (48 h)		Bellas (2006)
	Mussel	*Mytilus edulis*	Embryonic development	74 (48 h)		Bellas (2006)
PTPB	Crustacea	*Portunus trituberculatus*	Mortality	100 (24 h)		Mochida et al. (2012)
		Tigriopus japonicus	Mortality	6.6 (24 h)		Mochida et al. (2012)
		Artemia salina	Mortality		54 (48 h)	Okamura et al. (2009)
	Algae	*Chaetocerus calcitrans*	Growth	4.4 (72 h)		Mochida et al. (2012)
		Dunaliella tertiolecta	Growth	140 (72 h)		Mochida et al. (2012)
		Skeletonema costatum	Growth	2.2 (72 h)		Mochida et al. (2012)
		Tetraselmis tetrathele	Growth	2.9 (72 h)		Mochida et al. (2012)
	Fish	*Pagrus major* (juvenile)	Mortality		345 (96 h)	Mochida et al. (2012)
		Fundulus heteroclitus (larvae)	Mortality		420 (96 h)	Mochida et al. (2012)
		Oncorhynchus mykiss (juvenile)	Mortality		42 (28 days)	Okamura et al. (2002)
TCMTB	Crustacea	*Selenastrum capricornutum*	Mortality	433 (72 h)		Fernandez-Alba et al. (2002)
		Daphnia magna	Mortality	46 (48 h)		Fernandez-Alba et al. (2002)
		Vibrio fischeri	Mortality	58 (15 min)		Fernandez-Alba et al. (2002)
Zineb	Macroalga	*Hormosira banksii*	Germination	0.49 (72 h)		Myers et al. (2006)
		Hormosira banksii	Rhizoid growth	1.51 (72 h)		Myers et al. (2006)

Table 3.13 EC_{50} and LC_{50} of Zn pyrithione (ZnPT)

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
ZnPT	Crustacea	*Balanus amphitrite* (larvae)	Mortality		210 (24 h)	Bao et al. (2011)
		Elasmopus rapax (juvenile)	Mortality		29 (96 h)	Bao et al. (2011)
		Tigriopus japonicus (adult)	Mortality		170 (96 h)	Bao et al. (2011)
		Skeletonema costatum	Mortality	1.6 (72)		Onzuka et al. (2010)
	Cyanobacteria	*Chroococcus minor*	Growth	50 (7 days)		Bao et al. (2011)
		Synechococcus sp.	Growth	22 (96 h)		Bao et al. (2011)
	Coral	*Acropora tumida* (larvae)	Mortality		180 (24 h)	Bao et al. (2011)
	Sea anemone	*Aiptasia* sp.	Mortality		410 (96 h)	Bao et al. (2011)
	Polychaete	*Hydroides elegans* (larvae)	Mortality		7.6 (48 h)	Bao et al. (2011)
	Microalgae	*Skeletonema costatum*	Growth	1.7 (96 h)		Bao et al. (2011)
		Thalassiosira pseudonana	Growth	0.51 (96 h)		Bao et al. (2011)
		Pyrocystis lunula	Growth	44 (24 h)		Bao et al. (2011)
		Hormosira banksii	Germination	0.19 (72 h)		Myers et al. (2006)
		Hormosira banksii	Rhizoid growth	0.24 (72 h)		Myers et al. (2006)
		Dunaliella tertiolecta	Growth	8.0 (72 h)		Onzuka et al. (2010)
		Tetraselmis tetrathele	Growth	19 (72 h)		Onzuka et al. (2010)
		Chaetoceros calcitrans	Growth	4.5 (72 h)		Onzuka et al. (2010)
	Fish	*Oryzias merastigma* (larvae)	Mortality		43 (96 h)	Bao et al. (2011)
		Oncorhynchus mykiss (juvenile)	Mortality		4.6 (28 days)	Okamura et al. (2002)
		Tigriopus japonicus	Mortality	280 (24 h)		Onzuka et al. (2010)
		Pagurau major	Mortality	98.2 (24 h)		Onzuka et al. (2010)

Table 3.14 EC_{50} and LC_{50} of Cu pyrithione (CuPT)

Chemical	Taxon	Species	Endpoint	EC_{50} (μg/l)	LC_{50} (μg/l)	References
CuPT	Crustacea	*Balanus amphitrite* (larvae)	Mortality		63 (24 h)	Bao et al. (2011)
		Elasmopus rapax (juvenile)	Mortality		11 (96 h)	Bao et al. (2011)
		Tigriopus japonicus (adult)	Mortality		30 (96 h)	Bao et al. (2011)
		Skeletonema costatum	Mortality	1.5 (72 h)		Onduka et al. (2010)
	Cyanobacterium	*Chroococcus minor*	Growth	50 (7 days)		Bao et al. (2011)
		Synechococcus sp.	Growth	22 (96 h)		Bao et al. (2011)
	Coral	*Acropora tumida* (larvae)	Growth		28 (24 h)	Bao et al. (2011)
	Sea anemone	*Aiptasia* sp.	Mortality		2,000 (96 h)	Bao et al. (2011)
	Polychaete	*Hydroides elegans* (larvae)	Mortality		5.7 (48 h)	Bao et al. (2011)
	Microalgae	*Skeletonema costatum*	Growth	1.8 (96 h)		Bao et al. (2011)
		Thalassiosira pseudonana	Growth	0.70 (96 h)		Bao et al. (2011)
		Pyrocystis lunula	Growth	23 (24 h)		Bao et al. (2011)
		Dunaliella tertiolecta	Growth	7.3 (72 h)		Onzuka et al. (2010)
		Tetraselmis tetrathele	Growth	12 (72 h)		Onzuka et al. (2010)
		Chaetoceros calcitrans	Growth	3.2 (72 h)		Onzuka et al. (2010)
	Fish	*Oryzias merastigma* (larvae)	Mortality		8.2 (96 h)	Bao et al. (2011)
		Oncorhynchus mykiss (juvenile)	Mortality		1.3 (28 days)	Okamura et al. (2002)
		Tigriopus japonicus	Mortality	23 (24 h)		Onduka et al. (2010)
		Pagurau major	Mortality	9.3 (96 h)		Onduka et al. (2010)

3.3.2 Mixture Toxicity

Usually, alternative biocides have been used as mixtures of these compounds. Therefore, the adverse effect of alternative biocides to aquatic organisms must be evaluated as a mixture of compounds that are actually included in antifouling biocide products. Various evaluating methods for the mixture toxicity have been proposed. Here, three representative methods are described.

Mixture toxicity index (MTI) has been often used to evaluate the toxicity of a mixture. To calculate the MTI, toxic unit (TU) was determined by the sum of the ratios of antifouling chemical concentrations to their effective concentrations (24-h LC_{50}) (Verslyche et al. 2003).

$$\text{Expected toxicity (TU)} = \Sigma Cmi,a/Cmi,e$$

where Cmi,a is nominal concentration; Cmi,e is effective concentration (24-h LC_{50}).

The MTI (mixture toxicity index) was determined, according to the following equation, on the basis of the methodology originally described by Konemann (1981).

$$\text{MTI} = 1 - (\log M/\log M0)$$

where M is the sum of the concentrations that was expressed as equal fractions of the 24-h LC_{50} of each component (M = sum TUi); $M0$ is M divided by the largest fraction in the mixture ($M0 = M/\max(\text{TUi})$). MTI = 0 gives antagonism and no addition, partial addition gives $0 < \text{MTI} < 1$, strict addition and synergism gives $\text{MTI} > 1$.

Koutsaftis and Aoyama (2007) evaluated the effect of the binary mixtures for brine shrimp, *Artemia salina*, by MTI. The binary mixtures, two agents consisting of 0.2, 0.4, 0.5, and 0.8 times the 24-h LC_{50} for one chemical with the other chemical constituting the remaining percentage by its 24-h LC_{50} times the fraction amount, determined the combination ratio. For the mixtures of ZnPT and CuPT, all propositions of mixtures indicated synergistic effect. The mixtures of chlorothalonil and CuPT gave antagonistic effect in all propositions of mixture. For chlorothalonil and ZnPT, CuPT and diuron, diuron and ZnPT, and diuron and chlorothalonil, effects for brine shrimp depend on the proportions of the mixture.

Fernandez-Alba et al. (2002) evaluated the toxicity of mixtures using *Vibrio fischeri*, *Selenastrum capricornotum*, and *Daphnia magna*. A binary mixture of Irgarol 1051 and diuron, and Irgarol 1051 and TCMTB, showed the synergistic effect for these three species. A binary mixture of sea nine 211 showed antagonistic effect for *V. fischeri* and additive effect for *S. capricornotum* and *D. magna*. Although a binary mixture of Irgarol 1051 and chlorothalonil showed synergistic effect for *V. fischeri* and *S. capricornotum*, they showed antagonistic effects for *D. magna*. A binary mixture of Irgarol 1051 and dichlofluanid showed additive

effect for *V. fischeri* and *S. capricornotum* and showed synergistic effect for *D. magna*. Thus, effects of mixtures were various for organisms.

In the other method, Abotts' formula has been often used to compare expected and observed inhibitions:

$$C\text{exp} = A + B - (AB/100)$$

where *C*exp is expected inhibitions (%); *A* or *B* is inhibitions caused when compounds act alone.

The ratio of inhibition (RI) for each mixture of compounds was calculated as follows.

$$\text{RI} = \text{observed inhibition}/C\text{exp}$$

where RI values > 1 are synergism; RI values $= 1$ are additivity; RI values < 1 are antagonism.

Gatidou and Thomaidis (2007) evaluated the effect of a mix of the antifouling biocides and their metabolites or copper by this method. A binary mixture of Irgarol 1051 and GS26575 revealed additive effects on the growth of a species of green alga, *Dunaliella tertiolecta*. Coexistence of Irgarol 1051 and copper had shown additivity as well. A binary mixture of GS26575 and copper resulted in additive effects when a low concentration of copper (2000 μg/l) combined with GS26575 and synergism when a high concentration of copper (4000 μg/l) was used. Combination of diuron with either DCPMU or DCA resulted in synergistic effects. Copper, when either diuron or DCPMU was used together, showed antagonistic effects. A binary mixture of both mixtures (Irgarol 1051-GS26575 and diuron-DCA) for a species of diatoms, *Navicula forcipata*, were similar to those observed for *Dunaliella tertiolecta*. All binary concentration levels of diuron and DCA were found to show synergistic effects for *Navicula forcipata*.

Mochida et al. (2006) investigated the joint toxicity by using LC_{50} values of pyrithiones and copper. As a result, the joint toxicity of the ZnPT and Cu mixture was more than the additive toxicities of CuPT and Cu in toy shrimp, *Heptacarpus futilirostris*.

3.4 Conclusion

After the worldwide ban of TBT, various alternative biocides have been detected in the marine environment.

Fortunately, detection frequencies of alternative biocides in biological samples were low; however, higher concentrations of these alternative biocides were detected in water and sediment. Although the concentrations of alternative biocides detected in coastal water samples from various areas were lower than their toxicity level for aquatic organisms, these compounds were transferred to deep-sea areas.

Some of the alternative biocides were easily degraded in water; however, little information has been available for the behavior and toxicity of degradation products in the aquatic environment. Further studies are needed to clarify the fate and effect of alternative biocides containing the degradation products.

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Part II
Contamination by Organotins and Organotin-Induced Imposex in Gastropod Mollusks

Chapter 4
Contamination by Organotins and Its Population-Level Effects Involved by Imposex in Prosobranch Gastropods

Toshihiro Horiguchi

Abstract A history of the production and use of organotin compounds is briefly introduced. The worldwide use of tributyltin (TBT)- or triphenyltin (TPhT)-based antifouling paints since the mid-1960s has caused extensive contamination in the aquatic environment, especially in the marine environment, which led to contamination of aquatic organisms by these compounds and became a concern in terms of both seafood safety and ecotoxicology. Legislation of TBT- and TPhT-based antifouling paints began in Europe and the U.S.A in the 1980s and in Japan in 1990. An International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention) for the worldwide ban of TBT- and TPhT-based antifouling paints came into force on 17 September 2008. Organotins have various toxicities to vertebrates and invertebrates; imposex is known to be induced in many gastropod species by TBT and also by TPhT released from antifouling paints on ships and fishing nets. Reproductive failure may be brought about in severely affected stages of imposex, resulting in population decline or mass extinction. Population-level effects involved by imposex and similar phenomena are described in the rock shell (*Thais clavigera*), the ivory shell (*Babylonia japonica*), and the giant abalone (*Haliotis madaka*), with special reference to tissue distributions of TBT, TPhT and their metabolites.

Keywords Imposex • Population decline • Reproductive failure • Rock shell (*Thais clavigera*) • Ivory shell (*Babylonia japonica*) • Abalone • Tributyltin (TBT) • Triphenyltin (TPhT) • Accumulation • Tissue distribution

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Abbreviations

AFS Convention	an International Convention on the Control of Harmful Anti-fouling Systems on Ships
DBT	dibutyltin
DPhT	diphenyltin
GC-FPD	gas chromatography with flame photometric detection
MBT	monobutyltin
MPhT	monophenyltin
RXR	retinoid X receptor
TBT	tributyltin
TBT-MMC	tributyltin methyl methacrylate copolymer
TBTO	bis (tributyltin) oxide
TcHT	tricyclohexyltin
TPhT	triphenyltin
TPrT	tripropyltin

4.1 Introduction

Although there have been several books, or book chapters, whose subjects are related to contamination and effects in aquatic organisms caused by organotin compounds such as tributyltin (TBT) and triphenyltin (TPhT), here I briefly introduce a history of contamination by organotin compounds in the aquatic environment; legislation on production, import, and usage of organotin compounds for antifouling paints; and scientific research on adverse effects by organotin compounds in aquatic organisms, especially focusing on imposex in gastropod mollusks.

More than 800 organotin compounds are known, and most of them are of anthropogenic origin, with the exception of methyltins, which can also be produced by biomethylation (Hoch 2001). There are a larger number of organotin derivatives in commercial use. An increase in the variety of commercial applications markedly increased the worldwide production of organotin compounds from less than 5000 tons (t) in 1955 to about 50,000 t in 1992 (Hoch 2001). The major application of organotin compounds (approximately 70 %) is the use of mono- and di-alkyltin derivatives as heat and light stabilizer additives in polyvinyl chloride (PVC) processing (Hoch 2001). It is well known that mainly tri-substituted organotin species have biocidal properties (WHO 1980, 1990). Therefore, these tri-substituted organotins have been used as fungicides, miticides, molluscicides, nematocides, ovicides, rodent repellants, wood preservatives, and antifouling paints, primarily containing TBT, TPhT, and TcHT as toxic additives. Biocidic products make up about 20 % of the total annual organotin production (Hoch 2001; Bennett 1996). TBT is the main organotin species used in antifouling paints worldwide. In Japan, however, TPhT as well as TBT was used in antifouling paints

for vessels and fishing nets from the mid-1960s to 1989 (Horiguchi et al. 1994). The total annual production and import of TBT and TPhT in Japan was 6340 t in 1989; approximately 70 % was used in antifouling paints for vessels and 20 % as antifouling for fishing nets (Horiguchi et al. 1994). The rest was used for agriculture, wood preservation, and other industrial purposes in Japan (Horiguchi et al. 1994).

As a consequence of this worldwide use of TBT- or TPhT-based antifouling paints on vessels and fishing nets, contamination of the aquatic environment by these compounds became a concern. Legislation on tri-organotin-based antifouling paints started in France in 1982, the U.K. in 1987, and the U.S.A. in 1988: vessels shorter than 25 m (excluding those made from aluminum) were prohibited from using tri-organotin-based antifouling paints (Stewart 1996; Bosselmann 1996). Vessels longer than 25 m were permitted to use tri-organotin-based antifouling paints if the maximum release rate of tri-organotin was less than 4 μg cm^{-2} day^{-1} (Stewart 1996; Bosselmann 1996). Similar legislation was introduced in Canada, Australia, and New Zealand in 1989 (Stewart 1996). Environmental quality standards were established for TBT and TPhT in the U.K. in 1989: 20 ng/l for both TBT and TPhT in freshwater and 2 ng/l (TBT) and 8 ng/l (TPhT) in seawater (Stewart 1996; Maruyama 1992). Ambient water quality criteria were also established for TBT in the U.S.A. in 1988 (U.S. EPA 1988). In Japan, the regulatory system for TBT and TPhT compounds is different from those in other countries, such as the U.K. and U.S.A. Since 1990, regulations for TBT and TPhT compounds have been implemented for each chemical species of TBT and TPhT in accordance with the law concerning the Examination and Regulation of the Manufacture, etc. of Chemicals in Japan. Since January 1990, the manufacture, import, and use of TBTO have been completely prohibited by law. As of September 1990, however, other TBT (13 substances, including TBT-MMC) and TPhT (7 substances) compounds were allowed to be used, manufactured, or imported if their expected amounts were reported to the Ministry of International Trade and Industry (MITI). Although it was permissible to use TBT- or TPhT-formulated antifouling paints on fishing nets and on any kind of ship or boat (including those shorter than 25 m) at that time, the sale of TPhT products in the Japanese domestic market had essentially ceased in June 1989 under the administrative guidance of MITI. However, administrative guidance by Ministries and Agencies of the Government imposes no penalties and therefore differs from legal regulation. Such guidance systems are typical of regulatory systems in Japan. Similarly, the manufacture, import, and use of TBT compounds (excluding TBTO) had also been controlled by the administrative guidance of MITI, the Ministry of Transport, and the Government's Fisheries Agency from July 1990, but the mass media reported that the sale of TBT products to the Japanese domestic market had completely ceased by April 1997. No ambient water quality criteria have been established for TBT and TPhT in Japan (Horiguchi 2012). Meanwhile, in other Asian countries, for example, in Korea, since March 2000 the use of antifoulants that contain tri-organotins at more than 0.1 % has been prohibited on fishing nets and on small vessels (including fishing boats) using coastal waters and harbor facilities (see Chap. 7 in this volume).

To introduce effective international regulation of the use of tri-organotin-formulated antifoulants, a first proposal was made at the 29th Session of the Marine Environment Protection Committee (MEPC 29) of the International Maritime Organization (IMO) in March 1990. Following MEPC 29, a resolution on measures to control potential adverse impacts associated with the use of TBT compounds in antifouling paints was adopted at MEPC 30 in November 1990: For example, governments specifically consider the following actions, such as (a) eliminate the use of antifouling paints containing TBT compounds on non-aluminum-hulled vessels shorter than 25 m and (b) eliminate the use of antifouling paints containing TBT compounds that have average organotin release rates of more than 4 μg cm^{-2} day^{-1} (Horiguchi 2012). Unfortunately, however, adoption of the resolution at MEPC 30 in November 1990 did not result in the establishment of a new treaty or convention toward the total prohibition of the use of TBT compounds in antifouling paints for ships. At MEPC 38 in July 1996, Japan, the Netherlands, and some Northern European countries then proposed the need to establish a new treaty or convention aimed at worldwide total prohibition of the use of tri-organotin-formulated antifoulants, such as TBT compounds. In this proposal they took into account the temporal trends in contamination by TBT and TPhT in the marine environment, the adverse effects of TBT and TPhT as endocrine-disrupting chemicals, and the current status of development of alternatives to antifouling paints containing TBT compounds. MEPC set up a Correspondence Group for the Reduction of Harmful Effects of the Use of Antifouling Paints for Ships (chaired by the Netherlands) for the investigation. The results of the investigation performed by the Group were considered at MEPC 41 in March 1998. Finally, at its assembly in November 1999, the IMO decided to phase out TBT in antifouling paints over the period from 2003 to 2008. An International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention: 21 articles) was then adopted by the IMO on 5 October 2001 (Horiguchi 2012). According to the AFS Convention, all ships shall not apply or re-apply organotin compounds that act as biocides in antifouling systems after 1 January 2003, and all ships either (1) shall not bear organotin compounds that act as biocides in antifouling systems on their hulls or external parts or surfaces, or (2) shall bear a coating that forms a barrier to such compounds leaching from the underlying noncompliant antifouling systems after 1 January 2008. Because it had taken more time than expected for the AFS Convention to be ratified by member states, it finally came into force on 17 September 2008 (http://www.imo.org/). Continued monitoring is needed for marine/aquatic ecosystems to recover from the impacts of organotin pollution and to protect the marine/aquatic environment (Horiguchi 2012).

There are many reports on the levels of contamination by TBT and TPhT, including their metabolites, detected in the aquatic environment (e.g., review by Maguire 1996). For example, TBT at more than 1 μg/l has been detected in freshwater and seawater near marinas, harbors, and shipyards where severe contamination by antifoulants released from ships' hulls and old paint stripped from the hulls by surface blasting with water or abrasive slag fines has been observed (Maguire 1996; Batley 1996). A number of papers have also reported butyltin

and phenyltin contamination in aquatic invertebrates and vertebrates (Alzieu 1996; Takeuchi 1992). One study detected TBT at 750 ng/g and TPhT at 1770 ng/g (wet wt. basis) in the soft tissues of rock shell (*Thais bronni*) collected at Aburatsubo, Japan, in 1990 (Horiguchi et al. 1994). Concentrations of TBT and TPhT in muscle (i.e., edible part) of fishes and shellfishes were sometimes greater than ppm (= mg/l) levels around 1990, which meant a few pieces of those consumable seafoods reached or exceeded the levels of acceptable daily intake (ADI) of TBT [1.6 μg kg^{-1} (body wt.) day^{-1}] and TPhT [0.5 μg kg^{-1} (body wt.) day^{-1}] in Japan. Because TBT and TPhT are also very toxic to mammals, ADI values have been designated at 1.6 μg kg^{-1} (body wt.) day^{-1} for TBT and 0.5 μg kg^{-1} (body wt.) day^{-1} for TPhT (WHO 1980; Sugita 1992).

The worldwide usage of organotin compounds in antifouling paints for ships and fishing nets caused extensive contamination by organotins in the world: Concentrations of TBT in water, at maximum, reached parts per billion (ppb; = μg/l) levels near marinas, which was more than the threshold concentration of acute toxicities for sensitive species to TBT (Horiguchi and Shimizu 1992). Toxicities of organotin compounds to aquatic organisms are reviewed in several review papers and book chapters (e.g., reviews by Alzieu 1996; Hall and Bushong 1996). Lethal, developmental, behavioral, and reproductive toxicities as well as various other physiological toxic effects have been reported in aquatic invertebrates and vertebrates.

One of the typical adverse effects caused in aquatic organisms by organotin compounds, such as TBT and TPhT, is "imposex." The term imposex was defined by Smith (1971), meaning imposed sexual organs, to describe the syndrome of a superimposition of male genital tracts, such as penis and vas deferens, on female prosobranch gastropods, although the first report of masculinized female gastropod mollusks was made by Blaber (1970), describing a penis-like outgrowth behind the right tentacle in spent females of the dog whelk *Nucella lapillus* around Plymouth, U.K. Imposex is thought to be an irreversible syndrome (Bryan et al. 1986). Reproductive failure may be brought about in severely affected stages of imposex, resulting in population decline or mass extinction (Gibbs and Bryan 1986, 1996). Imposex is known to be induced in many species by TBT, and also by TPhT released from antifouling paints on ships and fishing nets (Bryan et al. 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1995, 1997a).

As of 2005, approximately 200 species of Caenogastropoda (formerly, Mesogastropoda and Neogastropoda) had been reported to be affected by imposex worldwide (Bech 2002a, b; Fioroni et al. 1991; Horiguchi et al. 1997b; Marshall and Rajkumar 2003; Shi et al. 2005; Sole et al. 1998; Ten Hallers-Tjabbes et al. 2003; Terlizzi et al. 2004); many of these gastropod species belong to the families Muricidae (e.g., *Nucella lapillus*, *Ocenebra erinacea*, *Thais clavigera*, and *Urosalpinx cinerea*), Buccinidae (e.g., *Babylonia japonica*, *Buccinum undatum*, and *Neptunea arthritica arthritica*), Conidae (e.g., *Conus marmoreus bandanus* and *Virroconus ebraeus*), and Nassariidae (e.g., *Ilyanassa obsoleta* and *Nassarius reticulatus*) of the Caenogastropoda (Fioroni et al. 1991; Horiguchi et al. 1997b).

Regarding Japanese gastropods, at least 39 species (7 mesogastropods and 32 neogastropods, at that time) have been found to be affected by imposex among 69 species examined (Horiguchi et al. 1997b; Horiguchi 2000). Although

imposex has been observed mostly in shallow-water species in previous surveys, detailed studies of species living at depths of 200 m or more should also be considered because of the discovery of imposex in alabaster false tun (*Galeoocorys leucodoma*) trawled from depths of 200–250 m off the Atsumi Peninsula, Japan, in 1999 (Horiguchi 2000).

The current status of contamination and imposex in prosobranch gastropods in Europe, China (including Hong Kong), and Korea is reviewed in the following chapters in this volume (Chaps. 5, 6, and 7). The mode of actions of organotins inducing the development of imposex is reviewed in another chapter (Chap. 9).

In this chapter, imposex and its population-level effects are reviewed in cases of the rock shell, *Thais clavigera*, and the ivory shell, *Babylonia japonica*. Endocrine disruption in abalone (*Haliotis madaka* and *H. gigantea*), which is similar to imposex, is also introduced.

4.2 Imposex and Organotins in the Rock Shell, *Thais clavigera*

The development of imposex is induced and promoted by certain organotins, such as tripropyltin (TPrT), TBT, tricyclohexyltin (TcHT), and TPhT in *Thais clavigera* (Horiguchi 2000). The effectiveness of these organotins promoting the development of imposex in *T. clavigera* is as follows: TPhT ≒ TBT > TcHT > TPrT (Horiguchi 2000). In case of TBT, imposex is induced in adult females of *T. clavigera* at an environmental TBT concentration of approximately 1 ng/l (Horiguchi et al. 1995). A variety of patterns of the development of penis and vas deferens was observed in imposex-exhibiting females of *T. clavigera* (Horiguchi 1993). Reproductive failure, mainly caused by a blockage of the vaginal opening (vulva) by the vas deferens formation or spermatogenesis in the ovary, together with a rare case of capsule gland split, was also observed in severely affected stages of imposex, resulting in population decline or mass extinction close to marinas and the inner part of the enclosed bay, although *T. clavigera* had a relatively longer period (approximately 2 months) of veliger larvae stages (Horiguchi 1993). The process of vas deferens and penis development is reviewed in *T. clavigera* as well as *B. japonica* in another chapter (Chap. 11).

County-wide surveys on imposex in *T. clavigera* have been conducted in Japan since 1990. Among rock shell (*T. clavigera*) samples collected between January 1999 and November 2001 from 174 locations along the Japan coast, imposex was observed at 166 locations, whereas no, or rare, cases were found at the remaining 8 locations. The percentage occurrence of imposex was as high as or close to 100 % in approximately half the affected locations surveyed. It is expected that spawning obstruction occurs in more than half the population of females when relative penis length (RPL) index exceeds 40, on the basis of the relationship between RPL index, vas deferens sequence (VDS) index, and the percentage occurrence of oviduct (vulva) blockage in females. Among the 174 locations, RPL index values exceeding

40 were found in 41 locations. High values of RPL and VDS indices were generally observed in the western part of Japan. Compared with the results of a previous survey (conducted between 1996 and 1999), the indices seemed to have decreased, but remained almost unchanged in some locations (Horiguchi 2004).

TPhT concentrations in tissues of the rock shell showed a decrease over time but varied distinctly between locations; relatively high pollution levels in a few locations were detected. Decreases in TBT concentrations were also distinct in general but the degree of decrease was lower than those in TPhT concentrations. Changes in concentrations over time were not observed in several locations. An increase in the concentrations of TBT was observed in two locations near fishing ports (Horiguchi 2004).

Specific accumulation of organotin compounds was examined in tissues of *T. clavigera*, using the rock shell specimens collected at a site neighboring a shipyard. These specimens had severe imposex symptoms, wherein penis length was rather long and vas deferens was well developed in females (i.e., imposex-exhibiting individuals), in which 91.0 % of individuals were recognized as sterile (Horiguchi et al. 2012). Because imposex of the rock shell *T. clavigera* is caused by TPhT as well as TBT (Horiguchi et al. 1997a), severe imposex symptoms can be attributed to severe contamination by TBT and TPhT.

Tissue concentrations of organotin compounds, such as butyltins and phenyltins, in imposex-exhibiting female and male *T. clavigera* specimens, determined by gas chromatography with flame photometric detection (GC-FPD), are shown in Figs. 4.1, 4.2, 4.3, and 4.4 (Horiguchi et al. 2012). Different tissue distributions were observed between butyltin and phenyltin compounds. The highest concentrations of TBT were detected in the osphradiums of females and the hearts of males

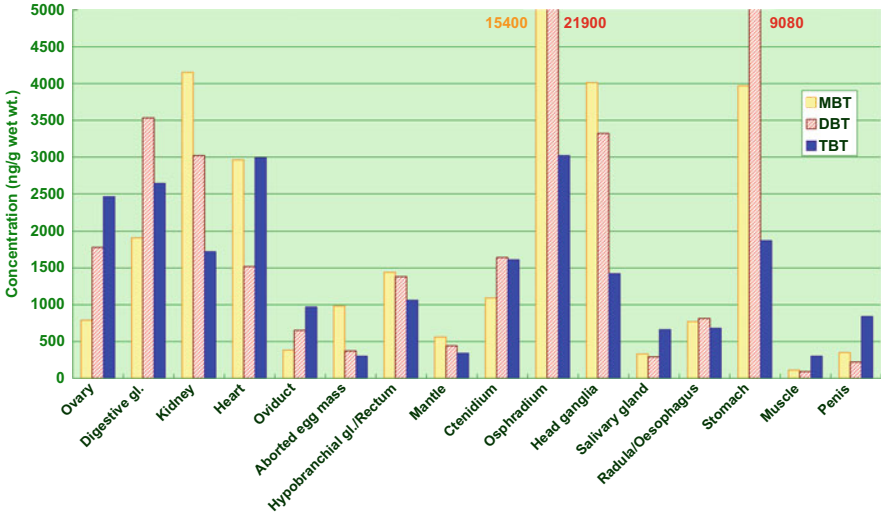

Fig. 4.1 Tissue distribution of butyltin compounds in imposex-exhibiting female rock shells (*Thais clavigera*) (Horiguchi et al. 2012)

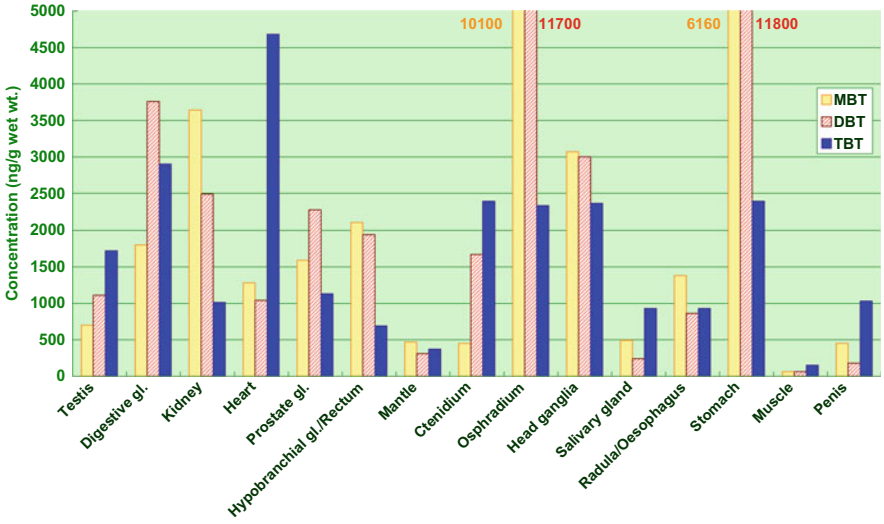

Fig. 4.2 Tissue distribution of butyltin compounds in male rock shells (*Thais clavigera*) (Horiguchi et al. 2012)

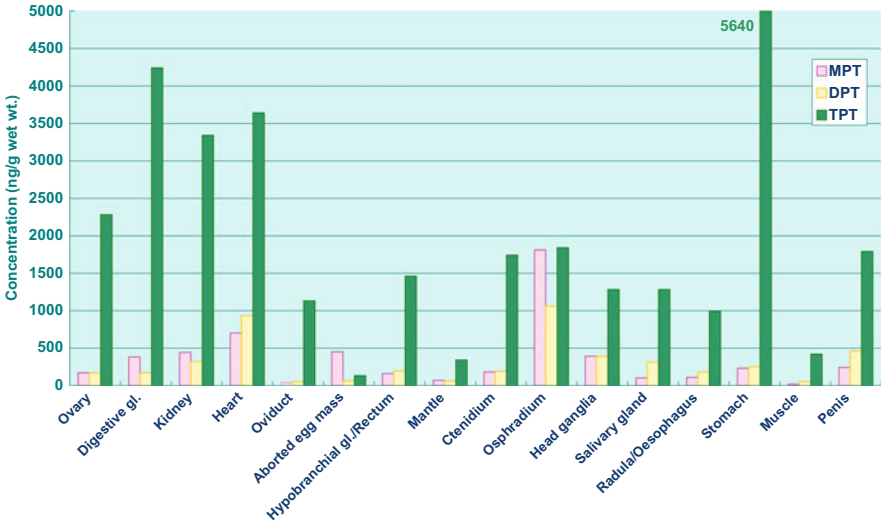

Fig. 4.3 Tissue distribution of phenyltin compounds in imposex-exhibiting female rock shells (*Thais clavigera*) (Horiguchi et al. 2012)

(Figs. 4.1 and 4.2). The highest concentrations of TPhT were detected in the stomachs of both females and males (Figs. 4.3 and 4.4). Concentrations of TBT and TPhT in tissues were not consistently higher in either females or males:

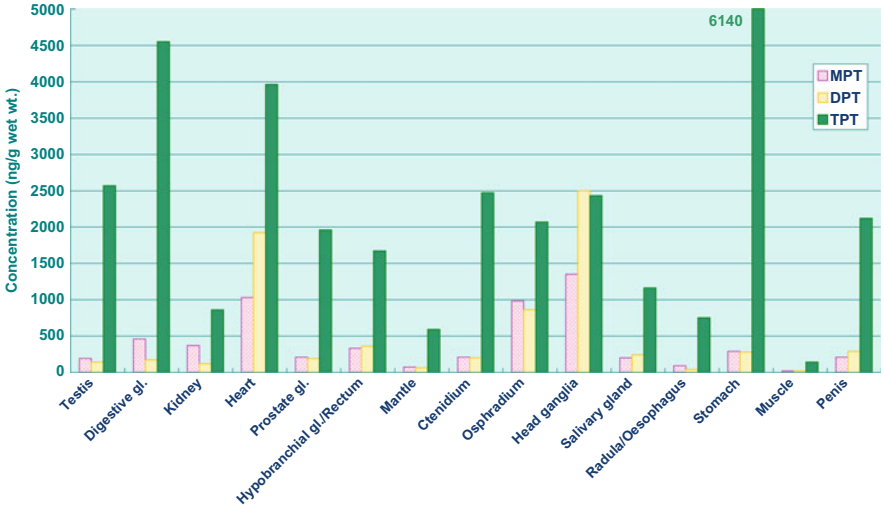

Fig. 4.4 Tissue distribution of phenyltin compounds in male rock shells (*Thais clavigera*) (Horiguchi et al. 2012)

sex-dependent difference was unclear for accumulation of TBT and TPhT in tissues, except for TPhT in kidney, in which TPhT concentration was more than three times higher in females than males. Regarding butyltins, the ratio of dibutyltin (DBT) and monobutyltin (MBT) to TBT was generally higher; TBT was predominant in ovary or testis, heart, oviduct (albumen, sperm-ingesting and capsule glands), ctenidium, salivary gland, muscle, and penis. Concerning phenyltins, however, TPhT was the most predominant in almost all tissues examined.

Based on the total body burden of TBT in *T. clavigera*, approximately one third or more of total TBT was accumulated in the digestive glands of both females and males (Horiguchi et al. 2012). Based on the total body burden of TPhT, approximately 40 % and one half of total TPhT accumulated in the digestive glands of females and males, respectively. The second highest tissue burden of TPhT was observed in the gonads of both females and males (Horiguchi et al. 2012).

Concentrations of TBT and TPhT in ovaries were 2460 and 2280 ng/g wet wt., respectively (Figs. 4.1, and 4.3), which were markedly higher levels than those reported in the literature (e.g., Laughlin 1996; Tanabe et al. 1998). Therefore, they may be an additional causal factor for the abortion of an egg capsule mass. Actually, marked accumulation of TBT or TPhT in the capsule gland, including aborted egg capsule mass, was reported in *Ocenebra erinacea* and *T. clavigera* (Gibbs et al. 1990; Horiguchi 1993). Regarding the total body burden of TBT, a similar accumulation pattern to *T. clavigera* was observed in *Babylonia japonica* (Horiguchi et al. 2006). Approximately half the total body TBT burden was accumulated in the capsule gland of *O. erinacea* (Gibbs et al. 1990), which may suggest a difference in organotin accumulation patterns among gastropod species.

Concentrations of TBT and TPhT in head ganglia, the central nervous system, of females and males were 1420 and 1280 ng/g wet wt. and 2370 and 2430 ng/g wet wt., respectively (Figs. 4.1, 4.2, 4.3, and 4.4), which were also rather high compared to those reported in the literature (Laughlin 1996; Tanabe et al. 1998). Although concentrations of TBT and TPhT in head ganglia were quite high in *T. clavigera*, the total tissue burden of those organotins was not high because of the relatively small ganglia tissue in *T. clavigera* (Horiguchi et al. 2012); this is also indicated with *B. japonica* (Horiguchi et al. 2006). Similar concentrations of TBT and TPhT were also detected in the ganglia of *Buccinum undatum* (Mensink et al. 1997). It is still obscure whether the neuroendocrine system is disturbed by marked accumulation of TBT and TPhT in head ganglia.

It is speculated that organotins, such as TBT and TPhT, in seawater and prey organisms are taken in via respiration at the ctenidium and digestion at the stomach, respectively, and then transported to various tissues or organs. Organotins are mainly metabolized at the digestive gland and excreted via kidney, although metabolism of organotins may be also performed by each tissue or organ. As remarkably high concentrations of organotins were also observed in heart and osphradium, except for tissues already mentioned, it is possible there are specific mechanisms of accumulation for organotins in heart and osphradium (Figs. 4.1, 4.2, 4.3, and 4.4).

Biological and ecological half-lives of TBT and TPhT were estimated as 22 days and 347 days, respectively, in *T. clavigera* (Horiguchi et al. 1995). The biological half-life of TBT was estimated to be between about 50 days and more than 100 days in *N. lapillus*, depending on conditions (Bryan et al. 1987). Relatively high tissue burdens of TBT and TPhT were observed in the reproductive organs (ovary, oviduct, and testis) and stomach, as well as in muscle and the ctenidium (Horiguchi et al. 2012).

As discussed in another chapter (Chap. 9), six hypotheses have been proposed to explain the mechanisms by which TBT induces imposex in gastropods: (1) an increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al. 1996); (2) an increase in testosterone levels caused by the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006); (3) TBT-mediated inhibition of excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason 1996); (4) TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983); (5) an increase in the level of an alanine-proline-glycine-tryptophan amide neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000); and (6) activation of RXR (Nishikawa et al. 2004). Among them, a hypothesis of activation of RXR seems definite, because there are several papers in which a hypothesis of activation of RXR is supported without any contradiction (Castro et al. 2007; Horiguchi et al. 2007, 2008, 2010a, b; Sternberg et al. 2008; Urushitani et al. 2011). However, it is still unclear whether transcription by RXR with organotins such as TBT and TPhT is activated in the presumptive penis-forming area behind the right tentacle of female *T. clavigera* or in the head ganglia, the central nervous system. Because

concentrations of TBT and TPhT in penises of female and male *T. clavigera* were much higher than those in the muscle (Figs. 4.1, 4.2, 4.3, and 4.4), it is likely that transcriptional activation of RXR by organotins is induced in the presumptive penis-forming area behind the right tentacle of female *T. clavigera*.

4.3 Collapse of Commercial Fisheries for the Ivory Shell *Babylonia japonica* in Japan: Reproductive Failure Involved by Imposex Possibly Induced by Organotins

The ivory shell *Babylonia japonica* (Caenogastropoda: Buccinidae), which inhabits sandy or muddy sediments in shallow water (approximately 10–20 m in depth) from the south of Hokkaido to Kyushu, Japan, is a scavenger in the inshore ecosystem, and traditionally a target species of commercial fisheries in Japan. Imposex seems to have been observed in the ivory shell since the 1970s (Kajikawa and Hamada, personal communication), and the total catch drastically decreased all over Japan in the late 1970s or early 1980s (Horiguchi and Shimizu 1992).

Much effort has been made to enhance the ivory shell stocks: seed production using adult ivory shells reared in hatcheries, with subsequent release of seeds/juveniles into the sea. Most seeds/juveniles of ivory shells released into the sea (approximately 90 % of total production in Japan) have been produced at a hatchery in Tomari, Tottori Prefecture, located in the western part of Japan (Horiguchi et al. 2006). In Tottori Prefecture, however, not only the total catch but also the number of egg capsules spawned by adult shells at the hatchery and seeds/juveniles artificially produced/released into the sea has decreased since the mid-1980s (Horiguchi et al. 2006) (Fig. 4.5). The total catch has drastically decreased since 1984, 2 years after the first observation of imposex-affected female ivory shells from Tottori Prefecture, involving the increase of both the percentage occurrence of imposex individuals and mean penis length in females (Hamada et al. 1988, 1989; Kajikawa 1984; Kajikawa et al. 1983) (Fig. 4.5). The number of egg capsules spawned by adult ivory shells at the hatchery, as well as the number of seeds/juveniles artificially released into the sea, has also decreased since the mid-1980s (Fig. 4.5). Introduction of adult ivory shells from another prefecture (Niigata Prefecture, Japan) to compensate for insufficient numbers of the normal brood stock also resulted in failure of the release of seeds/juveniles into the sea because of their high mortality at the hatchery before release (Fig. 4.5). Recovery of total catch of the ivory shell had not been observed in spite of such efforts to enhance the ivory shell stocks (Fig. 4.5). Finally, operation of the ivory shell hatchery for stock enhancement in Tottori had to be stopped, and the hatchery was closed in 1996 (Fig. 4.5). Therefore, possible reproductive failure involved by imposex in the ivory shell was suspected.

Horiguchi et al. (2006) examined the incidence of reproductive failure accompanied by imposex in the ivory shell, based on the histopathological observation of

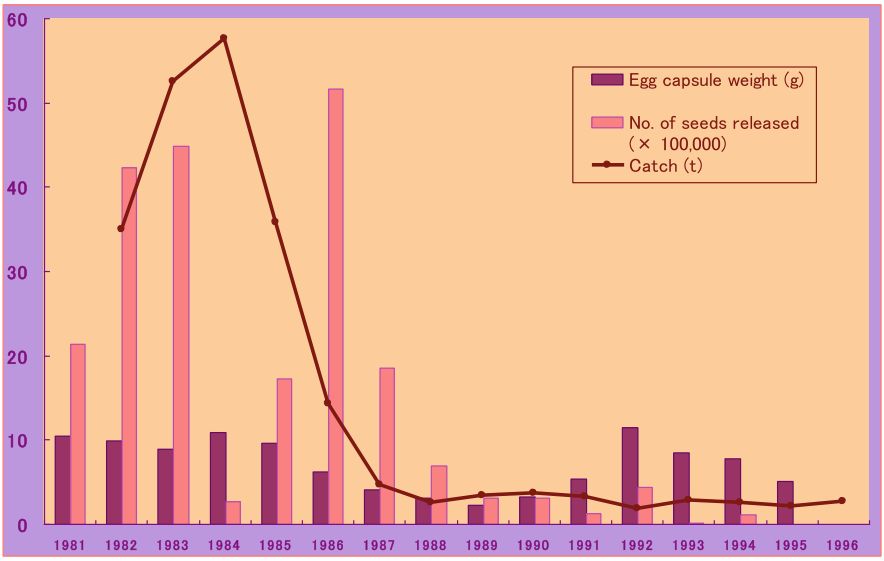

Fig. 4.5 Temporal trends in average weight of egg capsules spawned by an adult ivory shell, *Babylonia japonica*, at the Tottori hatchery, as well as numbers of seeds/juveniles released into the sea and the total catch off Tottori Prefecture, Japan

gonads, and investigated the relationship between organotin compounds and imposex in the ivory shell based on chemical analysis of organotin concentrations in the tissues. Horiguchi et al. (2006) also discussed the possibility that the marked decline in the ivory shell (*Babylonia japonica*) populations from Japan could have been brought about mainly by reproductive failure accompanied by imposex, induced by TBT and TPhT from antifouling paints.

The percentages of occurrence of imposex were 82.6 % and 88.9 % in *B. japonica* specimens collected from December 1988 to November 1989 and in June 1991, respectively. Both penis and vas deferens were found to be well developed in imposex-exhibiting females (Horiguchi et al. 2006). No oviduct blockage (i.e., occlusion of the vulva) by vas deferens formation, however, was observed in imposex-exhibiting female *B. japonica* (Horiguchi et al. 2006), a finding that differs from the imposex symptoms observed in *Nucella lapillus*, *Ocinebrina aciculata*, and *Thais clavigera* (Gibbs and Bryan 1986; Gibbs et al. 1987; Horiguchi et al. 1994; Oehlmann et al. 1996).

Temporal variations in the reproductive developmental score of the *B. japonica* population differed between females (including imposex-exhibiting females) and males (Horiguchi et al. 2006) (Fig. 4.6). Although the spawning season for *B. japonica* is late June to early August (Kajikawa et al. 1983), ovarian maturation seemed to be suppressed in females, compared to testicular maturation in males (Horiguchi et al. 2006) (Fig. 4.6), which is probably caused by the presence of immature females throughout the spawning season. During the spawning season, clearer ovarian maturation and spawning of many more egg capsules were observed in

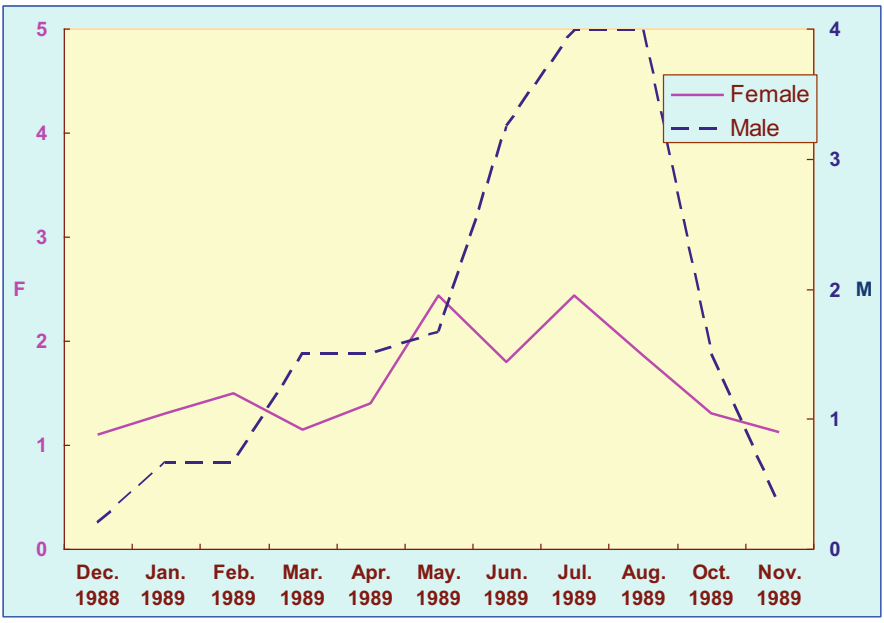

Fig. 4.6 Reproductive cycle of the ivory shell (*Babylonia japonica*) in 1989, represented by population reproductive development scores. Female (*F*) reproductive cells were scored on the basis of five categories, and those of males (*M*) were based on four categories. The female curve includes imposex-exhibiting females (Horiguchi et al. 2006)

B. japonica females in a population from Teradomari, Niigata Prefecture, Japan, compared to those from Tottori (Hamada and Inoue 1993, 1994, 1995). Testicular maturation in males from Tottori was clear in July and August, the spawning season for *B. japonica* (Horiguchi et al. 2006) (Fig. 4.6). Thus, the reproductive cycle was unclear in females but it was clearly observed in males (Horiguchi et al. 2006) (Fig. 4.6). This suppressed ovarian maturation during the spawning season could be the direct reason for the decreased number of egg capsules spawned by adult *B. japonica* at the hatchery and might accompany imposex in *B. japonica* (Gibbs et al. 1988).

Ovarian spermatogenesis (i.e., an ovo-testis) was observed in 6 (1 normal female and 5 imposex individuals) of 92 female or imposex *B. japonica* specimens examined, a frequency of about 6.5 % (Horiguchi et al. 2006) (Fig. 4.7). Most prosobranchs (including *B. japonica*) are known to be dioecious, although there are relatively few hermaphroditic prosobranchs in which the gonad produces eggs and sperm simultaneously (Fretter 1984; Uki 1989). Ovarian spermatogenesis has been observed in muricidae species (e.g., *N. lapillus*, *O. aciculata*, *T. clavigera*) and abalone (e.g., *Haliotis madaka*, *H. gigantea*) exposed to TBT or TPhT, although no penis formation is involved in spermatogenesis in ovaries of female abalone (see following) (Gibbs et al. 1988; Horiguchi and Shimizu 1992; Horiguchi et al. 2000, 2002, 2005; Oehlmann et al. 1996). Ovarian spermatogenesis observed even in an

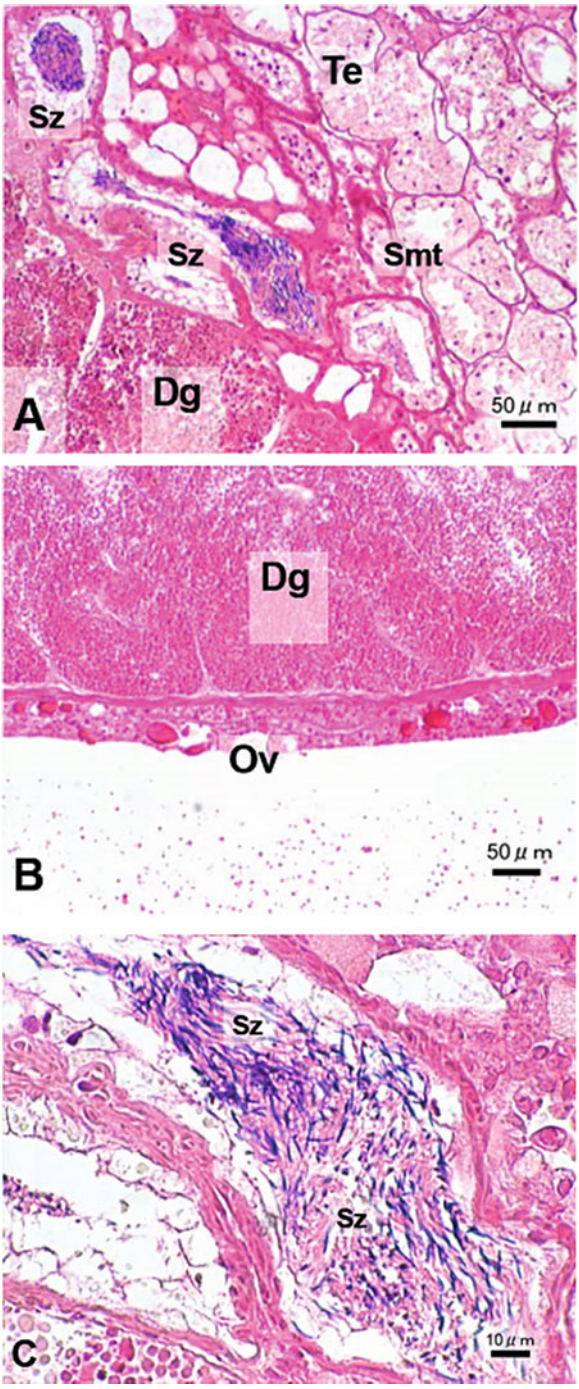

Fig. 4.7 Spermatogenesis in the ovary of a normal female *Babylonia japonica* (i.e., without penis and vas deferens). Testicular (**A**) and ovarian (**B**) tissues (i.e., ovo-testis) were observed in the gonad of a female *B. japonica*, which was classified originally as a female because of the presence of female accessory sex organs (e.g., a capsule gland) with neither penis nor vas deferens. Spermatogenesis was also observed in seminiferous tubules of the ovo-testis (**C**). *Dg* digestive gland, *Ov* ovary, *Smt* seminiferous tubule, *Sz* spermatozoon, *Te* testis (Horiguchi et al. 2006)

apparently normal female *B. japonica* without any penis or vas deferens formation (1 of 6, 16.7 %) may imply that the development of male-type genital organs (penis and vas deferens) and ovarian spermatogenesis in females exposed to TBT or TPhT might be controlled through different physiological pathways. This ovarian spermatogenesis may be one of the reasons why the spawning ability of female *B. japonica* decreased (Horiguchi et al. 2006).

Tissue concentrations of organotin compounds, such as butyltins and phenyltins, were determined by GC-FPD, and different tissue distributions were observed (Horiguchi et al. 2006) (Fig. 4.8). A marked accumulation of TBT was observed

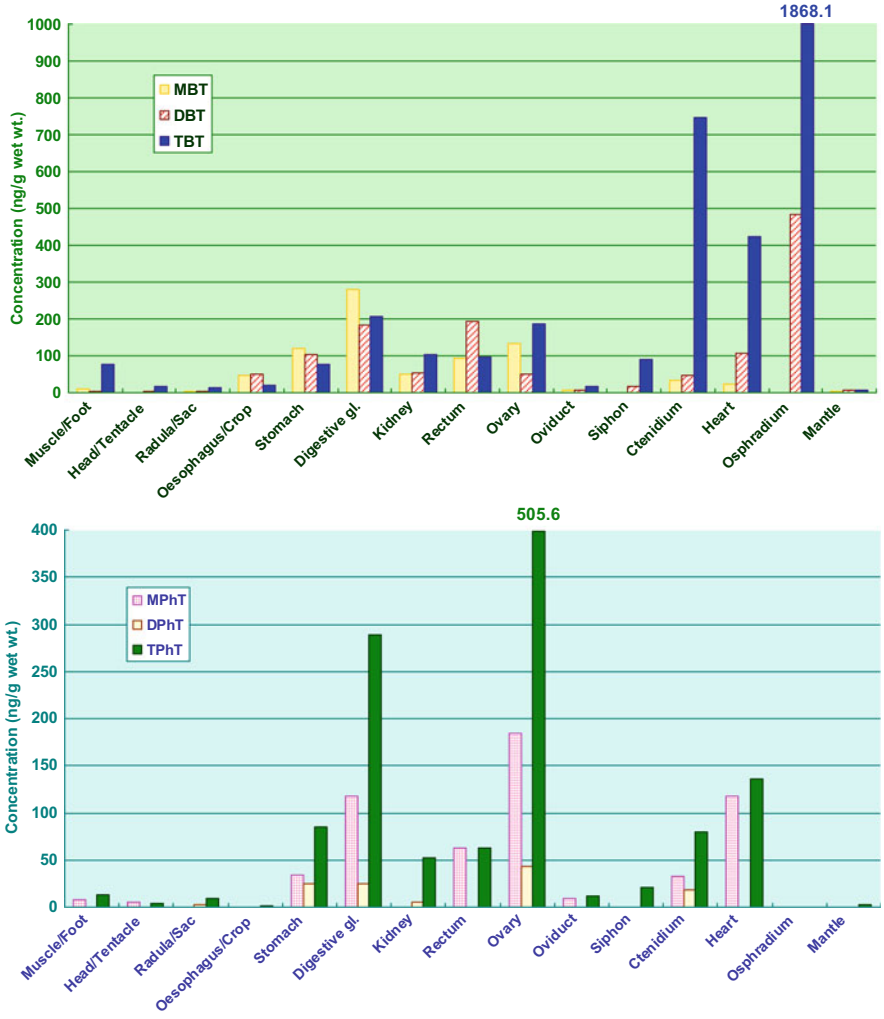

Fig. 4.8 Tissue distributions of organotin compounds in ivory shell (*Babylonia japonica*) from Yodoe, Tottori, Japan (June 1991): *top*, butyltins in females (including imposex individuals); *bottom*, phenyltins in females (including imposex individuals) (Horiguchi et al. 2006)

in the ctenidium, osphradium, and heart in both males and females, whereas the highest concentrations of TPhT were detected in the ovaries of females and the digestive glands of males (Horiguchi et al. 2006) (Fig. 4.8). Based on the total body burden of TBT in *B. japonica*, more than one third of total TBT accumulated in the digestive glands of both males and females, followed by the testis, ctenidium, muscle, and heart in males and the muscle, ovary, ctenidium, and head (including the central nervous system ganglia) in females (Horiguchi et al. 2006). Based on the total body burden of TPhT, approximately three fourths and more than one half of total TPhT accumulated in the digestive glands of males and females, respectively. The second highest tissue burden of TPhT was observed in the gonads of both males and females (Horiguchi et al. 2006).

Mortality of larvae and seeds or juveniles produced in the hatchery might also be caused by the accumulation of TPhT and TBT in ovaries, as well as contamination of seawater with TPhT or TBT (Coelho et al. 2001; Inoue et al. 2004; Lapota et al. 1993; Li et al. 1997; Nakayama et al. 2005; Ruiz et al. 1995; Treuner et al. 2009). Based on a survey of imposex and organotin concentrations in tissues of *T. clavigera* (Horiguchi et al. 1994), contamination with TBT and TPhT was relatively high along the coast of Tottori Prefecture, especially in Miho Bay, where the *B. japonica* specimens used in this study were collected.

Concentrations of TBT and TPhT were relatively high in the ovaries of females (Horiguchi et al. 2006) (Fig. 4.8). Both TBT and TPhT concentrations in gonads were positively correlated with penis length in females (Horiguchi et al. 2006) (Fig. 4.9), as was the case with *T. clavigera* (Horiguchi et al. 1994; Shim et al. 2000). Laboratory experiments revealed that both TBT and TPhT induced or

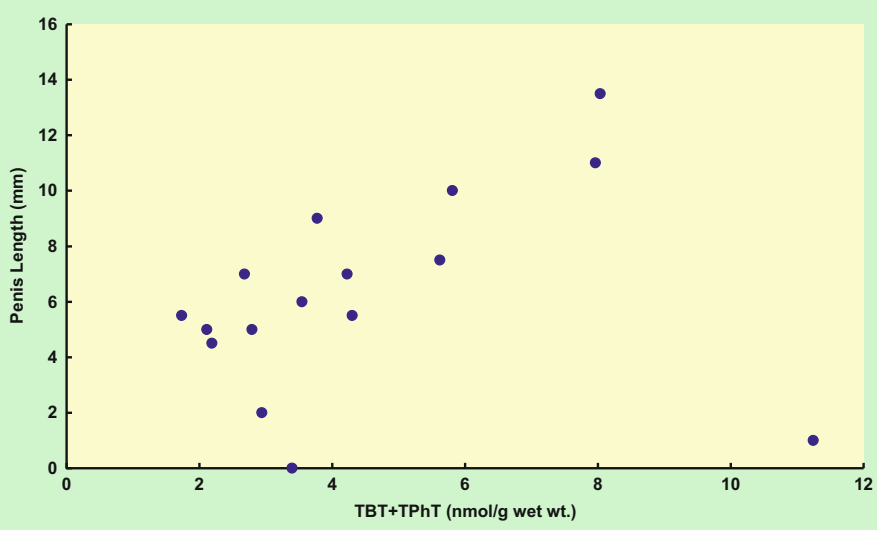

Fig. 4.9 Relationship between triorganotin (sum of TBT and TPhT) concentrations in gonads and penis length in female *Babylonia japonica* (Horiguchi et al. 2006)

promoted the development of imposex in *T. clavigera* (Horiguchi et al. 1995, 1997a); therefore, imposex could be caused by TBT or TPhT in *B. japonica* as well. Laboratory flow-through exposure experiments with *B. japonica* using TBT and TPhT are needed to estimate the threshold concentration for the development of imposex.

The planktonic stage of *B. japonica* is estimated to last approximately 4–5 days (Hamada et al. 1988, 1989), which suggests that the recruitment of veliger larvae from other populations inhabiting remote, less-contaminated areas is unlikely. Reproductive failure accompanied by imposex in females could result in extirpation of the *B. japonica* population within several years because the number of offspring produced by adult *B. japonica* in the population is likely to continue to decrease. The existence and duration of a free-swimming phase during larval development is an important factor in determining the linkage between impaired reproductive ability, caused by imposex, to population decline (Bryan et al. 1986; Gibbs and Bryan 1986; Gibbs et al. 1988, 1990, 1991; Horiguchi et al. 2006).

It could be concluded that reproductive failure (suppressed ovarian maturation and ovarian spermatogenesis) in adult females with imposex, possibly induced by TBT or TPhT from antifouling paints, could have brought about the marked decline in *B. japonica* populations that has been observed.

4.4 Ovo-Testis and a Disturbed Reproductive Cycle in Abalone, Possibly Linked with Organotin Contamination in a Site of Population Decline

A remarkable population decline has been observed in Japanese abalone since the 1970s (Fig. 4.10), although much effort (e.g., artificial production and release of juvenile abalone into the sea) has been made to enhance stocks (Imai et al. 2006). The proportion of artificially released individuals, which are distinguishable from natural stocks by the green color of the tips of the shells (Fig. 4.11), has exceeded 95 % of the total abalone captured in some areas, such as Jogashima (Kanagawa Prefecture) (Imai et al. 2006), suggesting that reproduction in natural abalone stocks is declining.

Reduced abalone recruitment may result from several factors, including mass mortality of larvae and juveniles (from sudden large changes in seawater temperature, food availability, increased predation, or increased incidence of disease), reduced egg production, low fertilization rate (possibly caused by pollutants in the marine environment), or overfishing (by commercial fishery). The causal factors for such population declines in abalone have been sought, but are still unknown (Imai et al. 2006).

On the other hand, imposex, the superimposition of male sexual organs on female prosobranch gastropod mollusks, bringing about reproductive failure in severely affected individuals, is known to be an endocrine disruption in gastropods,

Fig. 4.10 Temporal trend of total catch of abalone in Japan (1926–1999)

Fig. 4.11 External features of the shells of wild and artificially reared/released giant abalone (*Haliotis madaka*). *Left*: wild abalone. *Right*: artificially reared/released abalone

which is typically induced by TBT and TPhT from antifouling paints (Smith 1971; Gibbs and Bryan 1986; Gibbs et al. 1987; Horiguchi et al. 1997a).

The areas where abalone populations have decreased remarkably and the period when this occurred correspond broadly to sites contaminated with organotin compounds and sites with a history of marine pollution by organotins, respectively.

Therefore, it is hypothesized that endocrine disruption in abalone has been caused by organotins and has contributed to population decline (Horiguchi et al. 2000).

In their first survey, a total of 15 adult *Haliotis madaka* (giant abalone) individuals were collected monthly from two sites (Tsushima, Nagasaki Prefecture, as a reference site, whereas Jogashima, Kanagawa Prefecture, is representative of areas where abalone populations have declined drastically) between September 1995 and November 1996 (Horiguchi et al. 2000) for histological examination of gonads and chemical analysis of organotin residues.

The proportion of artificially released abalone in Jogashima was approximately 90 % in this study, much higher than that from Tsushima (less than 5 %). Morphological features of the gonads and digestive glands differed between specimens from the two sites, being either horn shaped (Tsushima) or blunt (Jogashima) (Horiguchi et al. 2000).

Temporal variations in the reproductive developmental score of the populations (the monthly mean value of the individual reproductive developmental scores, which represents the mean value of a histogram of these scores for the reproductive cells of each abalone) also differed between the two sites: gonad maturation of females and males was synchronous in abalone from Tsushima, but not in abalone from Jogashima ($p < 0.05$; Fig. 4.12). This observation may indicate differences in fertilization rates between abalone from Tsushima and Jogashima, because successful fertilization is considered to result from the synchronous release of eggs and sperm into seawater. Ovarian maturation also seemed to be suppressed in females from Jogashima, compared to Tsushima (Fig. 4.12), probably because of the presence of immature females in Jogashima throughout the spawning season. Testicular maturation seemed to be more frequently observed in male abalone from Jogashima than from Tsushima (Fig. 4.12). These gonadal features possibly suggest low reproductive success in giant abalone populations around Jogashima (Horiguchi et al. 2000).

Eleven of 54 females (approximately 20 %) from Jogashima were observed to be masculinized; most of the gonadal tissues were ovaries with a small amount of testis tissue (i.e., an ovo-testis) (Horiguchi et al. 2000) (Fig. 4.13). Either spermatogenesis (13 %) or seminiferous tubule-like structure formation (8 %) was observed (Horiguchi et al. 2000).

This phenomenon of ovo-testis formation is basically similar to imposex in muricidae and buccinidae gastropods, which is known to be induced by organotin compounds, such as TBT and TPhT from antifouling paints, although no penis formation is observed in abalone (Smith 1971; Gibbs et al. 1987, 1988; Horiguchi et al. 1997a). Approximately 200 species of prosobranch gastropods worldwide have been reported to be affected by imposex, as mentioned previously. Intersex, that is, the masculinization of female accessory sex organs, was observed in the periwinkle *Littorina littorea*, reportedly caused by TBT (Bauer et al. 1995). Both imposex and intersex involve reproductive failure in severely affected individuals (Gibbs and Bryan 1986; Gibbs et al. 1988; Oehlmann et al. 1996). Thus, organotin compounds, such as TBT, may also similarly affect the reproductive systems in abalone.

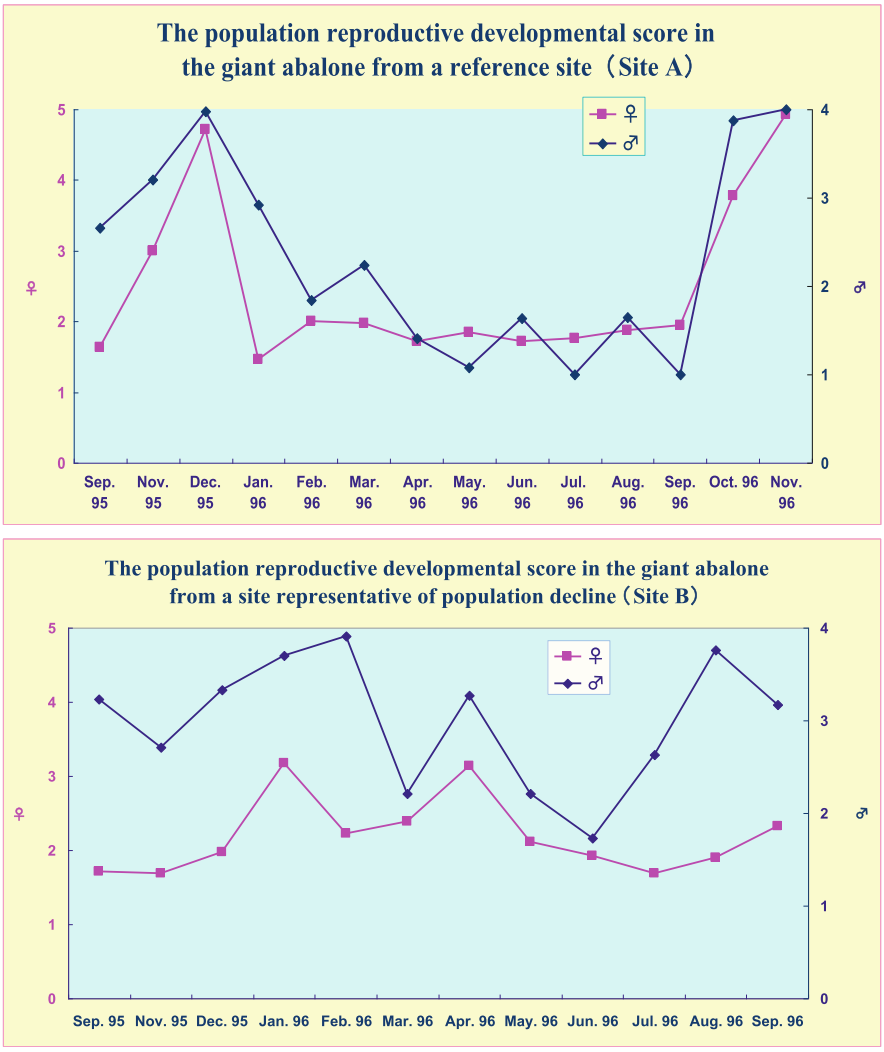

Fig. 4.12 Reproductive developmental scores of the two different populations of giant abalone (*Haliotis madaka*), from Tsushima (*top*) and Jogashima (*bottom*) (Horiguchi et al. 2000)

Concentrations of TBT and TPhT in the muscle of abalone from Jogashima ($n = 83$) of 4.9 ± 4.4 ng/g wet wt. and 6.3 ± 6.6 ng/g wet wt., respectively, were significantly higher than those from Tsushima ($n = 125$) ($p < 0.01$) of 0.8 ± 0.8 ng/g wet wt. and 0.6 ± 1.3 ng/g wet wt., respectively.

In addition, a 7-month in situ exposure experiment was conducted, using 40 abalone from Tsushima that were caged near a shipyard in Jogashima, from June 1998 to January 1999 (from the immature to the mature stage). The exposed abalone were fed brown algae, *Ecklonia cava*, once or twice a week during the experimental

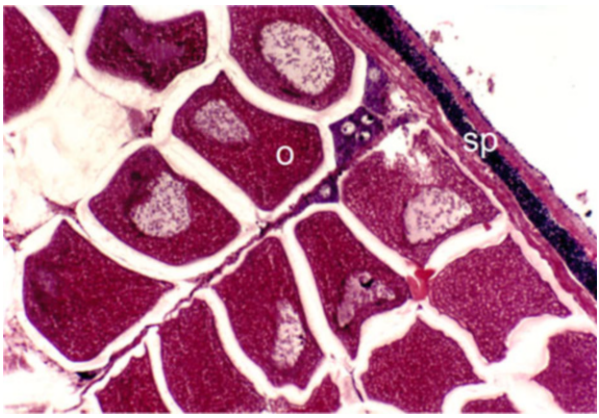

Fig. 4.13 Spermatogenesis in the ovary of giant abalone (*Haliotis madaka*) from Jogashima (Horiguchi et al. 2000). *sp* spermatozoa, *o* oocyte

Fig. 4.14 Spermatogenesis in the ovary of in situ exposed female abalone, near a shipyard in Jogashima for 7 months (Horiguchi et al. 2000)

period. They were collected in January 1999 for histological examination and chemical analysis. This 7-month in situ exposure experiment resulted in spermatogenesis in the ovary of approximately 90 % of exposed females (Horiguchi et al. 2000) (Fig. 4.14). TBT and TPhT levels in the muscle of the abalone varied from 0.9 ± 0.4 ng/g wet wt. and 1.3 ± 1.4 ng/g wet wt. ($n = 15$) to 5.0 ± 0.2 ng/g wet wt. and 21.5 ± 2.1 ng/g wet wt. ($n = 40$), respectively ($p < 0.01$) (Horiguchi et al. 2000).

Subsequently, 2-month flow-through exposure experiments of TBT and TPhT were conducted with the abalone *Haliotis gigantea* to examine whether TBT or TPhT induced spermatogenesis in females. Nominal concentrations of 100 ng/l of TBT and 100 ng/l of TPhT caused significant formation of spermatids, spermatozoa, and seminiferous tubule-like structures (spermatogenesis) in ovaries of exposed females (Horiguchi et al. 2002) (Fig. 4.15). Significantly more contracted primary oocytes were also observed in ovaries of females exposed to either TBT or

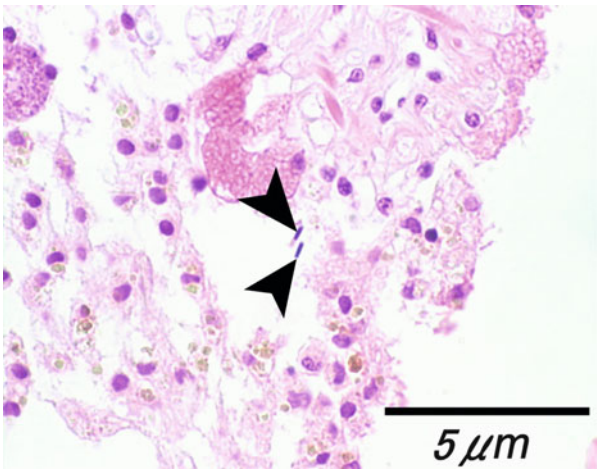

Fig. 4.15 Spermatogenesis in the ovary of female abalone exposed to TBT in the laboratory for 2 months (Horiguchi et al. 2002)

TPhT than in ovaries of controls (Horiguchi et al. 2002). No significant histological changes were observed in testis of exposed males (Horiguchi et al. 2002). This ovarian spermatogenesis caused by TBT and TPhT exposure seems similar to the masculinization of muricidae and buccinidae gastropods, such as imposex.

Remarkably high concentrations of TBT and TPhT were observed in the head (including ganglia of the central nervous system), compared to concentrations in muscles: 68.3 ± 4.8 ng TBT/g and 1406.4 ± 11.3 ng TPT/g in the head, compared to 2.4 ± 0.8 ng TBT/g and 126.1 ± 68.0 ng TPT/g in muscles (on a wet tissue basis) (Horiguchi et al. 2002). Accumulation of TBT and TPhT in the head may disturb reproductive hormonal regulators through neuropeptides released from ganglia: this may be one of the inducers for spermatogenesis in the ovaries of female abalone.

Thus, it was hypothesized that endocrine disruption, resulting in spermatogenesis in the ovary of giant abalone around the shipyard in Jogashima, was caused by TBT or TPhT, and that organotin compounds from antifouling paints could be one of the causal factors of the observed abalone population decline.

A few years later, histological examination of gonads as well as chemical analysis of organotin compounds in tissues of the giant abalone *Haliotis madaka* was conducted to evaluate continuing endocrine disruption in abalone populations in Japan (Horiguchi et al. 2005). Abalone specimens were collected from two areas, Tsushima as a reference site and Jogashima as a site representative of declining abalone populations where serious organotin contamination had been observed, each month from January 1998 to March 1999. Scores were given to the development stages of reproductive cells in the ovary and testis, the same as in Horiguchi et al. (2000), to evaluate the degree of sexual maturation by calculating the mean value of a histogram of these scores for the reproductive cells of each abalone (Horiguchi et al. 2005). The temporal variation in the degree of sexual maturation showed that female and male abalone from Tsushima matured synchronously, whereas those from Jogashima did not (Horiguchi et al. 2005), which was similar

to results of the previous study during September 1995–November 1996 (Horiguchi et al. 2000). Approximately 19 % of female abalone from Jogashima were masculinized with an ovo-testis (Horiguchi et al. 2005), which was also similar to the results of Horiguchi et al. (2000). Chemical analyses showed that concentrations of total butyltins [TBT, DBT, and MBT: Σ BTs] and total phenyltins [TPhT, diphenyltin (DPhT), and monophenyltin (MPhT): Σ PhTs] in the muscle of abalone from Jogashima ($n = 73$) of 7.8 ± 9.0 ng/g wet wt. and 4.5 ± 6.8 ng/g wet wt., respectively, were significantly higher than those from Tsushima ($n = 87$) of 4.7 ± 4.9 ng/g wet wt. and 0.8 ± 1.7 ng/g wet wt., respectively ($p < 0.05$ for Σ BTs; $p < 0.001$ for Σ PhTs) (Horiguchi et al. 2005). Thus, endocrine disruption as well as contamination by organotins in the giant abalone from Jogashima was still persisting, at least until March 1999.

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Chapter 5
Organotins and Imposex in Europe: A Pre-ban and Post-ban Perspective

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Abstract The use of tributyltin (TBT) and, to a lesser extent, triphenyltin (TPT) as biocides in antifouling paint formulations during the last decades of the twentieth century was responsible for the global contamination of the coastal and offshore areas of the European aquatic environment. This widespread contamination was responsible for extensive deleterious effects in nontarget organisms particularly in gastropods, which, as a consequence of TBT exposure, developed imposex in a dose-dependent manner. Over the last decades, more than 20 gastropod species registering the occurrence of imposex were used by European researchers to ascertain the degree of TBT pollution in the European coastal and marine environment. In this chapter, we will evaluate the status of organotin (OT) contamination in European countries, using imposex in gastropods as a surrogate. The temporal trends uncovered are evaluated under the light of the 2003 European ban (Directive 2002/62/EC and Regulation 782/2003) and the AFS Convention (entered into force in 2008). The reduction of TBT pollution is addressed under the same light. Overall, we conclude that significant reductions in imposex levels started to emerge after the European ban (2003), though those reductions were more pronounced from 2008 onward. The most recent surveys disclose that the recovery of the ecosystems from the "TBT nightmare" is ongoing.

Keywords AFS Convention • Antifouling paints • Directive 2002/62/EC • Imposex • Monitoring • *Nassarius reticulatus* • *Nucella lapillus* • Organotins • Tributyltin

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Abbreviations

AF	Antifouling
AFS Convention	International Convention on the Control of Harmful Antifouling Systems on Ships
EAC	Environmental Assessment Criterion
EC	European Commission
EcoQO	Ecological Quality Objective
IMO	International Maritime Organization
OSPAR	Convention for the Protection of the Marine Environment of the North-East Atlantic
OTs	Organotin compounds
PARCOM	Paris Commission
PVC	Polyvinyl chloride
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals (European Union Regulation)
RPLI	Relative penis length index
RPSI	Relative penis size index
TBT	Tributyltin
TPT	Triphenyltin
VDSI	Vas deferens sequence index

5.1 Introduction

Organotins (OTs) are industrial chemicals formed by a single tin atom covalently bound to one or several organic groups. Their classification is based on the number of organic substituents: monoorganotins ($R\mathrm{Sn}X_3$), diorganotins ($R_2\mathrm{Sn}X_2$), triorganotins ($R_3\mathrm{Sn}X$), and tetraorganotins ($R_4\mathrm{Sn}$), where X represents an inorganic or organic ligand (e.g., chloride, fluoride, oxide, hydroxide, carboxylate, or thiolate) and R is an organic alkyl or aryl group such as methyl, ethyl, butyl, propyl, phenyl, or octyl) (Hoch 2001; WHO 1990). OTs have an extremely diverse array of industrial manufacturing applications ranging from plastics, glasses, and pesticides to medical appliances and clothing (Sousa et al. 2013). Nevertheless, the bulk of industrial applications are comprised by stabilization of PVC (with 76 % of the total production in 2009), biocides (18 %), and catalysts for the manufacture of polyurethanes and silicones (5 %) (EVISA 2009). Generally, the plastic (PVC), polyurethane, and silicone industry uses mono and disubstituted organotins, whereas triorganotins, due to their high biological activity, are used as biocides in agriculture and in antifouling paints. In fact, it was the use of tributyltin (TBT) and, to a lesser extent, triphenyltin (TPT) as biocides in antifouling paint (AF) formulations (applied to protect ship's hulls and other submerged structures) that was responsible for OTs notoriety and for their recognition as priority environmental contaminants. Tributyltin was used in AFs for four decades in Europe (from the 1960s to 2000s) in

the shipping industry in order to reduce ships' biofouling and thus cut the costs associated with fuel consumption and dry dock operations. It is estimated that an unprotected ship hull can gather about 150 kg m^2 of fouling organisms during 6 months at the sea and that this fouling is responsible for an average increase in fuel consumption of 40–50 % (IMO 1999). The outstanding biocidal properties of TBT alongside with the enhanced properties and cost-effectiveness of the TBT-based AF paint formulations were responsible for the widespread use of these paints not only in Europe but around the world. In fact, according to the IMO, by the 1970s, most of the seagoing vessels used TBT-based AF paints (IMO 1999). With such generalized application, TBT was continuously introduced in the environment, and contrarily to what was expected from the initial laboratory experiments (which appeared to demonstrate that TBT could be easily degraded into the nontoxic tin form), its degradation in the marine environment was neither immediate nor easy (see, e.g., Omae 2006; Sousa et al. 2014). TBT in seawater adsorbs to particulate matter that will deposit in sediments where TBT degradation is very slow (half-lives of decades have been reported), and therefore, sediments are TBT ultimate sink (WHO 1990; Burton et al. 2004). The concentrations in sediments tend to be very high (in the ppm range inside dockyards and marinas), and upon diffusion and/or resuspension, TBT can be released back into the water column, and thus sediments will act as secondary sources of this contaminant (Sousa et al. 2014; Hoch 2001). Alongside with its widespread distribution and persistency, TBT is, unsurprisingly, extremely toxic toward a wide range of nontarget organisms from bacteria to humans (a compilation of the taxonomic groups affected by TBT can be found elsewhere (e.g., Sousa et al. 2014)). Herein only the effects of TBT toward mollusks, and in particular gastropods, will be addressed.

5.2 TBT as Suspect, Defendant, and Perpetrator in the Gastropod Catastrophe

The first deleterious effects of TBT toward nontarget organisms were reported for mollusks, namely, oysters and gastropods (see review by Alzieu 2000a). In the 1970s and early 1980s, the oyster production in Arcachon bay (France), an enclosed bay famous for its recreational marinas and oyster farming facilities, suffered tremendous losses due to shell calcification anomalies and a complete lack of reproduction. These problems were caused by TBT released from the numerous vessels anchored in the local marinas (Alzieu 2000b). As a response to the oyster culture collapse and the associated economical loss, the French government introduced in 1982 the first restrictive initiative, prohibiting the use of TBT in small boats (<25 m).

Still during the 1970s, the presence of a penis in females of the gastropod *Nucella lapillus* collected in the south of England (Plymouth Sound) was reported for the first time in the literature (Blaber 1970). However, only a decade later, the

work developed by Smith in the United Sates using the species *Nassarius obsoletus* allowed linking this phenomenon, coined as imposex (superimposition of male characters onto females of gonochoristic gastropods), to TBT exposure (Smith 1971, 1980, 1981a, b). In the late 1980s, the association of imposex and TBT was further confirmed by the work of Gibbs and Bryan through laboratory and field experiments with *Nucella lapillus* in England (Bryan et al. 1986, 1987; Gibbs et al. 1987, 1988). This team proposed the use of imposex as a biomarker of TBT pollution and developed the VDSI index (vas deferens sequence index) to evaluate the degree of masculinization in *N. lapillus* females (Gibbs et al. 1987; Gibbs 1999). They also described for the first time the local extinction of gastropod populations as a consequence of TBT pollution (Bryan et al. 1986). Due to these severe ecological impacts, the UK banned the sale of TBT-based paints for small boats and aquaculture structures in 1987. In the same year, the PARCOM[1] recommendation (87/1) called for a similar ban over its entire area (Northeast Atlantic). Two years later, motivated by the collapse of the oyster industry and the negative ecological impacts on gastropod populations, the European Community banned the use of TBT and TPT on small boats (<25 m) through the Directive 89/677/EEC. This directive was, under due process, transposed to the national law of the member states and consequently implemented and enforced.

Upon the implementation of this European partial ban, several studies were performed and conflicting results were obtained. Whereas some reports suggested a recovery of the gastropod and oyster populations at severely polluted sites (Evans et al. 1996, 1995; Alzieu 1998; Miller et al. 1999; Harding et al. 1997; Minchin et al. 1995), others concluded that TBT pollution was not decreasing, with harbors remaining as hotspots of contamination, and that at some locations imposex levels were even increasing (Bailey and Davies 1989; Morgan et al. 1998; Minchin et al. 1995, 1996; Davies and Bailey 1991; Santos et al. 2002a; Barroso and Moreira 2002). Further support for the inefficacy of the partial ban was provided by the work of Ten Hallers-Tjabbes et al. (1994) that reported for the first time the occurrence of imposex in offshore locations. Their work was pivotal in demonstrating that TBT pollution was not restricted to coastal waters (where marinas and harbors are located), but was also present in locations away from the influence from those sources. Furthermore, they were able to demonstrate that imposex increased with shipping density. Subsequent studies also demonstrated that different populations from other offshore locations around Europe were similarly affected by imposex (ten Hallers-Tjables et al. 2003; Santos et al. 2002b, 2004; Goméz-Ariza et al. 2006). These evidences were also confirmed for other locations around the world (see, e.g., Sousa et al. 2014). Hence, solving this global scale pollution problem required a concerted worldwide initiative that was launched in 2001 under the

[1]PARCOM: PARIS COMMISSION. The commission was a group of experts who advised North Sea countries on environmental policy and legislation. The Paris Convention of 1974 was unified with the Oslo Convention from 1972, updated, and extended by the OSPAR Convention in 1992 (www.ospar.org).

auspices of the International Maritime Organization (IMO). The International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention) called for a total ban on the use of TBT-based AF paints from 2003 onward. However, the entry into force of this convention could only occur one year after the ratification by 25 states representing a minimum of 25 % of the world's merchant shipping tonnage. This came to happen only on the 17 September 2008, one year after the convention ratification by Panama (the 25th state). However, the European Union, anticipating the difficulties in the implementation of the AFS Convention, decided to move forward and to create its own ban trough the Directive 2002/62/EC and the Regulation 782/2003. Hence, in Europe since July 2003, the application of organotin-based AF paints in ship hulls was interdicted, and from 2008 onward, the prohibition on the use of TBT-based AF paints was extended to all ships entering EU ports. Due to the timeframe of these legislative measures, the European ban can be regarded as an anticipation of the AFS Convention, and therefore, the impact of the European initiative can be used to predict the impact of the global ban.

5.3 TBT and Imposex: The Ultimate Mechanistic Instrument in Environmental Chemistry

As previously mentioned, the use of imposex to monitor TBT pollution was proposed by Gibbs and coworkers in 1987. This biomarker was originally developed for the gastropod *Nucella lapillus*, but subsequent research using other gastropod species from different locations validated its use to monitor environmental levels of TBT. In fact, up to date more than 200 gastropod species were recognized as being affected by imposex on a worldwide basis (Shi et al. 2005), and in Europe, at least 22 species have been used over the last decades to monitor TBT pollution (Fig. 5.1, Table 5.1).

Of all the species described, *N. lapillus* was the most used ($n = 54$ surveys), followed by *Nassarius reticulatus* ($n = 28$). Both these species are recommended by the OSPAR Commission to be used to monitor TBT biological effects (OSPAR 2008). *N. lapillus* is the prime choice, but where this gastropod is absent (e.g., inside ports and marinas), the Commission recommends the use of the netted whelk *N. reticulatus* or the periwinkle *Littorina littorea*. At offshore locations, the OSPAR Commission recommends the use of *Buccinum undatum* and *Neptunea antiqua*. Nevertheless, several studies demonstrated that *N. reticulatus* can also be successfully used to evaluate TBT pollution in offshore areas (Barroso et al. 2011; Rato et al. 2008, 2006; Sousa et al. 2007; Santos et al. 2004). In Europe as a whole, the OSPAR-recommended species (together with *Hexaplex trunculus*) were the most used in monitoring studies (Fig. 5.1, Table 5.1).

There is an asymmetrical distribution in what concerns the imposex monitoring work carried out by each European country. In fact, as it can be easily observed in

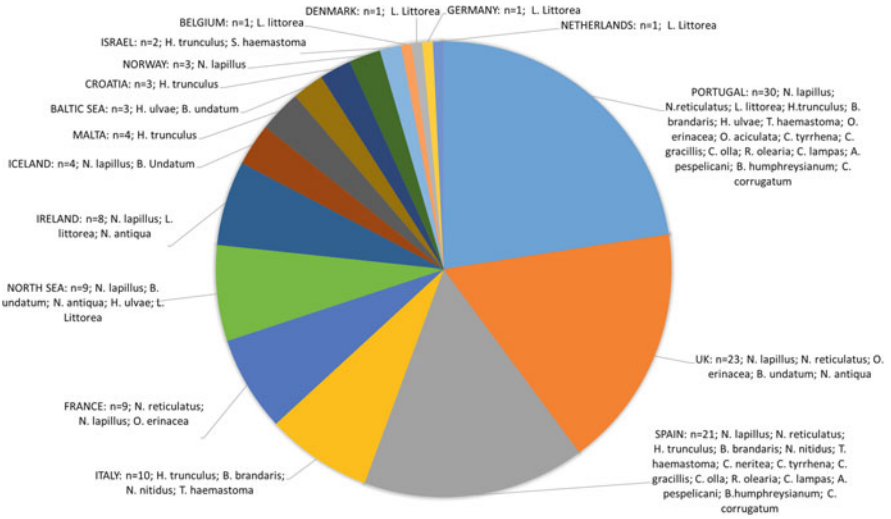

Fig. 5.1 Number of publications (n) per European country or region reporting monitoring results of imposex levels with the list of the top 10 bioindicator species surveyed in each country

Fig. 5.1, Portugal ranks in the top position concerning the number of imposex monitoring studies published ($n = 30$), followed by the UK and then Spain ($n = 23$ and 21, respectively). Regarding the number of species analyzed, Portugal and Spain exhibited the highest number (17 and 15, respectively). This considerably high number of species results mainly from a study conducted off the Iberian Coast (South of Portugal and Spain) in which 10 different species were collected and analyzed (Goméz-Ariza et al. 2006).

5.4 Profiling Imposex in a Post-ban World

In order to evaluate the effectiveness of the European ban and as an exercise to anticipate the effects of the AFS Convention on the recovery of the ecosystems from TBT pollution, several research groups took the initiative to monitor imposex levels before and after the implementation of the European legislation in 2003 (see Table 5.1 for a complete list of studies). *N. lapillus* and *N. reticulatus* were the preferential species used in the temporal trend evaluations, being the UK and Portugal the countries were most of those evaluations occurred. Overall, a significant reduction in the imposex indices VDSI and RPSI/RPLI[2] could be noticed after the introduction of TBT ban. However, the impact of the AFS 2008 global ban is

[2]RPSI (relative penis size index) = FPL(female penis length)^3 × 100/MPL(male penis length)^3) is used for *N. lapillus*, whereas for *N. reticulatus* the RPLI (relative penis length index) is used (RPLI = FPL × 100/MPL), due to the penis size and shape.

Table 5.1 Bioindicator species used in imposex monitoring studies performed in Europe, with the indication of the number of studies where the species were included, the geographical locations where those studies were conducted, and the year of the sampling campaign

Geographical area	Years	References
**Nucella lapillus*		
Firth of Forth, Scotland, UK	1975–1987	Bailey and Davies (1988)
SW England, UK	1984–1985	Bryan et al. (1986)
UK	1984–1986	Gibbs et al. (1987)
Scotland, UK	1987	Bailey and Davies (1989)
N Spain and Portugal	1987	Peña et al. (1988)
Dumpton Gap and Oldstairs Bay, UK	1987–1989	Gibbs (1993)
Shetland Islands, UK	1987, 1989, 1990	Davies and Bailey (1991)
Shetland, UK	1987–1995	Harding et al. (1997)
Ireland	1987–1993	Minchin et al. (1995)
N Wales, UK	1987, 1995, 2006	Oliveira et al. (2009)
W French coast, France	1988–1993	Huet et al. (1995)
Ireland and France	1988–1996	Oehlmann et al. (1998)
North Sea coastal waters	1991–1992	Harding et al. (1999)
Brittany, France	1992–1994	Huet et al. (1996a)
Brittany, France	1992–1994	Huet et al. (1996b)
NW Brittany, France	1992–1995, 1998, 2000, 2002	Huet et al. (2004)
Iceland	1992–2008	Guðmundsdóttir et al. 2011)
England; Clyde Sea; W Scotland; Norway	1993–1994	Evans et al. (1996)
Cork Harbor, Ireland	1994	Minchin et al. (1996)
Ireland	1994–1995	Minchin et al. (1997)
Portugal	1995–1996	Santos et al. (2000)
Ireland	1996	Minchin and Minchin (1997)
UK	1996–1997	Harding and Davies (2000)
Galicia, NW Spain	1996	Ruiz et al. (1998)
NW Spain	1996–1998	Barreiro et al. (1999)
Galicia, NW Spain	1997	Quintela et al. (2000)
Galicia, NW Spain	na	Quintela et al. (2002)
N Wales, UK	1997–1998	Son and Hughes (2000)
Firth of Forth, Scotland, UK	1997–1998	Miller et al. (1999)
Portugal	1997–2000	Barroso and Moreira (2002)
Ria de Aveiro, Portugal	1997–2007	Galante-Oliveira et al. (2009)
England and Wales, UK	1997–2014	Nicolaus and Barry (2015)
Ria de Aveiro, Portugal	1998	Barroso et al. (2000)

(continued)

Table 5.1 (continued)

Geographical area	Years	References
North Sea; Britain; France; Norway	1998	Evans et al. (2000a)
Scotland, UK	1998	Evans et al. (2000b)
Iceland	1998	Svavarsson (2000)
North Sea	1998–2000	Birchenough et al. (2002a)
Portugal	2000	Santos et al. (2002a)
Portugal	2000, 2003, 2006	Sánchez-Marín et al. (2016)
UK	2001	Birchenough et al. (2002b)
River Tyne, UK	2001	Smith et al. (2006)
Portugal	2003	Galante-Oliveira et al. (2006)
Galicia, NW Spain	2003	Ruiz et al. (2008)
Iceland	2003	Jörundsdóttir et al. (2005)
Norwegian coast	2003	Plejdrup et al. (2006)
SW Brittany, France	2003–2007	Huet et al. (2008)
SE England, UK	2004–2008	Morton (2009)
Ria de Aveiro, Portugal	2005–2007	Galante-Oliveira et al. (2010b)
Galicia, NW Spain	2006	Ruiz et al. (2010)
England, UK	2006–2008	Bray et al. (2012)
Portugal	2006, 2008	Galante-Oliveira et al. (2011)
Ireland	2007	Giltrap et al. (2009)
Ireland	2007–2011	Wilson et al. (2015)
Ria de Aveiro, Portugal	2013	Laranjeiro et al. (2015b)
**Nassarius reticulatus*		
Plymouth Sound, UK	1983–1985	Bryan et al. (1993)
Coast of Brittany and Normandy, France	1988–1991	Stroben et al. (1992)
NW Brittany, France	1992–1995, 1998, 2000, 2002	Huet et al. (2004)
Sado and Mira Estuaries, Portugal	1993–1995, 1997,1998	Pessoa et al. (2001)
Ria Formosa, Portugal	1996	Gibbs et al. (1997)
Ria de Aveiro, Portugal	1997–1999	Barroso et al. (2005)
NW Spain	1998–1999	Barreiro et al. (2001)
Ria de Aveiro, Portugal	1998	Barroso et al. (2000)
Off Oporto Coast, NW Portugal	1998	Santos et al. (2004)
Portugal	2000	Barroso et al. (2002)
Galicia, NW Spain	2000	Ruiz et al. (2005)
NW Portuguese continental shelf, Portugal	2002–2005	Rato et al. (2006)
Portugal	2003	Sousa et al. (2005)
Brittany, France	2004–2007	Wirzinger et al. (2007)

(continued)

Table 5.1 (continued)

Geographical area	Years	References
Ria de Aveiro, Portugal	2005	Sousa et al. (2007)
Galicia, NW Spain	2005	Ruiz et al. (2008)
Off Lisbon, Setubal, and Faro, Portugal	2006	Rato et al. (2008)
Portugal	2006	Rato et al. (2009)
NW Portuguese continental shelf, Portugal	2006, 2010	Barroso et al. (2011)
Cantabrian coast, N Spain	2006	Couceiro et al. (2009)
Basque coast, N Spain	2007	Rodríguez et al. (2009b)
Galicia, NW Spain	2008	Ruiz et al. (2010)
Vigo Harbor, NW Spain	2008, 2013	Laranjeiro et al. (2015a)
N Spain	2008	Rodríguez et al. (2010)
Portugal	2008	Sousa et al. (2009)
Ria de Aveiro, Portugal	2009	Laranjeiro et al. (2010)
Basque Coast, N Spain	2011	Cuevas et al. (2014)
Ria de Aveiro, Portugal	2013	Laranjeiro et al. (2015b)
**Littorina littorea (#)*		
Germany	1994–1995	Bauer et al. (1997)
Cork Harbor, Ireland	1994	Minchin et al. (1996)
Ireland	1994–1995	Minchin et al. (1997)
Ria de Aveiro, Portugal	1998	Barroso et al. (2000)
S Scheldt estuary, the Netherlands	1998	de Wolf et al. (2001)
North Sea	1998–2000	Birchenough et al. (2002a)
Denmark	2003	Rank (2009)
Belgium	2004	Van den Broeck et al. (2009)
Ria de Aveiro, Portugal	2013	Laranjeiro et al. (2015b)
**Buccinum undatum*		
North Sea	1991, 1992	Hallers-Tjabbes et al. (1994)
Iceland	na	Svavarsson et al. (2001)
North and Baltic Seas	1996–1998	Jakob and Jens (2002)
W Scotland, UK	1997	Poloczanska and Ansell (1999)
North Sea	1998–2000	Birchenough et al. (2002a)
North Sea	1999	Santos et al. (2002b)
North Sea	1999	ten Hallers-Tjables et al. (2003)
Neptunea antiqua		
W Scotland, UK	1997	Poloczanska and Ansell (1999)
Offshore Irish Sea, Ireland	1997–1998	Power and Keegan (2001)

(continued)

Table 5.1 (continued)

Geographical area	Years	References
North Sea	1998–2000	Birchenough et al. (2002a)
North Sea	1999	ten Hallers-Tjables et al. (2003)
Hexaplex trunculus		
Malta	1992	Axiak et al. (1995)
Malta	na	Axiak et al. (2000)
Malta	1992–1995	Axiak et al. (2003)
Malta	1993–2011	Axiak et al. (2012)
Italian Coastline, Italy	1995–1996	Terlizzi et al. (1998)
Italian Coastline, Italy	na	Terlizzi et al. (1999)
Ria Formosa, Portugal	1996	Gibbs et al. (1997)
Mediterranean Coast, Israel	1997	Gil et al. (2000)
Sicilian coast, Italy	1999–2000	Chiavarini et al. (2003)
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Marine Protected Areas, Italy	2002	Terlizzi et al. (2004)
N Mediterranean Sea, Italy	2002	Garaventa et al. (2006)
Lagoon of Venice, Italy	2002	Maran et al. (2006)
Lagoon of Venice, Italy	2002	Pellizzato et al. (2004)
Lagoon of Venice, Italy; Istrian Coast, Croatia	2002–2003	Garaventa et al. (2007)
Ria Formosa, Portugal	2003–2004	Vasconcelos et al. (2006)
Kaštela Bay, Adriatic Sea, Croatia	2004–2006	Stagličić et al. (2008)
Croatian Adriatic coast, Croatia	2005	Prime et al. (2006)
Bolinus brandaris		
NW Mediterranean, Spain	1996–2000	Ramon and Amor (2001)
Catalan Coast (NW Mediterranean), Spain	1997	Solé et al. (1998)
Sicilian coast, Italy	1999–2000	Chiavarini et al. (2003)
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
W Mediterranean, Spain	1999–2000	Ramón and Amor (2002)
Ria Formosa, Portugal	2008–2009	Vasconcelos et al. (2010a)
Ria Formosa, Portugal	2008–2009	Vasconcelos et al. (2010b)
Nassarius nitidus		
Venice Lagoon, Italy	2004–2005	Pavoni et al. (2007)
Basque Country, N Spain	2007	Rodríguez et al. (2009a)
Basque Coast, N Spain	2011	Cuevas et al. (2014)
Venice Lagoon, Italy	2013	Cacciatore et al. (2016)

(continued)

Table 5.1 (continued)

Geographical area	Years	References
Hydrobia ulvae		
German North Sea and Baltic Coast	1994–1995	Schulte-Oehlmann et al. (1997)
German North Sea and Baltic Coast	1994–1995	Schulte-Oehlmann et al. (1998)
Ria de Aveiro, Portugal	1998	Barroso et al. (2000)
Ria de Aveiro, Portugal	1998–2007	Galante-Oliveira et al. (2010a)
Thais haemastoma		
Azores (Portugal); Costa del Sol, Canaries (Spain)	1987–1988	Spence et al. (1990)
Sicilian coast, Italy	1999–2000	Chiavarini et al. (2003)
Ocenebra erinacea		
Cornwall, UK	1986–1996, 2006	Gibbs (2009)
NW Brittany, France	1992–1995, 1998,2000, 2002	Huet et al. (2004)
Ria Formosa, Portugal	1996	Gibbs et al. (1997)
Ocinebrina aciculata		
Ria Formosa, Portugal	1996	Gibbs et al. (1997)
Stramonita haemastoma		
Mediterranean Coast, Israel	1996, 1997	Gil et al. (2000)
Cyclope neritea		
Galicia, Spain	2005	Quintela et al. (2006)
Cassidaria tyrrhena		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Colus gracilis		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Cymbium olla		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Ranella olearia		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Charonia lampas		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Aporrhais pespelecani		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)

(continued)

Table 5.1 (continued)

Geographical area	Years	References
Buccinum humphreysianum		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)
Cymatium corrugatum		
Atlantic Ocean off the Iberian Peninsula	1999–2000	Goméz-Ariza et al. (2006)

(*) Species recommended by OSPAR to be used to monitor TBT biological effects (OSPAR 2008). (#)*Littorina littorea,* despite not exhibiting imposex (only intersex), is included here in accordance with the OSPAR list; (na): information not available

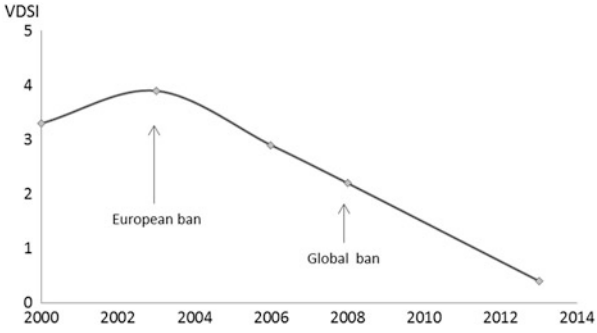

Fig. 5.2 Evolution of VDSI levels in *N. reticulatus* collected in Ria de Aveiro, Portugal (data extracted from Sousa et al. 2009; and Laranjeiro et al. 2015b)

more difficult to address as only a limited number of studies evaluated imposex levels after its entering into force (Nicolaus and Barry 2015; Laranjeiro et al. 2015b; Wilson et al. 2015; Cuevas et al. 2014; Cacciatore et al. 2016; Laranjeiro et al. 2015a).

In Portugal, for example, if the studies performed since 2000 are considered, a clear inflection of imposex levels in *N. reticulatus* (assessed by means of VDSI) from 2003 onward can be clearly observed (Fig. 5.2) and that tendency is maintained after 2008 until a recent past.

Similar decreasing trends were observed by Nicolaus and Barry (2015) that compiled *N. lapillus* imposex data from campaigns performed over a 22-year period (1992–2014) in England and Wales. VDSI values of the same sites sampled in 1997 and 2010 ($n = 56$) showed a statistically significant reduction from 2.89 to 0.42. Along the same lines, Wilson et al. (2015) found significant reductions in *N. lapillus* VDSI levels between 1987 and 2011 in the same sample sites located along the Irish coast. These results confirm the effectiveness of the European legislation (Directive 2002/62/EC and the Regulation 782/2003) aimed at reducing TBT pollution and permit us to foresee similar accomplishments for the AFS Convention.

In order to evaluate the modulation exerted by those reductions on the quality status of the ecosystem, most of the cited surveys used the assessment classes and

the Ecological Quality Objective (EcoQO) benchmark defined by the OSPAR monitoring guidelines (OSPAR 2008, 2007). Such guidelines were developed based on the VDSI levels of different gastropod species and are graded from A to F, where A corresponds to VDSI levels ≤ 0.3 in *N. lapillus* and *N. reticulatus* and F corresponds to VDSI levels in *N. reticulatus* between 3.5 and 4 and to the absence of populations in *N. lapillus*. The established EcoQO for imposex is achieved for VDSI levels below 2.0, and the objectives for the good chemical status of the surface waters correspond to the OSPAR-derived Environmental Assessment Criterion (EAC) of 0.1 ng TBT/L. In the survey performed by Laranjeiro et al. (2015b) in Ria de Aveiro, Portugal, the EcoQO objective was fully achieved in 2013; however, in the surveys performed in Ireland (2011) and England (2014), this objective was not achieved in 25 % and 11 % of the locations, respectively (Nicolaus and Barry 2015; Wilson et al. 2015). Nevertheless, these results can raise optimism and strengthen the idea that the impact of TBT in the European marine environment is undergoing a decreasing trend.

5.5 Final Remarks

TBT is considered as "the most toxic compound deliberately released into marine environment by man" (Goldberg 1986) and represents one of the better characterized occurrences of endocrine disruption in wildlife. In fact, imposex in gastropods is a very specific biomarker of TBT exposure and has been validated as a direct monitor of TBT environmental levels in several species.

The evidence gathered from the works published in Europe and here complied clearly demonstrates the efficacy of the legislation in improving the health of the ecosystems and highlights the utmost importance of the articulation between science and policy in order to protect, improve, and recover environmental health.

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Chapter 6
Current Status of Organotin Contamination and Imposex in Neogastropods Along Coastal Marine Environments of Southeast Asia and China

Kevin King Yan Ho and Kenneth Mei Yee Leung

Abstract Organotin compounds (OTs), in particular tributyltin and triphenyltin, have been contaminating various coastal marine environments around the world since their first application back in the 1920s. These compounds have been proven to adversely affect a wide range of marine organisms from microalgae to marine mammals, and they have a great potential of bioaccumulation via the food web. Some OTs such as triphenyltin can even be biomagnified through marine food chains. In Southeast Asia, OT contamination has been a widespread problem because most countries or regions do not implement local regulations to restrict the use of OT-based antifouling paints on seagoing vessels and fish farming facilities (e.g., open-sea cages), although some of them are members of the International Maritime Organization, which has enforced a global ban of such paints since September 2008. Contamination by OTs was the most severe in coastal waters where intensive shipping or mariculture activities could be found. To rectify the problem and safeguard the marine ecosystem and human health, long-term monitoring of OT contamination and enforcement of more stringent regulations on controlling the use and release of these pollutants in Southeast Asian countries are urgently needed.

Keywords Biomonitoring • Antifouling • Tributyltin • Triphenyltin • Ecological risk • Tissue burden • Endocrine disrupter • Marine ecosystem • South China • Hong Kong

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6.1 Overview

Organotin compounds (OTs), a group of organometallic compounds with a tin (Sn) atom covalently bonded to organic substituents, have been contaminating coastal marine environments worldwide ever since their first application as pesticides on high-value crops back in 1925 (Thompson et al. 1985; Hamlin and Guillette 2010). Among all OTs, the tri-substituted compounds, such as tributyltin (TBT) and triphenyltin (TPT), are found to have the greatest toxicological activity and, at the same time, the highest commercial value as effectual biocides (Fent 1996; Bao et al. 2011; Yi et al. 2012, 2014a, b). These compounds are then widely used in agricultural practices and textile industries as pesticides, fungicides, bactericides, and insecticides (Hoch 2001; Krupp et al. 2011; Lee and Chen 2011), as well as in antifouling systems on ship hulls and submerged fish farming facilities (Fent 1998). In Southeast Asia, TPT acetate and TPT chloride are even used as molluscides in the shrimp farming industry (Gräslund and Bengtsson 2001). To date, phenyltin compounds are still widely used in various industrial products, and their contamination is widespread in China because monophenyltin has been frequently detected in sediment samples from coastal waters of South China (Zhang et al. 2013). The global production of OTs in the industry had been increasing annually to couple with their growing demand, from <5000 tonnes in 1955 to 35,000 tonnes in 1986, and a further increase to 50,000 tonnes in 1992 (WHO 1980; Huang et al. 1993; Hoch 2001). Although data of global OT production are lacking and the demand is thought to have been reduced in recent years, the production and usage of these compounds still remain prominent. For instance, China consumes at least 7500 tonnes of OTs every year (Jiang 2001; Cao et al. 2009), and produces at least 200 tonnes of TPT annually (Hu et al. 2009). Between 1994 and 1996, Japan also exported up to 140 tonnes of TPT for antifouling paints, although their internal use of TPT had ceased after 1990 (WHO 1999).

OTs, in particular butyltin and phenyltin compounds, are highly persistent in the marine environment, extremely toxic, and highly bio-accumulative. Therefore, these two groups of compounds are considered as being the highest ecological concern (Huang et al. 1993; Fent 1996). Goldberg (1986) expressed that "TBT was perhaps the most toxic substance ever deliberately introduced to the marine environment by mankind." The adverse effects of OTs have called for management actions against these compounds on international stages. The Marine Environment Protection Committee (MEPC) of the International Maritime Organization (IMO) adopted a resolution in 1990 that recommended the elimination of the use of TBT-containing antifouling paints on non-aluminium-hulled vessels of less than 25 m in total length. In 1999, another resolution was adopted to call for a global ban of these paints. The legally binding convention, called the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention), was adopted in the IMO assembly in November 2001 (IMO 2008). The convention was then open for signature, and finally on 17 September 2007, Panama signed the convention as the 26th country ratifying the convention, and in total representing

38 % of the total tonnage of the world's fleet. The convention was finally entered into force on 17 September 2008 after a 1-year grace period (Sonak et al. 2009). Under the AFS Convention, all vessels cannot apply or re-apply OTs in their antifouling systems. All ship hulls should not bear OTs, and they should have a coating to cover the external parts of hulls to prevent such compounds from leaching into the marine environment (Cheung et al. 2010). Up until 31 August 2014, 68 countries or areas have ratified the AFS Convention, accounting for more than 83 % of the world's total tonnage (IMO 2014). Among the Southeast Asian countries or areas, only China, Malaysia, Singapore, and Macau (as associate member) have ratified the convention. Hong Kong has not yet signed the convention and Taiwan is not a member state of IMO. Thailand, the Philippines, East Timor, Indonesia, Brunei, Cambodia, Laos, and Myanmar have not ratified the convention either, and they all have very limited local restrictions of the use of OT-based antifouling systems.

Taking Hong Kong as an example, although Hong Kong is a member state of IMO it has not ratified the AFS Convention at the moment (IMO 2014). The only regulation regarding the use of OTs as antifouling paints was a partial ban on TBT-based antifouling paints on small vessels (i.e., <25 m in length) and open-sea cages, which was enacted in 1992 (Ko et al. 1995; ACE 1996). At the same time, TBT was deregistered from the list of pesticides under the Pesticide Ordinance Cap. 133, and the use of TBT was only allowed to those with a special permit (EPD 2008). However, there were still 11 registered boatyard users of TBT in 1995 (Ko et al. 1995), possibly continuously contaminating the marine environment of Hong Kong.

Taiwan did not sign the AFS Convention because it is not a member state of IMO (IMO 2014). However, there are some regulations concerning OTs locally. A prohibition of the use of pesticides containing TPT acetate was proposed by the Council of Agriculture in Taiwan in 1997. Triphenyltin compounds were then completely banned in agricultural practices in 1999. Tributyltin compounds were prohibited in the antifouling systems on small boats (<25 m in length) in 2003 (Meng et al. 2009). Other than these, no other regulations regarding OTs in water, sediment, flora, and fauna have been implemented in Taiwan so far (Hung et al. 2001).

On the other hand, as China is one of the countries ratifying the AFS Convention, it has the responsibility to implement national regulations to prohibit the use of OTs in antifouling systems concerning seagoing vessels and fish farming facilities (e.g., open-sea cages). However, such restrictions have not been implemented in any parts of China (Jiang et al. 2001; Cao et al. 2009). Together with the uncontrolled inland freshwater inputs of OTs (Lau et al. 2012), the contaminations have already caused widespread pollution problems in China, and the measured concentrations of OTs were far higher than international standards (Jiang 2001). Thus, academics have been calling for various actions to control the use and release of these pollutants into the marine environments of China for more than 10 years. These proposed actions include a continuous large-scale monitoring of OTs, an implementation of appropriate regulations to control the use of OT-containing antifouling

paints, an inclusion of OTs into water quality guidelines, an establishment of risk assessment procedures for these compounds, and an enhancement of the monitoring and educational programmes associated with the control of the use and release of these pollutants (Jiang 2001; Jin 2008; Cao et al. 2009; Gao et al. 2013).

Other Southeast Asian countries at present have no specific regulations on the use and release of OTs, including Malaysia (Tong et al. 1996) and Singapore (Tan 1997; Basheer et al. 2002). No relevant information can be found in the Philippines, East Timor, Indonesia, Brunei, Cambodia, Laos, and Myanmar. However, there is growing evidence showing that coastal marine waters of the southern Asian region have been extensively contaminated by OTs, posing health risks to the marine ecosystems and humans in this region.

6.2 Current Status of Organotin Contamination

As most Southeast Asian countries or regions do not have comprehensive regulations on the control of the use and release of OTs, contamination by OTs is commonly detected in water, sediment, and biota throughout this region (Tables 6.1, 6.2, and 6.3). Most documented studies have been concentrated on the distribution of TBT and other butyltin compounds in the water column, sediments, and biota. Although there is a growing concern about the contamination by TPT and other phenyltin compounds as well as their adverse impacts to marine organisms (Lee et al. 2005; Xie et al. 2010; Ho and Leung 2014b), the total number of relevant studies on phenyltin compounds is limited and scanty. Thus, historical comparisons of OT contamination would be limited to butyltin compounds.

6.2.1 Seawater

Table 6.1 summarises the concentrations of OTs measured in seawater samples collected in coastal environments of Southeast Asia and China during the past 27 years. In Hong Kong, a general decreasing trend of TBT was recorded during the past 20 years. Lau (1991) first measured the concentration of TBT in seawater, which reached 1000 ng l^{-1}. In sediment pore water, TBT concentration was detected up to 610 ng l^{-1} (Cheung et al. 2003). For sewage and stormwater discharge, the maximum concentration of butyltins detected was 12.2 ng l^{-1}, and TBT was only measured at 1.1 ng l^{-1} at maximum (Kueh and Lam 2008). However, the levels of TPT and other phenyltin compounds in seawater were only uncovered by a recent unpublished study, which revealed that the seawater was severely contaminated by TPT in different coastal areas of Hong Kong, in particular, at typhoon shelters (Ho et al. 2016). The total OT concentration ranged from 20.5 to 41.9 ng l^{-1}, at which TPT was the dominating compound among OTs, accounting for 45–63 % of total OTs (Ho et al. 2016). The concentrations of

Table 6.1 Concentrations of various organotin compounds in seawater in various locations of Southeast Asia and China: studies conducted between 1988 and 2014 of butyltins including mono-, di-, and tri-butyltin (TBT) and phenyltins encompassing mono-, di- and tri-phenyltin; total organotins are a sum of these six compounds

Country/ area	Location	Concentration	Year of study	References
Hong Kong	Various locations	TBT: <90–1000 ng l^{-1}	1988–1989	Lau (1991)
	Victoria Harbour	TBT: n.d.–2740 ng l^{-1}	1989	Chiu et al. (1991)
	Yam O, Tsing Yi	TBT: 120–610 ng l^{-1}	NA	Cheung et al. (2003)
	Various water control zones	Butyltins: <2–5 ng l^{-1}	2004	Kueh and Lam (2008)
	Sewage and river discharge	Butyltins: 0.0–12.2 ng l^{-1}	2004	Kueh and Lam (2008)
	Various locations	Total organotins: 20.5–41.9 ng l^{-1}	2013–2014	Ho et al. (2016)
China	Pearl River Delta	TBT: 21–39 ng l^{-1}	1996	Chau (2005)
	Coastal cities of Northern China	Butyltins: 224.2 ng l^{-1}	NA	Jiang (2001)
	Various locations along the coastline	Butyltins: 18.5–1273 ng Sn l^{-1}	1998–1999	Jiang et al. (2001)
	Shantou	Butyltins: 338.76 ng l^{-1}	2001–2002	Huang et al. (2005)
	Various locations along the eastern and southern coastline	Butyltins: 224.2 ng Sn l^{-1}	2002	Zhou et al. (2002)
	Pearl River Delta	TBT: 21.7–38.5 ng l^{-1}	NA	Fu et al. (2003)
	Bohai Bay	TBT: 0.0–14.7 ng l^{-1}	2002	Gao et al. (2004)
	Huiyang	Butyltins: 3290.2 ng l^{-1}	2002	Huang et al. (2005)
	Xiamen	Butyltins: 373.0 ng l^{-1}	2002	Huang et al. (2005)
	Xiamen	TBT: 2.2–160 ng Sn l^{-1}	2006	Wang et al. (2008)
	Shekou Harbour, Shenzhen	Butyltins: 191 ng l^{-1} (TBT up to 152 ng l^{-1})	2007	Deng et al. (2008)
Taiwan	Kaohsiung	Butyltins: 90 ng l^{-1}	1997–1998	Ou and Dong (1998) and Dong et al. (2004)
	Southern waters	Butyltins: 23.1–96.0 ng Sn l^{-1}	2002	Liu et al. (2002)
		(TBT was below detection limit)		
Malaysia	Various locations	TBT: <1.39–115 ng Sn l^{-1}	1991–1992	Tong et al. (1996)
	Strait of Malacca	Butyltins: <0.24–5.1 ng Sn l^{-1}	1996	Hashimoto et al. (1998)
Bay of Bengal	Various locations	Butyltins: <0.12–0.2 ng Sn l^{-1}	1996	Hashimoto et al. (1998)

NA data not available, *n.d.* not determined

Table 6.2 Concentrations of organotins in sediment in various locations of Southeast Asia and China from studies conducted during 1988–2014 of butyltins including mono-, di-, and tri-butyltin and phenyltins encompassing mono-, di-, and tri-phenyltin (dw, dry weight); total organotins are a sum of these compounds

Country/area	Location	Concentration	Year of study	References
China	Pearl River Delta	1.7–379.7 ng g^{-1} dw	1997–1999	Zhang et al. (2003)
	Bohai Bay	Butyltins: 1.32–2.16 ng g^{-1} dw	2011	An et al. (2013)
		Phenyltins: 5.14–7.21 ng g^{-1} dw		
	Xiamen	n.d.–26 μg Sn kg^{-1} dw	2006	Wang et al. (2008)
	Shantou	Butyltins: 5.1–35.9 ng g^{-1} dw	2002	Huang et al. (2005)
	Huiyang	Butyltins: 37.6–106 ng g^{-1} dw	2002	Huang et al. (2005)
Hong Kong	Various locations	n.d.–1160 ng g^{-1}[a]	1988–1989	Lau (1991)
	Various locations	2.4–2837 ng g^{-1} dw as TBT	NA	EPD (1998)
	Yam O	2–560 ng g^{-1} dw	NA	Cheung et al. (2003)
	Tsing Yi	3.2–100 ng g^{-1} dw	NA	Cheung et al. (2003)
	Victoria Harbour	129,486 ng g^{-1} dw as TBT	1994	Ko et al. (1995)
		53,000 ng g^{-1} dw as Sn		
	Aberdeen	44,709 ng g^{-1} dw as TBT	1994	Ko et al. (1995)
		18,300 ng g^{-1} dw as Sn		
	Various locations	TBT: 1.5 (east of Hong Kong Island)–1163 (Tsing Yi) ng g^{-1} dw as Sn	1998	Ma et al. (1998)
	Hebe Haven	MBT: 6.5 ng g^{-1} dw as Sn	1998	Ma et al. (1998)
		DBT: 37.5 ng g^{-1} dw as Sn		
		TBT: 39.0 ng g^{-1} dw as Sn		
Taiwan	Kaohsiung	57 ng g^{-1} dw	1997–1998	Ou and Dong (1998) and Dong et al. (2004)
Malaysia		Butyltins: 5.9–1266 μg kg^{-1} dw	2006	Harino et al. (2009)
		Phenyltins: 0.45–184 μg kg^{-1} dw		
		Butyltins: 14–1400 ng g^{-1} dw	1997–1998	Sudaryanto et al. (2004)
Thailand		MBT: 7–410 ng g^{-1} dw	1995	Kan-Atireklap et al. (1997)
		DBT: 2–1900 ng g^{-1} dw		
		TBT: 4–4500 ng g^{-1} dw		
		Butyltins: 3.3–1907 μg kg^{-1} dw	2004	Harino et al. (2006)
		Phenyltins: 0.9–44.1 μg kg^{-1} dw		
Vietnam		Butyltins: 1.55–43.6 μg kg^{-1} dw	2002	Midorikawa et al. (2004)
		Phenyltins: 2.4–9.8 μg kg^{-1} dw		

NA data not available

[a]The original literature did not mention whether the measured value was based on wet weight or dry weight

OTs were generally higher in summer than those in winter, probably because of the increased rainfall, surface runoff, and sewage discharge during summer.

Being the largest developing country in Southeast Asia and with wide applications of OTs, China has been reported to have heavy OT contamination in its seawater. Jiang et al. (2001) first extensively quantified the concentrations of butyltins (i.e., summation of MBT, DBT, and TBT) in various water bodies along the coastline of China and detected these compounds at concentrations up to 1273 ng Sn l^{-1}, in which TBT was the dominant compound, ranging from below detection limit (0.5 ng Sn l^{-1}) to 977 ng Sn l^{-1}. Many important coastal cities in Southeast China were threatened by OTs. Xiamen, for instance, was shown to have concentrations of OTs in seawater ranging from 62.9 to 1128.1 ng l^{-1} (total butyltins) and from 2.2 to 160 ng Sn l^{-1} (TBT), respectively, in two separate studies (Huang et al. 2005; Wang et al. 2008). The southern part of China, being an area with rapid economic growth, is often regarded as the "world's factory." Because of the intensive marine and estuarine traffic associated with busy marine trades and fishing activities, coastal areas of South China generally receive greater contamination of OTs than other parts of China. For example, in Shekou Harbour of Shenzhen, the average concentration of total butyltins in seawater was 191 ng l^{-1}, and TBT remained the dominant compound among butyltins at concentrations up to 152 ng l^{-1}, reflecting the continuous input of TBT into the system (Deng et al. 2008). Although the average concentration of total butyltins in South China was comparable to those measured in northern parts of China (Jiang 2001), the highest concentration of TBT in southern waters was much higher than its residual standards of the U.S.A. and Canada by 8- and 80-fold, respectively (Deng et al. 2008). The results indicated the severity of OT contamination in the South China region. Water samples collected from the Pearl River Delta also showed severe contamination by TBT at concentrations ranging from 21.7 to 38.5 ng l^{-1} (Fu et al. 2003), which were much higher than the standards given by the U.K. (2 ng l^{-1}; Cleary 1992) and the U.S.A. (10 ng l^{-1}; Federal Register 1989).

In Taiwan, Tang and Wang (2009) discovered a negative relationship between seawater TBT concentrations and the distance between a sampling point and an adjacent fishing port or harbour. Meng et al. (2009) summarised OTs concentrations detected in coastal waters of Taiwan and showed that the highest concentration of TBT, at 480 ng l^{-1}, was found in a commercial harbour of Kaohsiung (Jang 2004). In coral reef areas, the concentrations of TBT were comparatively low, ranging from not detected to 17 ng l^{-1} (Lee 2002). Phenyltin compounds were not detected in all samples.

In other parts of Southeast Asia, contaminations of OTs in seawater were poorly studied. Harino et al. (2008) summarised two studies of OT contamination in seawater from Malaysia. One of these studies revealed TBT at concentrations ranging from <1.39 to 115 ng Sn l^{-1}, and the highest concentrations were observed at Selangor within the Strait of Malacca (Tong et al. 1996). In Singapore, TBT concentrations in seawater ranged from 0.43 to 3.2 μg l^{-1}, and high concentrations

Table 6.3 Concentrations of organotins in biota tissues collected from various locations in Southeast Asia and China from studies conducted during 1990–2014 of butyltins (dw) including mono-, di-, and tri-butyltin and phenyltins encompassing mono-, di-, and tri-phenyltin; total organotins are these six compounds

Taxa	Species	Location	Concentration	Year of study	Reference
Gastropod	Rock shell, *Reishia clavigera*	Shantou, China	Butyltins: 47.40 ng g^{-1} ww	2001	Huang et al. (2005)
		Xiamen, China	Butyltins: 24.9–124.3 ng g^{-1} ww	2001–2002	Huang et al. (2005)
		Huiyang, China	Butyltins: 44.55 ng g^{-1} ww	2002	Huang et al. (2005)
		Pearl River Delta	TBT: <18.8 ng g^{-1} ww	2003	Huang et al. (2005)
		Hong Kong	Butyltins: <0.05–914.7 ng Sn g^{-1} dw	2004	Leung et al. (2006)
		Hong Kong	Butyltins: 19.1–177.0 ng Sn g^{-1} dw	2005–2006	Qiu et al. (2011)
		Shenzhen	Butyltins: 12.2–122.4 ng Sn g^{-1} dw	2006	Chan et al. (2008)
		Daya Bay and Dapeng Bay of Shenzhen	Butyltins: 15.8–70.8 μg kg^{-1} dw	2013	Ho and Leung (2014b)
			Phenyltins: 5047.3–23,420.5 μg kg^{-1} dw		
	Rock shell, *Thais* sp.	Sarawak, East Malaysia	MBT: 87.5–173 μg kg^{-1} ww	NA	Mohamat-Yusuff et al. (2014)
			DBT: 18–269 μg kg^{-1} ww		
			TBT: 1–33 μg kg^{-1} ww		
			MPT: up to 154 μg kg^{-1} ww		
			DPT: up to 538 μg kg^{-1} ww		
			TPT: up to 25 μg kg^{-1} ww		
	Veined rapa whelk, *Rapana venosa*	Bohai Bay	Butyltins: 14.1–28.81 ng g^{-1} dw	2011	An et al. (2013)
			Phenyltins: 49.04–78.02 ng g^{-1} dw		
	Babylon shell, *Babylonia areolata*	Hong Kong	Butyltins: 21.6 μg kg^{-1} dw	2012	Ho and Leung (2014a)
			Phenyltins: 1729.7 μg kg^{-1} dw		
	Neptunea cumingi	Lianyungang	Butyltins: 37.3 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Natica clausa	Dalian	Butyltins: 45.04 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various snails	Yantai	Butyltins: 20.2–63.4 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various snails	Qingdao	Butyltins: 19.8–29.4 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various snails	Tianjin	Butyltins: 27.1–32.0 ng Sn g^{-1} ww	NA	Zhou et al. (2001)

Bivalve	Clam	Hong Kong	Up to 672 ng g^{-1} dw	NA	Lau (1991)
	Clam, *Meretrix* spp.	Vietnam	Butyltins: 2.0–110.0 μg kg^{-1} ww	2002	Midorikawa et al. (2004)
			Phenyltins: 0.3–14.8 μg kg^{-1} ww		
	Pacific oyster, *Crassostrea gigas*	Taiwan	TBT: up to 1510 ng g^{-1} dw	1996–1997	Hung et al. (1998)
			TPT: 102–590 ng g^{-1} dw		
	Green-lipped mussel, *Perna viridis*	Hong Kong	9.07–114.5 ng g^{-1} Sn ww	NA	Chiu et al. (1991)
		Malaysia	3.6–900 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2004)
		Cambodia	Butyltins: 2.4–150 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		China	Butyltins: 30–500 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		Indonesia	Butyltins: 3.7–64 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		Malaysia	Butyltins: 3.5–960 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		Philippines	Butyltins: 0.8–74 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		Vietnam	Butyltins: 2.1–100 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2002)
		Thailand	Butyltins: 16–74 μg kg^{-1} ww	2004	Harino et al. (2006)
			Phenyltins: 11–106 μg kg^{-1} ww		
		Various north-eastern cities of China	Butyltins: 23.4–162.4 ng Sn g^{-1} ww	2004	Yang et al. (2006)
	Mytilus edulis	Various northeastern cities of China	Butyltins: 27.0–119.2 ng Sn g^{-1} ww	2004	Yang et al. (2006)
	Mytilus galloprovincialis	Qinhuangdao	Butyltins: 119 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Solen sp.	Beijing	Butyltins: 26.4 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Beijing	Butyltins: 21.5–218.2 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Dalian	Butyltins: 30.9–18,007 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Tianjin	Butyltins: 33.7–100.4 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Qingdao	Butyltins: 22.0–204.7 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Yantai	Butyltins: 23.8–70.0 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Various bivalve species	Lianyungang	Butyltins: 24.6–441.8 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Unidentified mussel sample	China (Pearl River)	Butyltins: 62.9 ng g^{-1} ww	1997–1999	Zhang et al. (2003)

(continued)

Table 6.3 (continued)

Taxa	Species	Location	Concentration	Year of study	Reference
Crustacean	Unidentified shrimp sample	China (Pearl River)	Butyltins: 16.4 ng g^{-1} ww	1997–1999	Zhang et al. (2003)
	Hemigrapsus penicillatus	Tianjin	Butyltins: below detection level	NA	Zhou et al. (2001)
	Orientomysis koreana	Tianjin	Butyltins: 31.4 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
Fish	Various fish species	Thailand	Butyltins: 2.9–16 ng g^{-1} ww	1990–1992	Kannan et al. (1995)
	Various fish species	Vietnam	Butyltins: ND–1.7 ng g^{-1} ww	1990–1992	Kannan et al. (1995)
	Various fish species	Indonesia	Butyltins: 0.41–19 ng g^{-1} ww	1990–1992	Kannan et al. (1995)
	Various fish species	Taiwan	Butyltins: 0.49–18 ng g^{-1} ww	1990–1992	Kannan et al. (1995)
	Asian seabass, *Lates calcarifer*	Bangkok, Thailand	Butyltins: 18.4 ng g^{-1} ww	1994	Kannan et al. (1995)
	Red seabream, *Pagrus major*	Taiwan	Butyltins: 18.5 ng g^{-1} dw	1997	Hung et al. (1998)
			Phenyltins: not detected		
	Peacock grouper, *Cephalopholis argus*	Taiwan	Butyltins: 46.2 ng g^{-1} dw	1997	Hung et al. (1998)
			Phenyltins: not detected		
	Asian seabass, *Lates calcarifer*	Taiwan	Butyltins: 54.6 ng g^{-1} dw	1997	Hung et al. (1998)
			Phenyltins: not detected		
	Black parrotfish, *Chlorurus (= Scarus) cedema*	Taiwan	Butyltins: 39.8 ng g^{-1} dw	1997	Hung et al. (1998)
			Phenyltins: not detected		
	Various fish species	Malaysia	3.6–210 ng g^{-1} ww	1997–1998	Sudaryanto et al. (2004)
	Ponyfish, *Leiogenathus splendens*	Taiwan	Butyltins: 236–2501 ng g^{-1} ww (whole body)	1997–1998	Dong et al. (2004)
	Lizardfish, *Trachinocephalus myops*	Taiwan	Butyltins: 36–159 ng g^{-1} ww (muscle)	1997–1998	Dong et al. (2004)
			3058–11,473 ng g^{-1} ww (liver)		
	Unidentified fish sample	China (Pearl River)	Butyltins: 11.0–34.0 ng g^{-1} ww	1997–1999	Zhang et al. (2003)
	Narrow-barred Spanish mackerel, *Scomberomorus commerson*	Taiwan	Butyltins: 258.7–1363.9 ng g^{-1} dw	2002–2003	Lee et al. (2005)

	Japanese seabream, *Pagrus major*	Taiwan	Butyltins: 82.1–769.1 ng g^{-1} dw	2002–2003	Lee et al. (2005)
	Croceine croaker *Larimichtchys croceus*	Taiwan	Butyltins: 169.1–208.3 ng g^{-1} dw	2002–2003	Lee et al. (2005)
	Milkfish, *Chanos chanos*	Taiwan	Butyltins: 180.2 ng g^{-1} dw	2002–2003	Lee et al. (2005)
	Yellow-back seabream, *Taius tumifrons*	Taiwan	Butyltins: 310.6–410.0 ng g^{-1} dw	2002–2003	Lee et al. (2005)
	Blue-and-gold fusilier, *Caesio caerulaurea*	Taiwan	Butyltins: 351.1 ng g^{-1} dw	2002–2003	Lee et al. (2005)
	Tonguesole, *Paraplagusia blochii*	Hong Kong	Butyltins: 61.2 μg kg^{-1} dw	2013	Ho and Leung (2014a)
			Phenyltins: 2264.6 μg kg^{-1} dw		
	Auxis tapeinosoma	Beijing	Butyltins: 31.2 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Trichiurus haumela	Beijing	Butyltins: 25.9 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Engraulis japonicus	Qingdao	Butyltins: 22.3 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Engraulis japonicus	Yantai	Butyltins: 42.6 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
	Muraenesox cinereus	Yantai	Butyltins: 29.6 ng Sn g^{-1} ww	NA	Zhou et al. (2001)
Mammal	Bryde's whale, *Balaenoptera edeni*	Thailand	Butyltins: 0.058–0.213 mg kg^{-1} ww	1997–2002	Harino et al. (2007a)
			Phenyltins: 0.028–0.872 mg kg^{-1} ww		
	False killer whale, *Pseudorca crassidens*	Thailand	Butyltins: 0.071–4.86 mg kg^{-1} ww	1997–2002	Harino et al. (2007a)
			Phenyltins: 0.022–1.14 mg kg^{-1} ww		
	Pygmy killer whale, *Kogia breviceps*	Thailand	Butyltins: 0.141–0.647 mg kg^{-1} ww	1997–2002	Harino et al. (2007a)
			Phenyltins: 0.136–0.413 mg kg^{-1} ww		
	Short-finned pilot whale, *Globicephala macrorhynchus*	Thailand	Butyltins: 0.191–0.546 mg kg^{-1} ww	1997–2002	Harino et al. (2007a)
			Phenyltins: 0.058–0.067 mg kg^{-1} ww		
	Sperm whale, *Physeter macrocephalus*	Thailand	Butyltins: 0.094–0.285 mg kg^{-1} ww	1997–2002	Harino et al. (2007a)
			Phenyltins: 0.106–0.413 mg kg^{-1} ww		
	Dugongs, *Dugong dugon*	Thailand	Butyltins: 14–14,468 μg kg^{-1} ww	1997–2002	Harino et al. (2007b)
			Phenyltins: <1–30 μg kg^{-1} ww		

NA data not available

of TBT were found in sites near Jurong Island where there are intensive shipping activities (Basheer et al. 2002).

6.2.2 Sediment

Concentrations of OTs measured in sediment in Southeast Asia and China during the past 27 years are presented in Table 6.2. Taking Hong Kong as an example, TBT concentrations in sediment fluctuated over time but generally showed a decreasing temporal trend. The TBT concentration in sediment was detected up to 1690 ng g^{-1} dry weight (dw) in the early 1990s (Lau 1991). Later, the concentration of TBT in a sediment sample reported by Ko et al. (1995) was detected up to 53,000 ng Sn g^{-1} dw (=129,486 ng TBT g^{-1} dw). Cheung et al. (2003) detected TBT concentrations up to 560 μg kg^{-1} in sediments from Yam O. However, in recent years, only a trace amount of TBT was detected in sediment by the Environmental Protection Department of the Hong Kong SAR Government (EPD 2008).

In China, data on OT contamination in sediment are relatively limited, especially in the northern part of China. Zhang et al. (2003) measured TBT concentrations in the sediment collected from Pearl River Delta and found concentrations ranging from 1.7 to 379.7 ng g^{-1} dw, which were lower than in other parts of the world. The highest concentrations of TBT were found in locations close to shipyard and harbours. There was a downward-sloping gradient of TBT concentrations from Pearl River to the estuary, suggesting that the source of TBT was from the intensive riverine shipping activities and sewage discharges, and there was a dilution effect of seawater to the contaminants in the estuary. Huang et al. (2005) investigated the concentrations of OTs in the sediment collected from three harbours along the coast of Southeastern China and found TBT levels between 0.3 and 174.7 ng g^{-1} dw. Xiamen Harbour was the most contaminated site among the three harbours, probably because of the discharge of contaminated freshwater and suspended solids from Jiulong River, and such turbid water in Xiamen Harbour resulted in a slower photodegradation rate of TBT therein.

In Taiwan, both butyltin and phenyltin compounds were detected in marine sediment (Meng et al. 2009). Butyltins, in particular TBT, were dominant over phenyltins. TBT was detected as high as 25,300 ng g^{-1} wet weight (ww) in a sample collected from the harbour area of Kaohsiung (Jang 2004), whereas in the estuary area of Love River, Kaohsiung, the concentration of TBT could also reach 20,200 ng g^{-1} ww. The concentration of TPT was as much as 1811 ng g^{-1} dw in the same estuary location (Chi 2004). Tang and Wang (2009) found that a fishing port was a hotspot of TBT contamination in both seawater and sediment compartments, whereas TPT was merely detected in sediment but not in seawater.

Among several Southeast Asian countries, TBT has been consistently detected in sediment samples collected from Malaysian waters for more than a decade (Tong et al. 1996; Sudaryanto et al. 2004). Harino et al. (2009) reported that TBT concentrations in sediment were higher in Johor Strait among the coastal waters

of Peninsula Malaysia, where there is a major shipping channel. Harino et al. (2012) reviewed the contamination of OTs in Southeast Asia and concluded that higher concentrations of TBT were persistent in sediments found in Malaysia and Thailand, particularly in the coastal areas. Specifically, the highest concentration of TBT at 1246 μg kg^{-1} dw was detected in sediment in an industrial area proximal to shipyards in Thailand. This finding echoed the previous study conducted by Kan-Atireklap et al. (1997) in Thailand in which high TBT concentrations, up to 4500 ng g^{-1} dw, were found in the marine sediment. Phenyltin compounds were also detected at concentrations up to 35 μg kg^{-1} dw in sediment in Thailand (Harino et al. 2012). In Vietnam, TBT remained the dominant OT compounds at concentrations ranging from 0.8 to 28 μg kg^{-1} dw, and hotspots of TBT contamination include trading ports and coastal sites near industrial areas (Midorikawa et al. 2004).

6.2.3 Biota

A wide range of marine organisms, from microalgae to fish, were found to accumulate OTs in their body tissues (Bao et al. 2011; Yi et al. 2012, 2014a, b). Bioconcentration, the direct uptake of OTs from water or sediment, has been clearly shown in marine algae (Yi et al. 2012). OTs can be bioaccumulated along a food chain through diet (Stäb et al. 1996; Yi et al. 2012). TBT has shown to be biomagnified as various studies documented higher TBT concentrations in animals occupying higher trophic levels, such as in marine mammals, fish-eating birds, and humans (Iwata et al. 1997; Kannan and Falandysz 1997). Concentrations of TPT were generally the lowest in seawater, followed by those detected in marine sediment, whereas its concentrations in marine invertebrates and fishes were often higher than those in the two aforementioned environmental compartments (Yi et al. 2012). Previous studies showed that TPT can be biomagnified across lower trophic levels of food chains, and the biomagnification factor of TPT ranged from 2.2 to 5.4 across trophic levels from plankton to fish (Hu et al. 2006; Murai et al. 2008; Fortibuoni et al. 2013). As there were only limited data for TPT concentrations at higher trophic level organisms, the biomagnification of this compound in top predators such as sharks, marine mammals, and seabirds is still unclear at present.

Although OTs were introduced as an antifouling agent to kill the target fouling organisms on ship hulls and submerged mariculture facilities, OTs have been found to induce toxic effects to non-target marine species since the 1970s (Antizar-Ladislao 2008). Being one of the most documented endocrine-disrupting effects of OTs, imposex (i.e., the superimposition of male sexual characteristics–penis and vas deferens–on female gastropods) has been widely observed in females of many gastropod species worldwide. The whelk *Reishia clavigera* (formerly known as *Thais clavigera*; see Claremont et al. 2013) is one of the most extensively used biomonitors of OT contamination in the Asia–Pacific region (Horiguchi et al. 1994; Shim et al. 2000; Leung et al. 2006; Qiu et al. 2011; Ho and Leung 2014b; Ho et al.

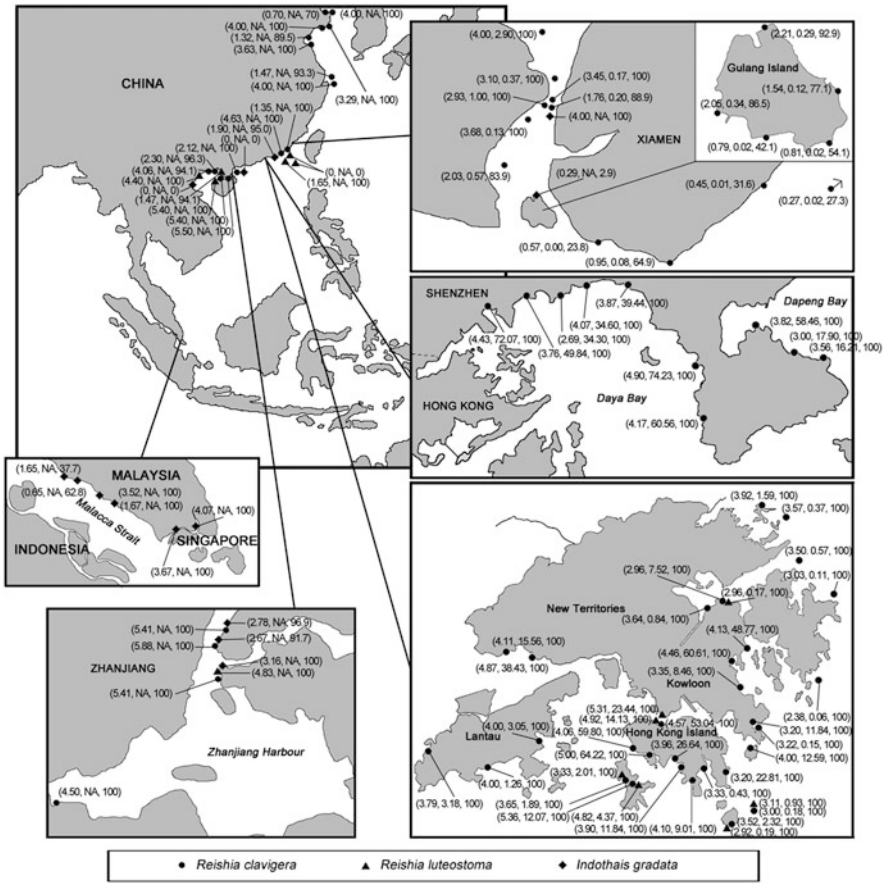

Fig. 6.1 Maps of Southeast Asia showing various studies of imposex status of three whelk species [*Reishia clavigera* (*circles*), *R. luteostoma* (*triangles*), and *Indothais gradata* (*diamonds*)] between 2000 and 2014. Data were extracted from Shi et al. (2005), Leung et al. (2006), Tang et al. (2009), Mohamat-Yusuff et al. (2010), Qiu et al. (2011) and Ho and Leung (2014b). *Values inside brackets* indicate vas deferens sequence index, relative penis size index, and proportion of imposex-affected females, respectively. *NA* data not available

2016) because of its high sensitivity towards OTs and its wide distribution across the region. This species could develop imposex at TBT concentrations as low as 1 ng l^{-1}, and TPT would have similar effect on this species as did TBT (Horiguchi et al. 1995). This species inhibits intertidal rocky shores from the southeast coast of China to Hokkaido, Japan (Tong 1986). Therefore, such a wide geographic distribution enables field monitoring of this species across the region. Across the Southeast Asian region, Hong Kong (Leung et al. 2006; Qiu et al. 2011), Taiwan (Liu et al. 1997; Hung et al. 2001), and Mainland China (Tang et al. 2009; Xie et al. 2010; Ho and Leung 2014b) have adopted the use of *R. clavigera* as a biomonitor species for monitoring the contamination of OTs in coastal marine environments (Fig. 6.1).

Apart from *R. clavigera*, three other whelk species that originally were placed in the genus *Thais* were also used as biomonitoring species for OT contamination in Southeast Asia. These species appeared to be sensitive towards OTs because the females were observed to be affected by imposex at locations with even slight contamination by OTs (Fig. 6.1): *Reishia luteostoma* (= *Thais luteostoma*) (Ellis and Pattisina 1990; Proud and Richardson 1997; Shi et al. 2005; Leung et al. 2006), *Indothais gradata* (= *Thais gradata*) (Swennen et al. 1997; Shi et al. 2005; Mohamat-Yusuff et al. 2010), and *Menathais tuberosa* (= *Thais tuberosa*) (Mohamat-Yusuff et al. 2011). Several other gastropod species, which belong to families Muricidae and Nassariidae, were also used as biomonitor species for OT contamination: *Nassarius siquijorensis* (Proud and Richardson 1997; Li 2000; Shi et al. 2005), *N. livescens* (Swennen et al. 1997), *N. festivus* (Chan and Morton 2003), and *Ergalatax contracta* (= *Ergalatax contractus*) (Li 2000; Shi et al. 2005). However, they inhabit narrower habitat ranges and are less sensitive towards OTs compared to *R. clavigera*, and hence they have been only used occasionally in regional monitoring studies in the past.

To assess the severity of imposex development on gastropods, two major indices are commonly used. The vas deferens sequence index (VDSI), first developed to quantify the severity of imposex development in *Nucella lapillus* (Bryan et al. 1986; Gibbs et al. 1987), is now widely applied for the assessment of imposex in other gastropod species. A generalized scheme describing the general imposex development in prosobranch gastropods was proposed by Stroben et al. (1995), and this scheme was further developed by Shi et al. (2005) to help assess imposex development in gastropods species other than *N. lapillus*. Cheung et al. (2010) summarised the classification of imposex stages of *R. clavigera* and gave pictorial descriptions on each imposex stage, and Horiguchi et al. (2012) further consolidated the classification of imposex stages of *R. clavigera* by using anatomical and histological observations. The same index has also been used for assessment in *R. luteostoma* (Shi et al. 2005; Leung et al. 2006), *Indothais gradata* (Shi et al. 2005), and *Menathais tuberosa* (Mohamat-Yusuff et al. 2011).

Relative penis size index (RPSI; sometimes relative penis length index is used instead, e.g., in Horiguchi et al. 1994) is another imposex index that is thought to be more objective in terms of measuring the relative sizes of penis between females and males. However, it was criticised that RPSI could vary seasonally (Chan et al. 2008; Tang et al. 2009). RPSI, together with the incidence of imposex (i.e., the proportion of imposex-affected female gastropods), were commonly used to assess the status of imposex in the aforementioned species (Ellis and Pattisina 1990; Leung et al. 2006).

Across the Southeast Asian region, certain large-scale biomonitoring studies have been conducted using *R. clavigera* as the biomonitor for the assessment of OT contamination (Fig. 6.1). A study conducted on the east coast of China, from Dalian to Hainan, showed that OT contamination was still prevalent across the region, especially in areas with intense marine traffic. In a study conducted by Shi et al. (2005) along the east coast of China, the imposex incidence in *R. clavigera* was more than 90 % in most of the survey locations. During 2004–2013, all sites in

Hong Kong and Shenzhen recorded 100 % imposex incidence in *R. clavigera* (Leung et al. 2006; Chan et al. 2008; Qiu et al. 2011; Ho and Leung 2014b; Ho et al. 2016), and 5 of 16 sites in Xiamen also showed 100 % imposex incidence (Tang et al. 2008, 2009).

Apart from *R. clavigera*, other gastropods (such as *R. luteostoma* and *I. gradata*) were also used as biomonitors of OT contamination as they exhibit imposex upon exposure to OTs (Fig. 6.1). The disruption of the reproductive system by exposure to OTs has been observed in other gastropods. For instance, the dysfunction of ovaries caused by ovarian dysmaturity and spermatogenesis were observed in the ivory shell *Babylonia japonica* (Horiguchi et al. 2006) and in the abalone *Haliotis gigantea* (Horiguchi et al. 2002).

A growing number of studies started incorporating measurement of tissue concentrations or tissue burdens of OTs as a direct way to quantify the effect of OT contamination in an organism (Table 6.3). However, studies measuring tissue concentrations of TPT and phenyltin compounds were far fewer than those measuring TBT and butyltin compounds. In gastropods, numerous studies have shown that tissue concentrations of OTs increased with decreasing distance to potential sources of OTs, such as piers, shipyards, and fish villages. This relationship has been documented worldwide, not only in Southeast Asia (Leung et al. 2006; Wang et al. 2008; Choi et al. 2009; Guðmundsdóttir et al. 2011).

Tissue concentrations of OTs in gastropods could vary seasonally. Hung et al. (2001) demonstrated that in *R. clavigera* the tissue concentration of butyltins was higher than that of phenyltins in winter, but phenyltin concentration was higher in summer. Qiu et al. (2011) found that the tissue butyltin concentrations were higher in winter than those in summer. Therefore, to understand the temporal dynamics of accumulation and depuration of OTs in gastropods, long-term monthly- or bimonthly-based monitoring surveys are recommended.

Apart from using gastropods as biomonitors, other species from different trophic levels have been quantified for their tissue concentrations of OTs because these compounds can be bioaccumulated along the marine food chain and may affect humans if they consume contaminated seafood. For instance, mussels were often used to quantify the degree of chemical contamination (e.g., metals and synthetic organic chemicals; Leung et al. 2011, 2014a, b) in marine environments. The green-lipped mussel *Perna viridis* had been used to assess the OT contamination in the Strait of Malacca, Malaysia, and it was found that TBT was the predominant compound whereas TPT was not found in the mussel samples (Harino et al. 2009).

Fishes are also often used to assess the OT contamination in the marine environment because of their wide distributions and linkage with human consumption as seafood. In Taiwan, several studies have assessed the OT contamination in various fish species. Dong et al. (2004) investigated the tissue-specific butyltin concentrations in the benthic ponyfish *Leiognathus splendens* and the lizardfish *Trachinocephalus myops* and found that the concentrations varied seasonally. Lee et al. (2005) showed that certain demersal fishes exhibited high concentrations of TPT in their tissues, including the Japanese seabream *Pagrus major* and the narrow-barred Spanish mackerel *Scomberomorus commerson*. Their team also

demonstrated that the tissue concentrations of OTs in fishes showed seasonal patterns, in which butyltins were dominant in winter and phenyltins dominated in summer. Recently, Ho and Leung (2014a) quantified the amounts of OTs in 11 species of seafood in Hong Kong and found that certain benthic fish species, such as the tonguesole *Paraplagusia blochii*, could have higher tissue concentrations of OTs than other species, posing a higher health risk to humans at an average level of fish consumption.

OTs, in particular TPT, could induce immunotoxic effects in marine mammals and humans (Nakayama et al. 2009; Yi et al. 2012). With a large log K_{ow} value, ranging from 3.0 to 5.0 (Yi et al. 2012), it has been demonstrated that both TBT and TPT can be biomagnified through the food chain in coastal ecosystems to a certain extent (Strand and Jacobsen 2005; Hu et al. 2006), although the biomagnification of TPT through the food chain at higher trophic levels is still not clearly demonstrated.

For measuring OTs in marine mammals, the liver is the most commonly used target tissue type because concentrations of total butyltins in the liver were demonstrated to be much higher than those in other tissues such as muscle and blubber (Iwata et al. 1997). Nakayama et al. (2009) quantified the concentrations of various OTs in the liver of the finless porpoise *Neophocaena phocaenoides* collected in Hong Kong waters. The results showed that both butyltins and phenyltins concentrations were 10- to 20-fold higher than those measured in Japan, indicating that Hong Kong has been heavily contaminated with these OTs. It had been showed that OTs could increase the susceptibility of parasitic infection in the porpoises (Nakayama et al. 2009). Tanabe et al. (1998) quantified the concentrations of OTs in the livers of the finless porpoise from China, and the long-snouted spinner dolphin (*Stenella longirostris*) and Fraser's dolphin (*Lagenodelphis hosei*) from the Philippines, and reported that the highest tissue concentration was 890 ng g^{-1} ww. Choi et al. (2013) investigated the temporal variation (2003–2010) of TBT concentrations in the liver of the finless porpoise *Neophocaena asiaeorientalis* from the Yellow Sea near Qingdao, and found that the highest total butyltin concentration was 1432 ng g^{-1} ww, but that the concentrations were declining over the years.

Harino et al. (2007a, b) measured the concentrations of OTs in marine mammals, including whales and dugongs from Thailand, and found that tissue-specific butyltin concentrations were generally higher than those of phenyltins. Most OTs accumulated in the liver rather than other parts of the body such as blubber, lung, kidney, and muscle. In particular, the false killer whale from Phuket had the highest concentrations of butyltin compounds, up to 5 mg kg^{-1} ww.

6.3 Conclusions and Perspectives

The published data clearly showed that contamination of OTs in the coastal marine environments of Southeast Asia was undoubtedly severe. However, the current legislations in this region were far from adequately protective of the marine ecosystem. For those countries or areas with implementation of partial restrictions

on the use of OT-based antifouling paints (such as Hong Kong and Taiwan), more stringent regulations should be enacted locally or nationally to strengthen controls on the use and release of these chemical contaminants into the marine environment. For those countries or areas currently without any such restrictions, a comprehensive baseline survey on the contamination status of OTs should be conducted in different compartments of the marine environment, including seawater, sediment, and biota, with a view to fully determining the severity of OTs contamination in that region, evaluating the ecological risks associated with OTs, and making appropriate management strategies to better protect the ecosystem. The countries or areas in this region, especially those that already have had reasonably amounts of study on OTs, should be an integral part of information exchange, knowledge transfer, and research collaboration to jointly tackle such a pressing environmental issue in the region.

The industry is now phasing out the use of OT-based antifouling paints and searching for promising alternatives of booster biocides such as Irgarol 1051, copper pyrithione, zinc pyrithione, and triphenylborane. However, these synthetic booster biocides have been accused of being toxic to a variety of non-target marine organisms (Yebra et al. 2004; Zhang et al. 2008; Okamura et al. 2009; Bao et al. 2012). There are also uncertainties regarding their environmental impacts, including their bioaccumulation potential and synergistic interactions with other chemical pollutants (Yebra et al. 2004; Bao et al. 2013, 2014). Apart from comprehensively studying their environmental behaviours, the development of naturally biodegradable biocides and self-cleaning surfaces (e.g., trypsin-based coatings; Shi et al. 2011) would also help strike a balance between environmental protection and antifouling performance.

With the advances in technology, several molecular methods have been developed to help assess the severity of effects of OTs on biota, particularly on imposex development on marine gastropods, including gene and protein expression assays (Horiguchi et al. 2007). Recently, the use of the transcriptomic approach to reveal the toxic mechanisms of chemicals towards marine organisms has become popular and economical. For example, two transcriptomic libraries of *Reishia clavigera* (Ho et al. 2014; Ip et al. 2016) and *Perna viridis* (Leung et al. 2014a), which are common biomonitors in Asia, have been developed and made available in the GenBank for reference. These genomic resources could help environmental researchers to better understand the underlying molecular toxic mechanisms of OTs on marine organisms.

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Chapter 7
Current Status of Contamination by Organotins and Imposex in Prosobranch Gastropods in Korea

Hyeon-Seo Cho and Toshihiro Horiguchi

Abstract Organotin compounds are considered to be dangerous chemicals because of their deleterious effects on non-target marine organisms. In 2003, the use of TBT-based antifouling paints was totally banned in Korea, and the International Maritime Organization (IMO) proposed to extend the ban to almost all ocean-going vessels. In this study, the concentrations of organotins in the coastal environment are analyzed to illustrate the differences of these analyzed items in the periods before and after the IMO and Korean regulations, focusing on organotins concentrations in molluscan soft tissues and the imposex phenomenon in the rock shell (*Thais clavigera*), a gastropod species sensitive to organotin compounds, collected from the Korean coasts from 1995 to 1997 and 2002, and from 2005 to 2009. TBT and TPhT were dominant organotins. Higher organotin concentrations were observed in areas with frequent shipping activities, including regions adjacent to harbors or shipyards, than in areas away from shipping activities. Concentrations of TBT, TPhT, and their metabolites in tissue, imposex frequency, and relative penis length index and sterility ratio of rock shell specimens collected from the southern coast after the regulations for TBT- and/or TPhT-based antifouling paints were in place showed lower values than those before the regulations. Because of the continued occurrence of imposex in the rock shell populations after the regulations were established, it is necessary to carry out further studies to monitor organotin concentrations in tissues and imposex frequency of rock shell specimens along with evaluations of the organotins residues in seawater and sediment in Korean coastal areas.

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Abbreviations

MBT	monobutyltin
DBT	dibutyltin
TBT	tributyltin
MPhT	monophenyltin
DPhT	diphenyltin
TPhT	triphenyltin
IMO	International Maritime Organization
RPLI	relative penis length index

7.1 Introduction

Organotin compounds have been used worldwide in antifouling paints to prevent adherence of sedentary organisms to ship hulls and other structural surfaces immersed in seawater. Organotins such as tributyltin (TBT) and triphenyltin (TPhT) are considered to be dangerous chemicals because of their deleterious effects on non-target marine organisms. Particularly, the imposex phenomenon, a superimposition of male genital tracts (penis and vas deferens) on female gastropods, has been reported to occur at low concentrations of certain organotins such as TBT and TPhT. As of 2004, approximately 150 species of gastropods had been reported to be affected by imposex worldwide (Horiguchi 2009).

In Korea, national restrictions on the use of TBT-based antifouling paints were introduced in 2000 for small boats, and these paints were totally banned in 2003. In addition, the International Maritime Organization (IMO) proposed to extend the ban to almost all ocean-going vessels from 2003. In the case of persistent substances in the environment, such as organotin compounds, their fate in the environment before and after the regulations were instituted is an important issue. In this study, the concentrations of organotins in the coastal environment are described and discussed to illustrate the differences of these analyzed items in the periods before and after the IMO and Korea regulations in 2003, focusing on their concentrations in molluscan soft tissues and imposex phenomenon of the rock shell (*Thais clavigera*), a gastropod species sensitive to organotin compounds, collected from the Korean coasts from 1995 to 1997 and 2002, and from 2005 to 2009.

7.1.1 Materials and Methods

7.1.1.1 Sample Collection

We collected the rock shell, *Thais clavigera* (Muricidae, Gastropoda), at 43 sites along the Korean coast between October 1995 and August 1997, and at 37 sites from March to August 2002 (Fig. 7.1). From 2005 to 2009, the nationwide survey was carried out in 45 sampling sites from coastal areas of the West Sea, South Sea, and East Sea (Fig. 7.2). The sampling sites were slightly different between each sampling year, but most of the selected sampling points were located in the coastal areas where contamination by organotin compounds derived from shipping activities was expected. Rock shell specimens were collected in the intertidal bedrock areas in the Korean coast. The rock shell *Thais clavigera* is one of the most sensitive species to TBT or TPhT (Horiguchi et al. 1995) and therefore was used as a bioindicator for biological effects by TBT or TPhT (Horiguchi et al. 1997). The appearance of imposex has been also reported in other gastropods to reflect the degree of contamination of organotin compounds (mainly, TBT) released from antifouling paints (Gibbs et al. 1991; Stewart et al. 1992; Stroben et al. 1992; Wilson et al. 1993; Curtis 1994; Evans et al. 1994; Horiguchi et al. 1994).

Additionally, surface seawater, sediment, and several shellfishes, including the blue mussel (*Mytilus edulis*), Pacific oyster (*Crassostrea gigas*), grand jackknife clam (*Solen grandis*), manila clam (*Ruditapes philippinarum*), dosinia (*Dosinia japonica*), venus clam (*Meretrix lusoria*), surf clam (*Mactra veneriformis*), ark clam (*Tegillarca granosa*), comb pen shell (*Atrina pectinate*), blood clam (*Scapharca broughtonii*), Chinese freshwater mussel (*Lanceolaria grayana*), trough clam (*Spisula sachalinensis*), and sandy beach clam (*Gomphina melanaegis*) were also collected at several sites on the coastal area of Korea from June to November 2000. We also investigated the seawater and sediment contamination levels by organotins in Busan and Ulsan harbors, using seawater and sediment samples collected at both harbors in 2002. Busan and Ulsan harbors are known as

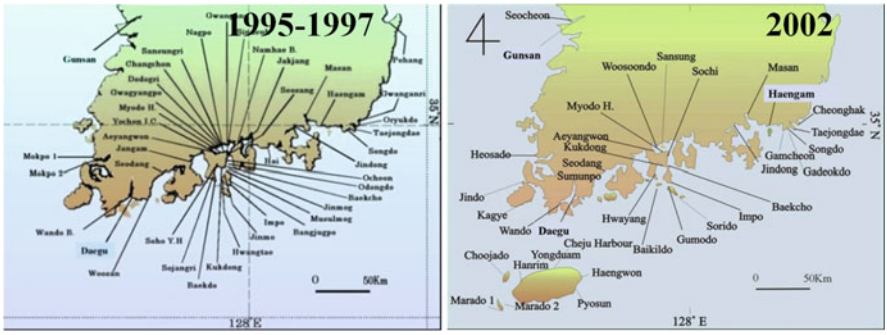

Fig. 7.1 Map showing the sampling sites of rock shells, *Thais clavigera*, collected from the Korean coast from 1995 to 1997 and in 2002

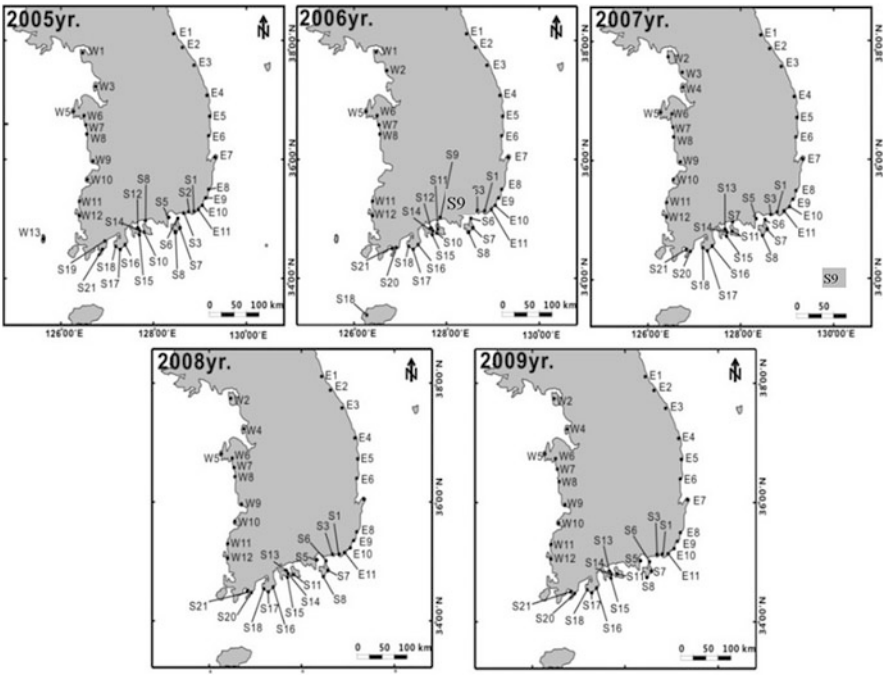

Fig. 7.2 Map showing the sampling sites of rock shells, *Thais clavigera*, collected from the Korean coast from 2005 to 2009 (Details on sampling location are shown in Table 7.1)

the biggest trade and industrialized harbors in Korea, respectively, which could have been the hotspot areas of organotins in Korea. Samples were kept at the freezer at −20 °C until chemical analysis for determination of organotin compounds.

7.1.2 Imposex Determination

According to the methods of Gibbs et al. (1987) and Horiguchi et al. (1994), sex was identified by the presence of female or male accessory sex organs: albumen gland, sperm-ingesting gland, and capsule gland for females, and prostate gland for males. Imposex was judged in female rock shells, based on the occurrence of a penis and development of vas deferens. Imposex was evaluated using the following indices: (1) percentage occurrence of imposex (imposex frequency = the number of imposex-exhibiting females/total number of females); (2) relative penis length index [RPLI (%)], which as calculated as the ratio (multiplied by 100) of the mean penis length in females to that in males at each location (Gibbs et al. 1987; Horiguchi et al. 1994); and (3) sterility, which as calculated as the ratio of total number of sterile females whose oviducts were blocked by vas deferens formation to total number of females (Horiguchi et al. 1994).

Table 7.1 Sampling sites of rock shell, *Thais clavigera*, collected from the Korean coast from 2005 to 2009

Eastern coast		Southern coast		Western coast	
E1	Sokcho, Gangwon-do	S1	Taejongdae, Busan metropolitan city	W1	Yeongjongdo, Incheon metropolitan city
E2	Jumunjin, Gangwon-do	S2	Gadeokdo, Busan metropolitan city	W2	Jamjindo, Incheon metropolitan city
E3	Samcheok, Gangwon-do	S3	Ungchon, Gyeongsangnam-do	W3	Jumundo, Incheon metropolitan city
E4	Jukbyeon, Gyeongsangbuk-do	S4	Deokdong, Gyeongsangnam-do	W4	Jebudo, Gyeonggi-do
E5	Hupo, Gyeongsangbuk-do	S5	Deokmyung, Gyeongsangnam-do	W5	Shinjindo, Chungcheongnam-do
E6	Yeongdeok, Gyeongsangbuk-do	S6	Guyeong, Gyeongsangnam-do	W6	Seosan, Chungcheongnam-do
E7	Guryongpo, Gyeongsangbuk-do	S7	Daegye, Gyeongsangnam-do	W7	Cheonbuk-myeon, Chungcheongnam-do
E8	Ulsan, Ulsan metropolitan city	S8	Haegeumgang, Gyeongsangnam-do	W8	Daecheon Harbor, Chungcheongnam-do
E9	Onsan, Ulsan metropolitan city	S9	Samchunpo, Gyeongsangnam-do	W9	Gunsan, Jeollabuk-do
E10	Ganjeolgot, Ulsan metropolitan city	S10	Seosang, Gyeongsangnam-do	W10	Gyeokpo, Jeollabuk-do
E11	Ilgwang, Busan metropolitan city	S11	Jicjang, Gyeongsangnam-do	W11	Gyema Harbor, Jeollanam-do
		S12	Industrial complex, Jeollanam-do	W12	Doripo, Jeollanam-do
		S13	Myodo, Jeollanam-do	W13	Heuksando, Jeollanam-do
		S14	Sindeok, Jeollanam-do		
		S15	Odongdo, Jeollanam-do		
		S16	Naro bridge, Jeollanam-do		
		S17	Dohwa, Jeollanam-do		
		S18	Poogyang, Jeollanam-do		
		S19	Usan, Jeollanam-do		
		S20	Maryang, Jeollanam-do		
		S21	Daegu, Jeollanam-do		

7.1.2.1 Determination of Organotin Compounds

After the imposex examination, tissue concentrations of six organotin compounds including monobutyltin (MBT), dibutyltin (DBT), tribultyltin (TBT), monophenyltin (MPhT), diphenyltin (DPhT), and triphenyltin (TPhT) were

analyzed. The extraction procedure is as described in Horiguchi et al. (1994) and Choi et al. (2013), with some slight modifications. Briefly, organotins (butyltins and phenyltins) in homogenized biological samples were extracted with 0.1 % toropolone/benzene and 0.1 N HBr/ethanol. Then, extracts were derivatized with propylmagnesium bromide and cleaned up by silica gel column chromatography. Organotin compounds in these samples were measured by gas chromatograph-flame photometric detector (GC-FPD) (HP 5890A, Shimadzu GC-17A) or GC-mass selective detector (GC-MSD) (Shimadzu QP5050A, QP2010), and expressed as chloride, based on the internal standard method. Pretreatment procedures of organotin compounds in seawater and sediment were basically the same as mentioned but slightly modified (Ministry of the Environment, Japan). QA/QC was performed, using the certified reference materials of NIES CRM No. 11 and No. 12 (National Institute for Environmental Studies, Japan) for chemical analysis of butyltins and phenyltins in biological and sediment samples.

7.2 Results and Discussion

7.2.1 Organotin Pollution and Imposex in the Rock Shell in 1995 to 2002

From 1995 to 2002, imposex symptoms were evaluated in rock shell specimens from all sampling locations, but organotin concentrations in their soft tissues were determined only for some selected locations. From 1995 to 1997, organotin concentration (ng Sn/g wet wt.) ranges in rock shell specimens from 17 selected sampling locations were ND–90.6 (mean, 34.6) for TBT, ND–202.8 (mean, 87.2) for butyltins (BTs), ND–1086.0 (mean, 125.8) for TPhT, and ND–1163.9 (mean, 140.5) for phenyltin (PhTs). In 2002, organotin concentrations (ng Sn/g wet wt.) in rock shell specimens from 21 selected sampling locations ranged from 0.9 to 991.0 (mean, 121.5) for TBT, 1.0–2810.5 (mean, 381.5) for butyltins (BTs), ND–170.5 (mean, 26.7) for TPhT, and ND–276.2 (mean, 58.8) for PhTs. The mean compositions (%) of TBT among BTs were 38.2 % and 31.9 % for the period of 1995 to 1997 and 2002, respectively. The mean compositions (%) of TPhT among PhTs were 87.5 % and 51.3 % for the period of 1995–1997 and 2002, respectively.

In the first imposex survey conducted in Korea from 1995 to 1997, the frequency of imposex in rock shell populations was 100 % in almost all sites surveyed along the Korean coast. No imposex populations, that is, all females being normal in each population, were only observed in the population from Deukryang Bay located in the southwestern part of the South Sea, whereas the frequency of imposex was in the range 67–88 % close to the western part of the bay. The frequency of sterile individuals (with vaginal openings blocked by vas deferens formation) was higher (60 % or more) in the eastern part than in the western part of the South Sea. No sterile females were observed on the open-sea side and some other areas.

In 2002, the occurrence frequency of imposex in rock shell populations was 100 % in most of the sites surveyed along the South Sea coast. Although the frequency of occurrence of imposex in the rock shell populations from the Jeju coast was 0–100 %, no imposex populations were observed at two of eight sites surveyed in Jeju. Geographic distribution of the frequency of sterile individuals was similar to that of the first survey, and a higher frequency (60 % or more) of sterile individuals was found in the eastern part than the western part of the South Sea. No sterile females were found at 6 of 19 sites along the South Sea coast and at 5 of 8 sites surveyed in Jeju Island.

7.2.2 Concentrations of Organotins in Seawater, Sediment, and Other Shellfishes Collected in 2000 and 2002

In 2000, six organotin concentrations in shellfishes as well as seawater and sediment samples from the south and east coasts were relatively higher than those on the west coast. Average TBT concentrations in seawater and sediment samples were 8.1 ng Sn/l and 10.6 ng Sn/g wet wt., respectively. Average TBT concentrations in tissues of the shellfishes collected at the south and east coasts were 23.9 ng Sn/g wet wt. and 32.0 ng Sn/g wet wt., respectively. Species-specific accumulations of organotins in tissue were observed. The highest BTs (158.2 ng Sn/g wet wt.) and PhTs (20.1 ng Sn/g wet wt.) were found in the Pacific oyster. In addition, organotin concentrations in the Pacific oyster were generally higher than those in other shellfishes. MBT (mean, 2.8 ng Sn/g wet wt.), DBT (mean, 5.5 ng Sn/g wet wt.), TBT (mean, 50.5 ng Sn/g wet wt.), and TPhT (mean, 3.0 ng Sn/g wet wt.) were detected in all Pacific oyster specimens. The ranges of organotin concentrations (ng Sn/g wet wt.) detected in other shellfishes were as follows: ND–18.5 (mean, 2.7) for MBT, ND–21.4 (mean, 3.0) for DBT, ND–93.2 (mean, 17.3) for TBT, ND–0.6 (mean, 0.02) for MPhT, ND–0.2 (mean, 0.02) for DPhT, and ND–6.7 (mean, 1.1) for TPhT. Tributyltin (TBT) was a predominant butyltin species and accounted, on average, for 74.6 % of BTs in all analyzed shellfish specimens. Except for a comb pen shell and a trough clam sample, TPhT was predominant among PhTs and accounted, on average, for 89.4 % of PhTs in all analyzed shellfish specimens.

In the detailed survey for hotspot harbors conducted in 2002, TBT concentrations in the seawater of Busan and Ulsan harbors were in the range 1.3–12.7 (mean, 4.7) ng Sn/l and 4.8–46.6 (mean, 13.2) ng Sn/l, respectively. TBT concentrations in Nakdong River estuary were ND–1.6 (mean, 0.3) ng Sn/l. TPhT was also detected in seawater at several locations. The concentrations of TBT and TPhT in the sediment of Busan Harbor were 4.8–745.6 (mean, 187.2) ng Sn/g dry wt. and 0.4–10.7 (mean, 2.2) ng Sn/g dry wt., respectively. Much higher concentrations, of 203.1–556.7 μg Sn/g dry wt. of TBT and 2.2–3.9 μg Sn/g dry wt. of TPhT, were observed in sediment near a dockyard in Busan. The concentrations of TBT and

TPhT in sediment of Ulsan Harbor were 22.3–1042.9 (mean, 259.2) ng Sn/g dry wt. and 0.2–36.2 (mean = 5.6) ng Sn/g dry wt., respectively. TBT concentrations in sediment from the Nakdong River estuary were in the range 0.2–8.7 (mean, 1.6) ng Sn/g-dry wt. TBT concentrations in seawater and sediment samples varied regionally and also differed from those in shellfishes. High shipping activity, especially in the coastal/inshore areas, has brought about extensive contamination by organotin compounds, such as TBT and TPhT, suggesting the continuous contamination of organotin in the coastal waters of Korea in the sampling period.

7.2.3 Organotin Pollution and Imposex in the Rock Shell in 2005–2009

From 2005 to 2009, imposex symptoms and organotin concentrations in rock shell populations were analyzed using specimens sampled from all locations. In detail, the locations for sampling rock shell specimens were 39 in 2005, 37 in 2006, 2007, and 2009, and 36 in 2008. The range and mean concentrations of all six organotin compounds (ng Sn/g wet wt.) in rock shells collected from the West Coast were 6.6–36.1 (mean, 18.4) for 2005, 9.0–46.1 (mean, 24.1) for 2006, 2.3–57.4 (mean, 10.9) for 2007, 2.5–131.0 (mean, 24.8) for 2008, and 2.4–24.5 (mean, 13.6) for 2009. The range and mean concentrations of all six organotin compounds (ng Sn/g wet wt.) in rock shells collected from the South Coast were 12.8–138.0 (mean, 48.4) for 2005, 8.2–202.0 (mean, 49.7) for 2006, 4.8–45.7 (mean, 22.7) for 2007, 3.0–38.1 (mean, 21.7) for 2008, and 1.8–26.1 (mean, 6.4) for 2009. In the West Coast, the range and mean concentrations of all six organotin compounds (ng Sn/g wet wt.) in rock shells were 2.8–34.8 (mean, 13.9) for 2005, were 5.1–78.7 (mean, 20.0) for 2006, 4.8–35.8 (mean, 11.3) for 2007, 1.1–18.4 (mean, 6.0) for 2008, and 2.3–13.6 (mean, 6.5) for 2009.

The total of all six organotin concentrations in each sampling geographic area from 2005 to 2009 are shown in Fig. 7.3. On the east coast, the highest concentrations of organotins in rock shell specimens were found in Ulsan and Onsan. The concentrations of organotins in rock shell specimens collected from the south coast were found as two to three times higher than those in the west and east coast. On the south coast, the highest concentrations of organotins in rock shells were found in Busan and Yeosu. On the west coast, the highest concentrations of organotins in rock shells were found in Seosan, Daecheon Harbor, and Gyema Harbor, and relatively consistent organotin concentrations were found in other sampling locations.

In areas adjacent to a harbor or shipyard, concentrations of BTs consisted of ND–27.3 ng Sn/g wet wt. for MBT, ND–51.6 ng Sn/g wet wt. for DBT, and ND–34.2 ng Sn/g wet wt. for TBT. Concentrations of PhTs consisted of ND–4.4 ng Sn/g wet wt. for MPhT, ND–34.2 ng Sn/g wet wt. for DPhT, and ND–4.7 ng Sn/g wet wt. for TPhT. In addition, in other sampling areas, concentrations of BTs consisted

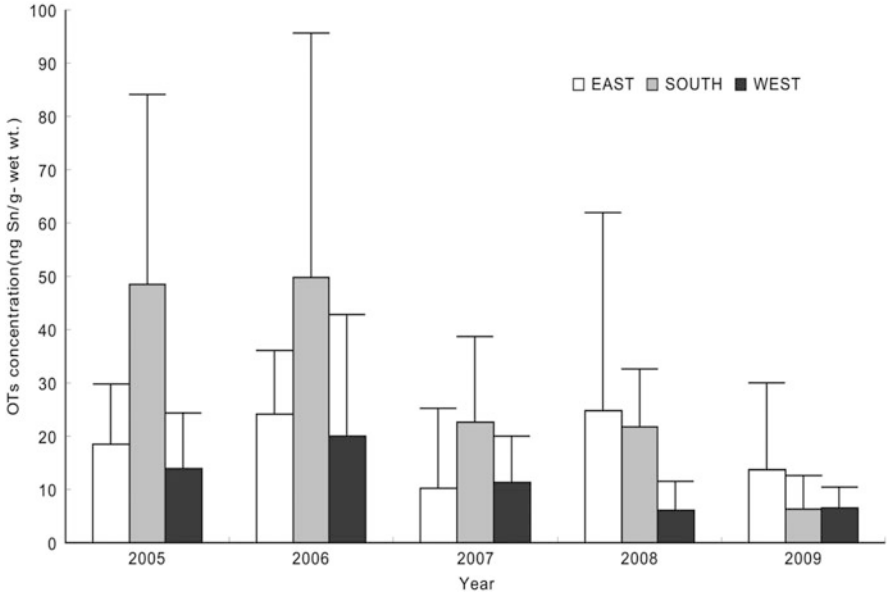

Fig. 7.3 Annual mean concentrations of all six organotin compounds in tissue of *Thais clavigera* from the Korean coast from 2005 to 2009

of ND–6.1 ng Sn/g wet wt. for MBT, ND–7.5 ng Sn/g wet wt. for DBT, and ND–8.0 ng Sn/g wet wt. for TBT, and concentrations of PhTs consisted of ND–1.4 ng ng Sn/g wet wt. for TPhT; MPhT and DPhT were not detected.

Higher concentrations of BTs than PhTs were found in both females and males of rock shell specimens. The total of all six organotin concentrations in females ranged from ND to 97.6 ng Sn/g wet wt. The concentrations of BTs in females showed the highest concentration in 2006 and a decreasing trend in 2007, 2008, and 2009. The average relative percent (%) composition in concentrations of MBT:DBT:TBT in total concentrations of BTs was 27.9:34.9:37.2, respectively. Of PhTs, only TPhT was detected in female individuals, and the concentration of TPhT increased from 2005 to 2007 and then decreased from 2007 to 2009.

Total of all six organotin concentrations in males ranged from ND to 105.0 ng Sn/g wet wt. BTs in males showed the highest concentration in 2006 and a gradually decreasing trend in 2007, 2008, and 2009. The average relative % composition in concentrations of MBT:DBT:TBT in total concentrations of BTs was 26.9:35.1:38.0, respectively. Regarding PhTs, the average relative % composition of MPhT:DPhT:TPhT was 12.7:0.1:87.2. In 2005, the highest composition ratio in total PhTs was found for MPhT, but was highest for TPhT in other sampling years.

7.3 Trends of Environmental Concentrations of Organotin Compounds and Imposex Phenomenon in Rock Shells Before and After the Korean Domestic and International Regulations for TBT- or TPhT-Based Antifouling Paints

The south coast is selected, because there are more data than from the east and west coasts, for comparing the concentrations of organotins before and after the Korean domestic and international regulations for TBT- and/or TPhT-based antifouling paints, based on local surveys from 1995 to 2009. It is worth noting that imposex symptoms were consistently observed in the rock shell populations collected at sampling locations along this south coast during the period (1995–2009), suggesting a sequence of organotin pollution related to vessel-related activities and its environmental impacts. Thus, the variation in environmental concentrations of organotins and imposex symptoms in the rock shell were investigated on the south coast on the basis of field surveys before (1995–1997 and 2002) and after (2005–2009) the Korean domestic and international regulations for TBT- or TPhT-based antifouling paints.

In the period 1995–2009, the range of six organotin concentrations detected in tissues of rock shell specimens collected from the south coast was 1.0–1651.0 ng Sn/g wet wt. The mean six organotins concentrations were found to be 193.0 ng Sn/g wet wt. for the periods of 1995–1996, 176.0 ng Sn/g wet wt. for 1997, 484.0 ng Sn/g wet wt. for 2002, 48.4 ng Sn/g wet wt. for 2005, 49.7 ng Sn/g wet wt. for 2006, 22.7 ng Sn/g wet wt. for 2007, 21.7 ng Sn/g wet wt. for 2008, and 6.4 ng Sn/g wet wt. for 2009. The highest organotin concentration was found for TPhT in 1995–1996 as 396.0 ng Sn/g wet wt. The concentrations of TPhT, however, rapidly decreased from 1995 to 2002 and from 2005 to 2009. On the other hand, the concentrations of TBT increased from 1995 to 2002 and then gradually decreased from 2005 to 2009.

All six organotin concentrations (ng Sn/g wet wt.) in tissues of each sex of rock shell specimens before the regulations were ND–873.0 (mean, 163.0) and 3.0–1791.0 (mean, 190.0) for females and males, respectively. Those concentrations after the regulations were 0.1–97.6 (mean, 15.5) and 0.1–105.0 (mean, 14.2) for females and males, respectively. Gender-specific difference in accumulation of organotins was unclear. Although there was a specific contamination by site, the mean organotin concentrations in tissues of female and male rock shell specimens from the south coast before the regulations were 11- and 13 fold higher than those after the regulations, respectively. The efficiency of the Korean domestic and international regulations for TBT- or TPhT-based antifouling paints seems clear. On the other hand, the proportions of detected concentrations of MBT, DBT, and TBT among BTs appeared to be consistent in both males and females before and after the regulations, and TBT accounted for a relatively higher proportion than other BTs. Additionally, TPhT was found to account for high proportions in total

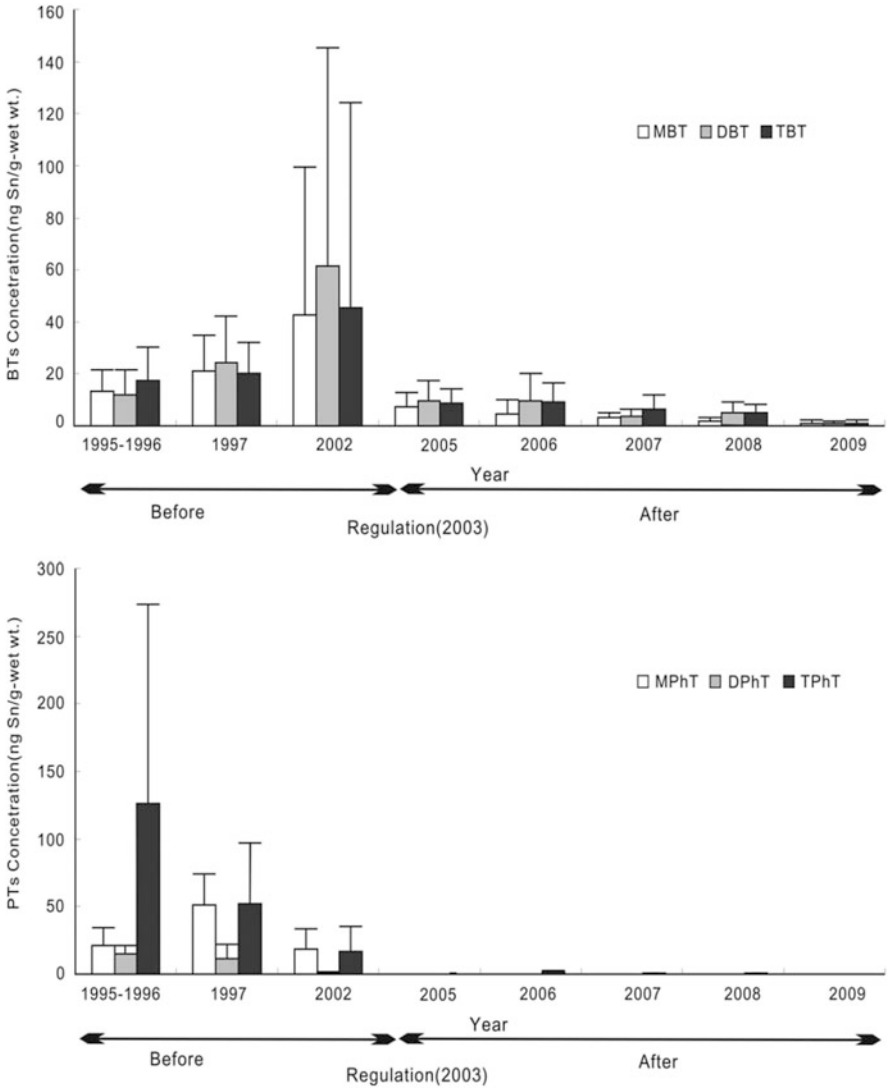

Fig. 7.4 Interannual variation of mean concentrations of butyltins (*upper*) and phenyltins (*lower*) in female *Thais clavigera* tissues from the southern coast, Korea

PhTs detected in both male and female rock shells, except for males in 2002 (Figs. 7.4 and 7.5).

Concentrations of six organotins in rock shell tissues collected from the south coast showed a gradually decreasing trend after the regulations and a relatively low residual concentration after 2007. However, the areas of Yeosu, Jinhae, and Busan had higher organotin concentrations than other areas, which may be attributed to the existence of local pollution sources. The interannual variation of imposex

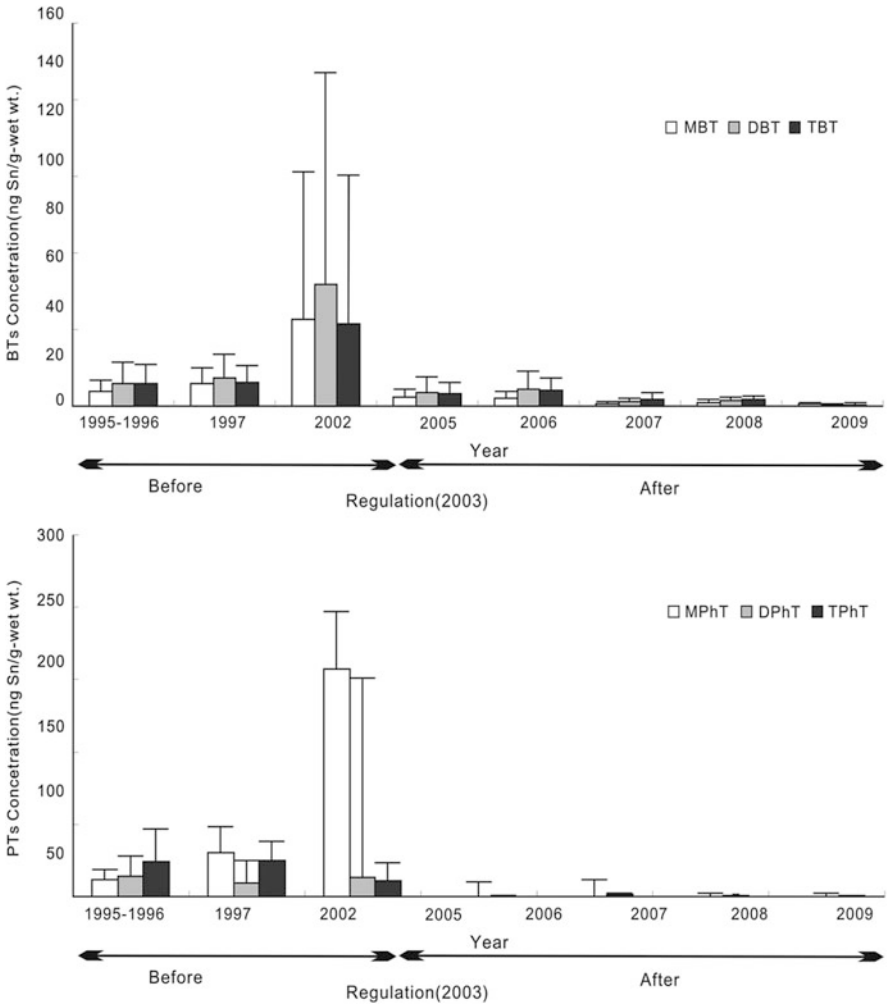

Fig. 7.5 Interannual variation of mean concentrations of butyltins (*upper*) and phenyltins (*lower*) in male *Thais clavigera* tissues from the southern coast, Korea

frequency, RPL index (RPLI), and sterility in the rock shells from the south coast is shown in Fig. 7.6. The imposex frequency in 1995– 2009 ranged from 0 to 100 %. Before the regulations, except for some selected control areas in 1995–1996, the imposex frequency was 100 % until 2005 and gradually decreased from 2005 to 2009. This high imposex frequency in the rock shell from the south coast of Korea suggests it may be the result of the high sensitivity of the rock shell to TBT, whose imposex is induced by very low concentration of TBT (approximately 1 ng/l) in seawater (Horiguchi et al. 1995). The low or slow recovery rate from imposex in the rock shell from the south coast may imply a point source of contamination by

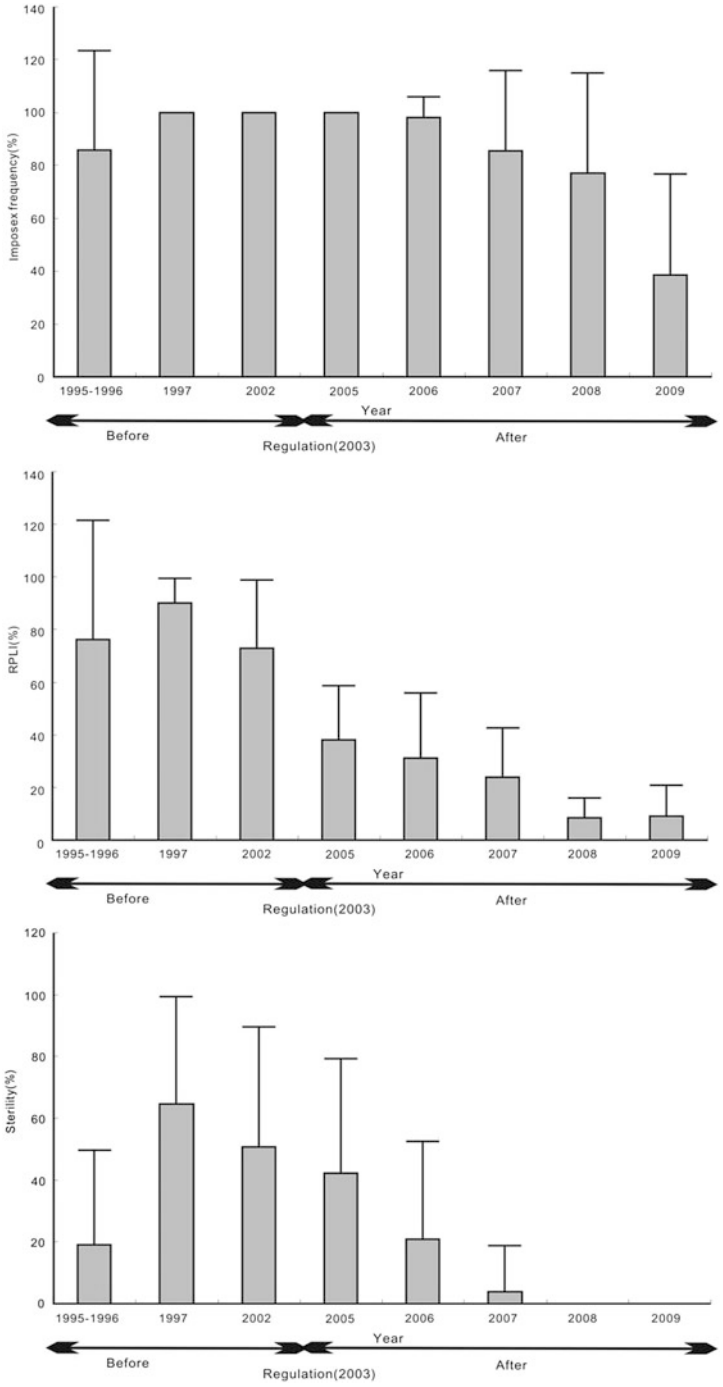

Fig. 7.6 Interannual variation of imposex frequency, RPL index (RPLI) and sterility in *Thais clavigera* from the southern coast, Korea

organotins resulting from the existence of many anchoring areas and shipping activities in this area.

The range of RPLI was 0–100 % in 1995 and 2009, and the mean of RPLI was 76.3 %, 93.0 %, 29.3 %, 38.1 %, 32.5 %, 23.6 %, 8.9 %, and 9.2 % in the period of 1995–1996, 1997, 2002, 2005, 2006, 2007, 2008, and 2009, respectively. RPLI before the regulations ranged from 0 to 149.0 % (mean, 62.4 %) and from 0 to 84.6 % (mean, 23.1 %) after the regulations. The mean RPLI after the regulations was found to be one third of that before the regulations.

The percentage occurrence of sterile females ranged from 0 to 100 % in the period from 1995 to 2009, and its mean value varied as 19.1 %, 64.7 %, 50.6 %, 42.4 %, 20.9 %, 3.8 %, 0 %, and 0 % in the period of 1995–1996, 1997, 2002, 2005, 2006, 2007, 2008, and 2009, respectively. The sterility ratio showed an increasing trend from 1995 to 2002, except for selected control sites in 1995, and showed a continuous decreasing trend after the regulations from 2005 to 2009.

7.4 Conclusions

TBT and TPhT were found to account for a high composition ratio in total butyltin and phenyltin concentrations, respectively. The higher concentrations of all six organotin concentrations were observed in areas with frequent shipping activities, including regions adjacent to harbors and shipyards than those in areas away from shipping activities.

In rock shell populations, the range of imposex frequency, RPLI, and sterility was 0–100 %, 0–84.6 %, and 0–100 %, respectively. Similar to the geographic distribution of all six organotin concentrations in seawater, the occurrence and degree of imposex were observed in rock shell specimens collected along the Korean coast (i.e., areas with frequent shipping activities such as harbors and shipyards, areas away from harbors, and ship-anchoring regions).

In summary, concentrations of TBT, TPhT and their metabolites in tissue, imposex frequency, and RPLI and sterility ratio of rock shell specimens collected from the Korean southern coast after imposition of the Korean domestic and international regulations for TBT- or TPhT-based antifouling paints showed lower values than those before the regulations. Thus, in general, efficiency of the Korean domestic and international regulations for TBT- and TPhT-based antifouling paints seems clear. However, it was also observed that these values were still high in areas adjacent to harbors or shipyards. Meanwhile, although concentrations of TBT and TPhT, which cause imposex in gastropods, continuously decreased after the regulations, increasing trends of concentrations of metabolites of TBT and TPhT (namely, MBT and DBT for TBT and MPhT and DPhT for TPhT) were found.

Because of the continued observation of the occurrence of imposex in rock shell populations, even after the regulations were established, it is necessary to carry out further field studies to monitor concentrations of organotins in tissues and imposex

frequency in rock shell specimens in parallel with evaluations of the organotin residues in seawater and sediment in the coastal waters of Korea. It is also necessary to put more efforts into understanding the possible ecological impacts by other antifouling substances (i.e., alternatives of TBT- and TPhT-based antifoulants) after the Korean domestic and international regulations for TBT- or TPhT-based antifouling paints were established.

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Part III
Fundamental Knowledge of Physiology and Mode of Action of Organotins to Induce the Development of Imposex in Gastropod Mollusks

Chapter 8
Neuropeptides and Their Physiological Functions in Mollusks

Fumihiro Morishita

Abstract Neuropeptides have essential functions in the neural regulation of physiological functions of various tissues and organs, as well as of animal behaviors. Many neuropeptides have been identified in mollusks, and investigation of their functions is currently proceeding. In this review, I attempt to give an overview of the neuropeptides in mollusks. Then, regulatory actions of neuropeptides are described with a special reference to reproduction. I chose three neuropeptides: egg-laying hormone (ELH) and caudodorsal cell hormone (CDCH), gonadotropin-releasing hormone (GnRH), and APGWamide. ELH and CDCH are well-investigated peptide hormones that trigger complex egg-laying behaviors in *Aplysia* and *Lymnaea*. GnRH, which is a key peptide that induces gonadal maturation and ovulation in mammals, also regulates gonadal maturation in bivalves and cephalopods. However, evidence suggests that GnRH also mediates other activities such as feeding and locomotion in mollusks. APGWamide, which regulates the male copulatory activity in freshwater snails, seems to have pheromonal actions in bivalves and cephalopods. These facts collectively emphasize the diverse actions of neuropeptides and peptide hormones on the regulation of reproduction in mollusks.

Keywords Neuropeptide • Peptide hormone • Nervous system • Endocrine system • Reproduction

Abbreviations

ACEP	*Achatina* cardioexcitatory peptide
AGP	atrial gland peptide
AKH	adipokinetic hormone
BCP	bag cell peptide
CDC	caudodorsal cell
CDCH	caudodorsal cell hormone

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CDCP	caudodorsal cell peptide
EC_{50}	effective concentration that induce 50 % response
EDC	endocrine-disrupting chemical
ELH	egg-laying hormone
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HPLC	high-performance liquid chromatography
LC-ESI-MS/MS	liquid chromatography–electrospray ionization tandem mass spectrometry
LUQ	left upper quadrant
NMDA	*N*-methyl-*D*-aspartate
PC2	prohormone convertase 2
PCR	polymerase chain reaction
Q-PCR	quantitative PCR
PTM	post-translational modification
RT-PCR	reverse transcription PCR
TEP	*Thais* excitatory peptide

8.1 Introduction

When the internal environments of animals are challenged, stabilization of homeostasis is essential for their survival. When the animal is fully grown-up, reproduction must be started at the proper timing. In various aspects of animal life, the nervous system and endocrine system have central roles in mounting the proper reactions to environmental stimuli, which lead to the survival and breeding of species. Since various chemicals including acetylcholine, biogenic amines, amino acids, nucleotides, steroids, and peptides mediate the regulatory actions of the nervous and endocrine systems, elucidation of the function of the signal molecules is necessary to understand the cellular and molecular mechanisms of neural and hormonal control of the physiological activities and behaviors in animals.

In structure and function, the diversity of neuropeptides and peptide hormones overwhelms that of other signal molecules. In fact, many peptides were identified in mollusks in the past four decades through the combination of fractionation of peptidic extracts by high-performance liquid chromatography (HPLC) and immunological or biological screening of the obtained fractions. Identification of novel peptides is accelerated nowadays by analysis using mass spectrometry supported by genetic information such as the genome sequence and expressed sequence tag (EST) data. Through the intensive investigations on the physiological functions of the identified peptides, it is well accepted that neuropeptides and peptide hormones have central roles in regulation of homeostasis and animal behaviors, including reproduction and feeding in mollusks.

Mollusks, as the consequence of evolution, are adapted to diverse environments such as land, freshwater, and seawater. They have evolved various strategies for

reproduction including gonochorism, hermaphroditism, and sex reversal. Accordingly, the cellular and molecular mechanisms that regulate reproduction are also diverse. In bivalves and primitive gastropods, eggs and sperm are released into the seawater and fertilization occurs externally (Fretter 1984; Mackie 1984). In those animals, a mechanism that triggers the synchronized gamete release is essential. In many gonochorismic (Fretter 1984) and hermaphroditic (Geraerts and Joosse 1984; Hadfield and Switzer-Dunlap 1984; Tompa 1984) gastropods, fertilization occurs internally, after copulation with the conspecific. In those animals, neural and hormonal systems that motivate courtship behaviors concomitant with sexual maturation are important for mating. Some mollusks exhibit protandrous sex reversal once in their lifetime, and others exhibit repetitive sex reversal every mating season (Fretter 1984; Mackie 1984). In those animals, changes in a particular hormone level may induce the sex reversal.

In the 1980s, it was recognized that organotin compounds such as triphenyl tin and tributyl tin in seawater induce the secondary formation of male reproductive organs or sperm formation (imposex) in female gastropod snails (Smith 1981), which disturbs reproduction of the afflicted snails (Birchenough et al. 2002; Horiguchi 2006; Titley-O'Neal et al. 2011). It was once proposed that malfunctioning of the neuropeptide system, including the penis-morphogenic factor (Geraerts et al. 1988) and APGWamide (Oberdorster and McClellan-Green 2002), is a key step for the induction of imposex by environmental organotin. Now, accumulating evidence suggests that the primary target molecule for organotin is a nuclear receptor, retinoid X receptor (RXR) (Nishikawa et al. 2004; Castro et al. 2007). Because RXR regulates gene expression, it is possible to assume that the endocrine-disrupting chemical (EDC) induces malfunctioning of the regulatory neuropeptide system by modifying the expression of neuropeptide precursor and receptor genes.

Unfortunately, we do not have enough evidence to explain all the neural and hormonal regulation of reproduction in mollusks. Nevertheless, accumulated data have demonstrated that neuropeptides and peptide hormones, such as egg-laying hormone (ELH) and caudodorsal cell hormone (CDCH), trigger egg-laying behaviors in the gastropods *Aplysia* and *Lymnaea*. Gonadotropin-releasing hormone (GnRH)-related peptides are also found in mollusks, and their functions are currently being investigated. Therefore, I believe that it is informative for all readers who are interested in imposex of mollusks to briefly summarize how neuropeptides and peptide hormones regulate mollusk reproduction.

In this review, I provide some basic information on the molluscan neuropeptides. Then, I refer to the structure and function of neuropeptides of prosobranch gastropods so far identified. Finally, I characterize three neuropeptides, namely, APGWamide, ELH and CDCH, and GnRH, to overview the regulatory system of the reproduction in mollusks, including bivalves and cephalopods. Apparently, it is beyond my capacity to cite all the papers on reproduction in mollusks. Readers can refer to other books and review articles on the peptide biology of mollusks (Chase 2002; Morishita and Furukawa 2006; Ketata et al. 2008; Koene 2010; Morishita et al. 2010).

8.2 Basic Information on Molluscan Neuropeptides

Most of the neuropeptides found in mollusks are oligopeptides, consisting of fewer than 20 amino acids (Muneoka et al. 2000; Morishita and Furukawa 2006). These peptides are synthesized in neurons as a part of the precursor protein. On the precursors of short peptides such as APGWamide, FMRFamide, and PRQFVamide, the same peptide sequences are aligned in tandem on the respective precursor. For example, the *Lymnaea* APGWamide precursor contains 10 copies of APGWamide (Smit et al. 1992), the *Aplysia* FMRFamide precursor contains 28 copies of FMRFamide (Taussig and Scheller 1986), and the *Aplysia* PRQFVamide precursor contains as many as 34 copies of PRQFVamide (Furukawa et al. 2003). This multiplicity of peptides on a single precursor is beneficial for effective production of signal molecules from one precursor protein, as well as a backup for replacement of the amino acid by genetic mutation.

On the precursors for longer neuropeptides such as myomodulin and enterin, several copies of similar but different peptides are found. For example, the *Lymnaea* myomodulin precursor contains 5 kinds of heptapeptides sharing the C-terminal Met-Leu-Arg-Leu-NH_2 structure (Kellett et al. 1996), whereas the *Aplysia* enterin precursor includes 21 kinds of deca- and undecapeptides, most of which share the C-terminal His-Xaa-Phe-Val-NH_2 structure (Furukawa et al. 2001). These peptides are collectively referred as the family peptide, such as the enterin family peptide.

One important point is that a minor difference in amino acids greatly modifies the bioactivity of the peptides. For example, ENe (ADLGFTHSFV-NH_2) and ENh (VPGYSHSFV-NH_2) are *Aplysia* enterin family peptides found in the same precursor. However, EC_{50} of the inhibitory action of ENh on the triturating stomach is around 10^{-10} M, whereas that of ENe is a little more than 10^{-7} M. Thus, minor differences in the peptide structure affect affinity to the receptor and tolerance to degradation by the peptidase of the peptide. Thus, neurons expressing the family peptide precursor release a cocktail of the peptides with different potencies.

Recently, it was found that G protein-coupled receptors (GPCR), including neuropeptide receptors, works in forms of monomer, homodimer, or heterodimer (Milligan 2009). Satake et al. (2013) proposed a hypothesis that neuropeptide receptors modify selectivity to the ligand by changing the partner for the dimerization. If a similar receptor system is applicable to mollusks, the aforementioned ENe peptide that is less effective on the triturating stomach than ENh, for instance, may have potent action on other tissues.

Newly synthesized precursor proteins in the cell body are subjected to the post-translational modification (PTM), during it passes through the trans-Golgi network. Matured peptide is ultimately packaged in the synaptic vesicles and transported to the nerve endings (Elekes and Rozsa 1984; Reed et al. 1988). PTM is a rather complicated enzymatic and nonenzymatic reaction that includes processing, disulfide-bond formation, and C-terminal amidation. For example, the processing enzymes prohormone convertase 2 (PC2) and furin were identified in *Aplysia* (Nagle et al. 1993, 1995) and *Lymnaea* (Smit et al. 1994; Spijker et al. 1999).

PC2 cleaves the precursor at the C-terminal side of the mono- or di-basic amino acids such as Arg and Lys, which liberate neuropeptides from the precursor. Furin cleaves precursor at the tetra-basic site on the precursor. It was demonstrated that the ELH precursor in *Aplysia* is cleaved by furin, and the N-terminal and C-terminal fragments thus generated are packaged into the different synaptic vesicles. Because those vesicles are transported to the different nerve endings (Fisher et al. 1988; Sossin et al. 1990; Li et al. 1994), a single neuron can release different peptides from different nerve endings.

A unique PTM in molluscan neuropeptides is D-/L-conversion of N-terminal penultimate amino acid (Kamatani et al. 1991; Kreil 1994a, b; Morishita et al. 1997), catalyzed by the peptidyl D-/L-isomerase (Kreil 1994a, b). This modification greatly affects both the bioactivity and half-life of the peptide. However, differing from processing or amidation, neither the amino acid sequence of the precursor nor structural analysis of peptides by standard mass spectrometry shows us which peptide contains D-amino acid. One practical approach to find a D-amino acid-containing peptide is comparison of elution time between the native peptides and synthetic peptides with or without D-amino acids, on reversed-phase or ion-exchange column HPLC (Fujimoto et al. 1991; Morishita et al. 1997). Although the D-/L-conversion of neuropeptide is a rare phenomenon, it is an important issue when the bioactivities of newly identified peptides are examined.

The synthesis of a precursor normally occurs in the cell body. However, local synthesis of peptide hormone is also known in *Aplysia* (Lee and Wayne 2004) and *Lymnaea* (Van Minnen et al. 1997). In these gastropods, translation machinery such as mRNA and ribosomes is transported to the nerve endings. Local synthesis of peptide is promoted by the depolarization of the nerve endings (van Minnen and Bergman 2003).

Neuropeptides packaged in the synaptic vesicles in the nerve endings are released by exocytosis, when the synapse membrane is depolarized (Sudhof 2012). The elevation of Ca^{2+} in the presynaptic element by Ca^{2+} influx through the voltage-dependent Ca^{2+} channel triggers the exocytosis (Geiger et al. 2009). In vertebrate neurons, the fusion of the neuropeptide-containing synaptic vesicle to the presynaptic membrane requires a high concentration of Ca^{2+}. Accordingly, neuropeptide release occurs when the neuron is discharged at high frequency (Leng and Ludwig 2008). However, Vilim et al. (Vilim et al. 2000) demonstrated that, at the neuromuscular junction between the buccal B16 neuron and anterior radula closer muscle of *Aplysia*, neuropeptide release occurs at a low-frequency discharge of the neuron. Thus, co-release of ACh and myomodulin is inducible at low-frequency neuronal discharge.

The mode of action of the released neuropeptides is variable in mollusks. The central actions of a neuropeptide include postsynaptic action, which modulates the excitability of neurons, and presynaptic action that modulates neurotransmitter release (Fossier et al. 1994). Most of the peripheral actions are the control of muscle contraction in the digestive (Sweedler et al. 2002; Furukawa et al. 2003) and cardiovascular (Buckett et al. 1990; Sasaki et al. 2004) systems and reproduction-associated ducts, as well as the hormonal actions (Newcomb and Scheller 1990;

Hermann et al. 1997). In one instance, a peptide in the pedal ganglion regulates locomotion of *Aplysia* by regulating ciliary movements on the surface of the foot (Hall and Lloyd 1990). In the female reproductive gland in land snails, neuropeptides promote ovulation by squeezing the acini around the oocyte (Chase et al. 2004).

Degradation of the released peptides is another important aspect in the effectiveness of neuropeptides. The neuropeptides released into the extracellular space or the hemolymph are normally digested by peptidases (Squire et al. 1991; Owens et al. 1992; Rothman et al. 1992). Apparently, localization and activity of the peptidase are important factors that determine the half-life of the peptides. Some peptides have protections against the peptidase by various modifications, such as C-terminal amidation (De Camargo et al. 1982) and D-amino acid (Morishita et al. 2003) or proline (Mentlein 1988) at the N-terminal penultimate residue. Accordingly, the half-life of neuropeptides with those modifications is longer than that of other peptides. On the other hand, deamidase, which inactivates D-amino acid-containing neuropeptide (Morishita et al. 2003), and dipeptidyl proline aminopeptidase, that degradates peptides with N-terminal Xaa-Pro structure, such as APGWamide, are reported (Henry and Zatylny 2002).

Despite the diversity of neuropeptides so far known, only a few neuropeptide receptors are currently identified. Neuropeptide receptors cloned in mollusks include receptors for conopressin (van Kesteren et al. 1995), LyCEP (Tensen et al. 1998), and leucokinin-like peptide (Cox et al. 1997) in *Lymnaea*, tachykinin (Kanda et al. 2007) and cephalotocin (Kanda et al. 2003) in *Octopus*, and FMRFamide in *Aplysia* (Lingueglia et al. 1995: Furukawa et al. 2006). The FMRFamide receptor is a ligand-gated Na^+ channel, whereas the others are GPCRs. Information on the spatial and temporal expression of GPCRs for neuropeptides on the target tissues, as well as that on the signal transduction cascade that is downstream to the GPCR, is indispensable to elucidate the functions of neuropeptides.

8.3 Peptides Identified in Prosobranch Gastropods

Early evidence for the existence of neuropeptides in prosobranchs came from immunological studies. For example, using antibodies to vertebrate neuropeptides, the presence of neuropeptide-Y and substance P was reported in a freshwater gastropod, *Viviparus ater,* and that of calcitonin gene-related peptide and small cardioactive peptide was reported in the green ormer, *Haliotis tuberculata* (Barlow and Truman 1992; Franchini et al. 1994; Duvail et al. 1997). However, the precise structures of the immunoreactive materials are not known so far.

In the 1990s, Muneoka and colleagues purified several bioactive peptides from a peptidic extract of the ganglia of the spindle snail, *Fusinus ferrugineus*, through fractionation by reversed-phase or cation-exchange HPLC, which was followed by bioassay with the radula retractor muscle of the animal. Identified peptides are

Table 8.1 Structures of neuropeptides isolated from prosobranch gastropods

Fusinus ferrugineus		*Thais clavigera*	
Name	Structure	Name	Structure
FRFamide	GSLFRFa	FRFamide	GSLFRFa
	SSLFRFa		SSLFRFa
FMRFamide	FMRFa	Tachykinin	FHPSAFFGSRa
FLRFamide	FLRFa	WWamide	WKSMSKVWa
	ALTNDHFLRFa	TEP-1	KCSGKWAIHACWGGNa
myomodulin	PMSMLRLa	TEP-2	KCYGKWAMHACWGGNa
	PMNMLRLa		
APGWamide	APGWa		
FEP	GFRMNSSNRVAHGFa		

Two cysteine residues in TEP-1 and TEP-2 are linked by intramolecular disulfide bond

a C-terminal amide

LSSFVRIamide, ALTNDHELRFamide, GSLFRFamide, SSLFRFamide, allatotropin-like tetradecapeptide (*Fusinus* excitatory peptide 4, FEP-4), myomodulin, and APGWamide (Kanda et al. 1990; Kuroki et al. 1990; Harada et al. 1993; Kuroki et al. 1993) (Table 8.1).

Bioactivities of those peptides are mainly examined on the isolated preparation of some muscular tissues of *Fusinus*. For example, FEP-4 and APGWamide potentiate electrically induced twitch contraction of the radula muscle of *F. ferrugineus* and the rapa whelk, *Rapana thomasiana* (Minakata et al. 1991; Harada et al. 1993), whereas FRFamide showed inhibitory action on the contraction of the radula retractor muscle (Kuroki et al. 1993). Detailed characterization of those *Fusinus* peptides, such as localization in the nervous tissues and molecular cloning of the precursor, has not been reported. Accordingly, the actions of those peptides on the gonad or reproduction-associated organs are largely unknown.

Another instance of the peptide identification in prosobranchs was our study on the rock shell, *Thais clavigera*. In this study, we identified tachykinin-related peptide, WWamide, FRFamide (Morishita et al. 2006a), and *Thais* excitatory peptide (TEP)-1 and -2 (Morishita et al. 2006b). Of those peptides, WWamide and FRFamide reduced the contractile activities of the esophagus and penial complex whereas others showed excitatory actions on those tissues. Both TEP-1 and TEP-2 induce contraction of the penial complex, as well as the esophagus, of *T. clavigera*. Molecular cloning of the TEP precursor revealed that TEP-1 and TEP-2 are encoded on a distinct precursor protein (Morishita et al. 2015), and the

two precursor genes are expressed in different subsets of neurons in the central nervous system. The biological significance of this distinct expression between TEP-1 and TEP-2 is currently unknown.

Recent progress in techniques for the analysis of nucleotide sequences enabled us to predict the amino acid sequences of neuropeptide precursors from the genome DNA. With this technique, Veenstra (2010) predicted the amino acid sequences of neuropeptide precursors in an owl limpet, *Lottia gigantea*. Predicted precursors included some molluscan neuropeptides such as ELH, myomodulin, APGWamide, and enterin, as well as some unique peptides such as bursicon that mediate cuticle tanning in insects (Luo et al. 2005). This approach greatly improved our knowledge on the structures of peptides in prosobranchs. However, their physiological functions in the limpet are largely unknown.

Recently, York et al. (2012) cloned several neuropeptide precursor cDNAs in the Indo-Pacific tropical abalone, *Haliotis asinina*. In this study, cDNA prepared from the cerebral and pleuropedal ganglia that regulate reproduction was subtracted by cDNA prepared from other ganglia. Thus, it is likely that the cloned precursors encode neuropeptides that mediate reproduction. The cloned precursors included APGWamide, myomodulin, and whitnin. The APGWamide precursor encoded 8 copies of authentic APGWamide, whereas the myomodulin precursor encoded 14 copies of distinct peptides sharing the C-terminal Leu-Arg-Leu-NH_2 structure. The whitnin-related precursors have been found in *Lottia* (Veenstra 2010), *Lymnaea* (Koert et al. 2001) and *Aplysia* (Moroz 2006), as well. On those precursors, the proctolin-related hexa-peptide (PKYMDT) is conserved in the middle region, while a 22-mer peptide with one intramolecular disulfide bond and a C-terminal amide is conserved in the C-terminal region. In *Lymnaea*, a serotonergic cerebral giant cell in the cerebral ganglion contains the peptide (Koert et al. 2001). However, the physiological functions of those peptides are currently unknown.

Because the timing of the spawning of *Haliotis asinina* is well synchronized with the lunar and tidal cycle, the authors determined the changes in the expression levels of the precursor mRNA by quantitative polymerase chain reaction (Q-PCR) at the different times of spawning (York et al. 2012). The results demonstrated that expression of neuropeptide precursors in the cerebral and pleuropedal ganglia was higher on the day of spawning than the days before or after spawning. This result suggests that external cues such as tidal cycle, and internal cues such as circadian rhythm, augmented the de novo biosynthesis of neuropeptides for spawning. The next question to be answered is how external or internal stimuli regulate the gene expression in *Haliotis*.

In the following section, I describe the regulation of reproductive activity by selected peptides in mollusks.

8.4 APGWamide

APGWamide was originally identified in a prosobranch, *Fusinus ferrugineus* (Kuroki et al. 1990). However, regulatory action of APGWamide, especially on male reproductive behavior, is well investigated in the freshwater snail *Lymnaea stagnalis*. In this snail, the penial complex is located just behind the right tentacle and is retracted into the body during the resting phase. Although *Lymnaea* is hermaphroditic, one snail behaves as a male that transfers sperm, and the other behaves as a female that receives sperm. When the male snail is motivated to mate, he climbs up on the shell of the partner female snail and approaches the female genital pore from behind (Van Duivenboden and Ter Maat 1988). Then, the preputium of the penial complex is everted and inserted into the female genital pore before intromission is completed.

APGWamide-containing nerve endings were found in the body wall around the male genital pore, as well as male sex-associated organs such as penial complex, vas deferens, and prostate gland (Croll and Van Minnen 1992; de Lange and van Minnen 1998). APGWamide inhibited the contraction of vas deferens that has been induced by a neuropeptide, conopressin, and an injection of APGWamide into the snail induced eversion of the preputium (De Boer et al. 1997). Moreover, the right hemisphere of the cerebral ganglion of *Lymnaea* contains a cluster of more than 100 neurons, which is immunoreactive to specific anti-APGWamide antibody (Croll and Van Minnen 1992). It was also suggested that axon terminals of these APGWamide-containing neurons are located in the aforementioned male reproductive organs. Moreover, the firing rate of the APGWamide-containing neurons in the right cerebral hemisphere is increased when the snail everts the preputium (De Boer et al. 1997). These results strongly suggested that APGWamide regulates male reproductive activity in *Lymnaea*. However, regulation of the reproductive activities of the snail may not be so simple, because nerve processes in the male reproductive organ contains various kinds of neuropeptides such as FMRFamide and myomodulin (De Lange et al. 1998).

As is in *Lymnaea*, localization of APGWamide-containing neurons in the right cerebral hemisphere was also found in *Aplysia californica*, and in the land snails *Helix pomatia* and *Achatina fulica* (Koene et al. 2000), but not in the common periwinkle *Littorina littorea* (Croll and Van Minnen 1992) or in the rock shell *Thais clavigera* (Fig. 8.1) (Morishita, unpublished data). Thus, it is likely that localization of the APGWamide neuron in the right cerebral hemisphere is characteristic of hermaphroditic gastropods.

Amino acid sequences of APGWamide precursor were available in the GenGank for a mussel, *Mytilus edulis* (Q25461), an oyster, *Crassostrea gigas* (EKC38991.1), an abalone, *Haliotis asinina* (AFN20271.1), a freshwater snail, *Lymnaea stagnalis* (1811269A), a sea hare, *Aplysia californica* (NP_001191561.1), and a sea slug, *Tritonia diomedea* (ABU82758.1). In the octopus *Octopus vulgaris*, cDNA encoding a part of the APGWamide precursor is available in the GenBank

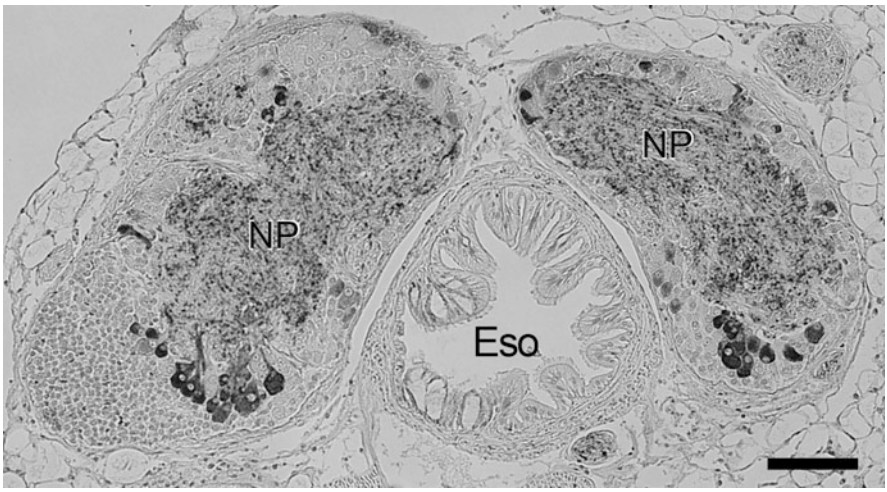

Fig. 8.1 APGWamide-containing neurons in the cerebral ganglion of *Thais clavigera*. A cross section of the cerebral ganglion was immunostained with anti-APGWamide antibody. Immunopositive neurons were found in the ventral side of the *right* and *left* hemispheres. Diffused positive signals were also found in the neuropile (*NP*). *Eso* esophagus. *Scale* 100 μm

(JR446524). The APGWamide precursor of *Lottia gigantea* was predicted by data mining on the genome sequences of this species (Veenstra 2010).

In the bivalves, APGWamide-containing neurons were demonstrated in the cerebral, pedal, and parietovisceral ganglia of a scallop, *Placopecten magellanicus*, an oyster, *Crassostrea virginica*, and *Mytilus edulis*, by immunohistochemistry with anti-APGWamide antibody. Immunopositive nerve processes were found in various tissues including the gonad of the scallop. However, the cloned precursor encoded RPGW-NH_2, TPGW-NH_2 and KPGW-NH_2, but not authentic APGWamide, in *M. edulis* (Favrel and Mathieu 1996). Presence of the three peptides in the *Mytilus* tissues was confirmed by precise mass spectrometry in the extract of the cerebral ganglion and pedal retractor muscle (Henry et al. 2000). With a similar technique, existence of APGWamide was also confirmed in the ganglionic extract of *Crassostrea gigas* (Bernay et al. 2006).

In *M. edulis*, synthetic peptides induced the contraction of pedal retractor muscle and anterior byssus retractor muscle (ABRM) (Henry et al. 2000). A simple interpretation of this result is that neurons containing RPGWamide, TPGWamide, and KPGWamide regulate the locomotion of the mussel. Functional relevance of APGWamide-related peptides to the regulation of reproduction is unclear in this animal. In *C. gigas*, APGWamide was also detected in the seminal fluid in the seminal duct, suggesting the pheromonal action of the peptide (Bernay et al. 2006). In this context, it is noteworthy that *C. gigas* initiates the open/closure response of the shell when the oyster is immersed in seawater containing APGWamide (Bernay et al. 2006), which is an important response for the effective release of gametes into the external seawater,

In cephalopods, APGWamide and TPGWamide were identified in the optic lobe and supra- and subesophageal masses of the brain and oviducal gland of the squid *Sepia officinalis* by liquid chromatography–electrospray ionization mass/mass spectrometry (LC-ESI-MS/MS) (Henry and Zatylny 2002). Because the two peptides inhibit the motility of the oviduct, involvement in female reproductive activity by modifying the transportation of oocytes through the oviduct is suggested. APGWamide was also found in seminal fluid obtained from the spermatophore, suggesting the exocrine or pheromonal actions of APGWamide in male *Sepia officinalis*. Involvement of APGWamide in the regulation of male reproductive activity is also suggested in a pygmy squid, *Idiosepius pygmaeus*. In this squid, APGWamide localizes in nerve processes in the male reproductive organs, as well as the brain regions such as the supraesophageal mass, palliovisceral lobe of the subesophageal mass, and olfactory lobe.

Henry et al. (1997) identified the dipeptide GW-NH_2 from the peptidic extract of the optic lobe of *Sepia officinalis* by the combination of HPLC fractionation and bioassay on the oviduct. The dipeptide showed potent inhibitory action on the oviduct. Minakata et al. (1991) examined the effects of APGWamide analogues on the ABRM of *M. edulis* and the radula retractor muscle of *Rapana thomasiana* and reported that the potency order was GWa> > APGWa > FAPGWa > PGWa. The peptides containing the N-terminal penultimate proline residue are digested by dipeptidyl proline aminopeptidase, which removes the N-terminal Xaa-Pro residue (Mentlein 1988). Thus, it is possible to assume that dipeptidyl proline aminopeptidase liberates GW-NH_2 from APGWamide. In fact, the optic gland of *S. officinalis* contains dipeptidyl proline aminopeptidase activity, because incubation of APGWamide with the extract of the gland generated GW-NH_2 (Henry et al. 1997). It is an interesting issue to be examined if generation of GW-NH_2 is occurring in the extracellular space or in the intracellular space as a step of post-translational modification.

In *Octopus vulgaris*, distribution of APGWamide was demonstrated by immunohistochemistry (Di Cristo et al. 2005). In this study, APGWamide-containing neurons were found in the inferior frontal lobe in the brain and posterior olfactory lobe on the optic tract, as well as the glandular cell of the oviducal gland. It is likely that the APGWamide neuron in the olfactory lobe regulates the activity of the optic gland, whereas APGWamide-containing cells in the oviducal gland secrete the peptide into the extracellular space to modify oviduct motility (Di Cristo and Di Cosmo 2007). Verification of this hypothesis is the next work to be conducted.

As already mentioned, the nucleotide sequence of cDNA encoding APGWamide precursor is found on the database among the transcriptome analysis data of *Octopus* (Zhang et al. 2012). The translated protein includes several copies of the APGW sequence flanked by the dibasic cleavage site and amidation signal in the C-terminal region of the polypeptide. However, this precursor seems to be a fragment, because it does not include the C-terminal signal peptide that is characteristic of the neuropeptide precursor. Therefore, more APGWamide, or a structurally related peptide such as TGPWamide as identified in *Sepia*, could be encoded in the missing N-terminal region.

8.5 Egg-Laying Hormone (ELH) and Caudodorsal Cell Hormone (CDCH)

ELH in *Aplysia californica*, and CDCH in *Lymnaea stagnalis*, are good examples that show us how peptide hormone regulates reproduction-associated behaviors. Egg laying in *Aplysia* is a series of behaviors including ovulation, packaging fertilized eggs into the egg string, and transportation of the string to the head through the genital duct and genital groove. Then, *Aplysia* attaches the egg string to a solid surface by waving the head to left and right. The bag cells, a cluster of neurosecretory cells in the abdominal ganglia, are crucial in initiation of this egg-laying behavior (Kupfermann and Kandel 1970). When an external or internal stimulus activates the bag cells, the cells initiate a series of synchronized discharges (Blankenship and Haskins 1979; Haskins and Blankenship 1979), which continues for some time even after the stimulation is terminated (afterdischarge) (Kupfermann and Kandel 1970).

Because the injection of the bag cell extract into matured *Aplysia* triggered egg laying (Kupfermann 1970), the bag cell contains signal molecules that trigger the egg laying. Those peptides include a 36-mer peptide, ELH (Chiu et al. 1979), and four short peptides, α-, β-, γ-, and δ-bag cell peptide (BCP) (Rothman et al. 1983a; Scheller et al. 1983; Nagle et al. 1990) (Figs. 8.2 and 8.3). ELH and BCPs are encoded on the same precursor (Fig. 8.2). The massive release of ELH and BCPs occurs from nerve endings of the bag cells during the afterdischarge of the cell (Nagle et al. 1988), which in turn induces egg-laying behaviors in *Aplysia* (Kupfermann and Kandel 1970; Pinsker and Dudek 1977; Dudek et al. 1979).

The central nervous system of *Aplysia* is covered by a ganglionic sheath made by the connective tissue. There is a space between the surface of the ganglion and the covering sheath, and the bag cells release ELH and BCPs into this space (Chiu and Strumwasser 1981). Because this space is connected to the circulatory system (Furgal and Brownell 1987), released peptides are delivered to the broad area of the central nervous system. For example, ELH induces ovulation in the ovotestis (Dudek and Tobe 1978; Dudek et al. 1980; Rothman et al. 1983b) and augments the neuronal activity of the R15 neuron in the abdominal ganglion (Mayeri et al. 1985; Levitan et al. 1987). The R15 neuron modulates the motility of the hermaphroditic duct that transports the egg codon to the genital pore (Alevizos et al. 1991). Recently, it was reported that ELH switches jaw movement in *Aplysia kurodai*, so that food ingestion is reduced (Narusuye et al. 2013). Feeding activity and reproductive activity are negatively correlated in mollusks, and this result suggests that ELH switches the two activities.

On the other hand, α- and β-BCPs reduce neural activity of the left upper quadrant (LUQ) neurons in the abdominal ganglion (Sigvardt et al. 1986) that regulate circulation and kidney function (Koester and Alevizos 1989). The peptides have an autocrine action that augments the excitability of the bag cell (Kauer et al. 1987; Brown and Mayeri 1989). This action of the BCPs is important for the prolonged discharge of the cells. During the afterdischarge, Ca^{2+} is mobilized

A) Alignment of ELH and related peptides in mollusks.

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Aplysia       ELH       ---ISINQDLKAITDMLLTEQIRERQ----RYLADLRQRLLEK---------
              Califin   ---ISINQDLKAITDMLLTEQIQARR----RCLDALRQRLLDL---------
Lymnaea       CDCH-I    ---LSITNDLRAIADSYLYDQNKLRE----RQEENLRRRFLEL---------
              CDCH-II   ----SITNDLRAIADSYLYDQHKLRE----QQEENLRRRFYELSLRPYPDNL
Lottia        ELH-1     AGRLSINGALSSLADLLVSENQRRDR----LESMELRQRLQYL---------
              ELH-2     -SRLSINQELKSLANLLVLRENKRRE----AQKTKLRSKLLSI---------
Haliotis      ELH       ---LSITNDLRAIADSYLYDQNMLRE----RQEENLRRRFLRL---------
Pinctata      ELH-1     -TYISLNGDMRSLAKMLMRHYGNRSVKRPVENYTSLRKKLYAL---------
              ELH-2     -QRLSVNSALASLADMVSADGHRRMK----EEMSSNHQRLLGL---------
Crassostrea   ELH-1     -GRLSLTADLRSLARMLEAHR-KRFIASRFP-YDSIRKKLFRY---------
              ELH-2     -QRLSVNGALSSLADMLAANGRQRMM----SEMAMNRQRLFGL---------
                           *:.  : :::                      : ::
```

B) Alignment of the bag cell peptides and caudo-dorsal cell peptides.

```
Aplysia       Peptide A IFVPNRAVKLSSDGNYPFDLSKEDGAQPYFMTPRLRFYPI
              α-BCP     ------------------------------APRLRFYSL
              β-BCP     --------------------------------RLRFH--
              γ-BDP     --------------------------------RLRFSD-
Lymnaea       α-CDCP    -------------------------------EPRLRFHDV
              β1-CDCP   --------------------------------RLRFH--
              β1-CDCP   --------------------------------RLRAS--
              β1-CDCP   --------------------------------RLRFN--
                                                        ***
```

Fig. 8.2 Alignments of egg-laying hormone (ELH) precursor-associated peptides. (**A**) Alignment of ELH and ELH-related peptides in mollusks. (**B**) Alignment of the bag cell peptides and caudodorsal cell peptides. Alignments were calculated by the ClustalW-multialign (Mobyle portal). *Asterisks*, *colons*, and *periods* beneath the sequences represent amino acid residues conserved among all data, those with high similarity, and those with moderate similarity, respectively

from the extracellular space through a Ca^{2+} channel, or from the intracellular Ca^{2+} store (Wayne and Frumovitz 1995; Magoski 2004). Increase in the Ca^{2+} level in the bag cells promotes the de novo synthesis of ELH (Wayne et al. 2004). Thus, coordination of ELH and BCPs is the key for egg-laying behaviors in *Aplysia*.

The atrial gland, an exocrine organ located on the large hermaphroditic tract of *Aplysia* (Painter et al. 1985), contains several egg laying-associated peptides, such as califin and atrial gland peptide (AGP)-A and -B (Table 8.2). AGP-A and AGP-B trigger the afterdischarge of the bag cell through the mediation of certain neurons in the pleural or cerebral ganglia (Heller et al. 1980; Painter et al. 1988), whereas califin regulates the excitability of neurons located in the left lower quadrant of the abdominal ganglion (Rothman et al. 1986). AGP-A and califin are encoded on the same precursor. The APG-B precursor is a C-terminal-truncated version of AGP-A precursor. Accordingly, the AGP-B precursor does not include the califin (Scheller et al. 1983; Nagle et al. 1986; Kurosky et al. 1997).

The overall structures of the AGP-A and AGP-B precursors are quite similar to that of the ELH precursor. However, 81 amino acids of the ELH precursor, which include β-, γ-, and δ-BCPs, are missing in the AGP-A and AGP-B precursors. The C-terminal regions of AGP-A and AGP-B are quite similar to that of α-BCP, and

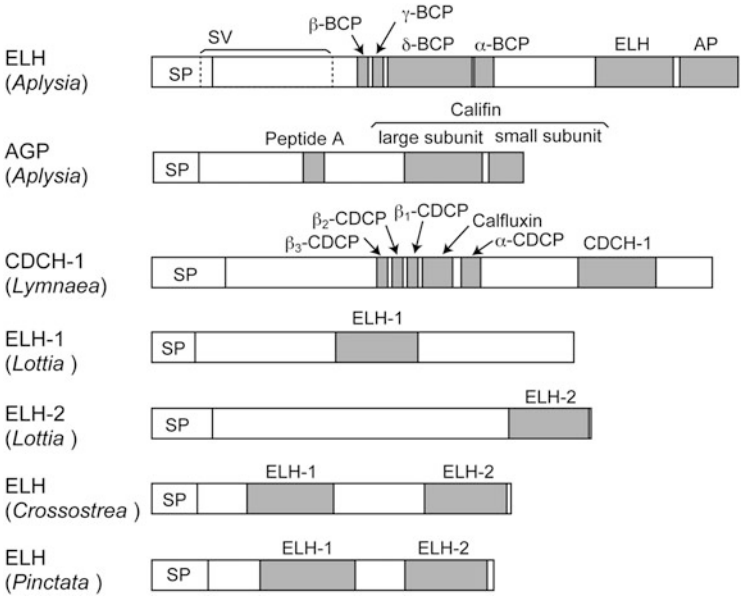

Fig. 8.3 Scale drawings represent localizations of egg-laying hormones and other associated peptides on the respective precursors. Drawings are based on the amino acid sequences of precursors for ELH in *Aplysia californica* (accession: P01362.2), *CDCH* in *Lymnaea stagnalis* (accession: P06308), *AGP* in *A. californica* (accession: P01360.2), ELH in *Lottia gigantea* (accession: XP_009066138.1), and ELH in *Crassostrea gigas* (Veernstra 2010). Note that in the *Aplysia* ELH precursor there is a splicing variant that lacks the N-terminal region (indicated by SV). *Shaded bars* represent the localizations of bioactive peptides. *AGP* atrial gland peptide, *AP* acidic peptide, *BCP* bag cell peptide, *CDCH* caudodorsal cell hormone, *CDCP* caudodorsal cell peptide, *ELH* egg-laying hormone, *SP* signal peptide

the N-terminal region of califin is identical to that of ELH (Figs. 8.2 and 8.3). As the ELH precursor gene, but not the AGP-A and AGP-B precursor, are found in other opisthobranch gastropods, it is likely that the peptide A/califin-precursor gene emerged from the duplication and deletion of the ancestral ELH precursor gene (Nambu and Scheller 1986).

In intact animals, external or internal stimuli, such as temperature and growth and maturation of the animal, initiate egg laying in *Aplysia*. How do these stimuli activate the bag cells and atrial gland? It was demonstrated that neurons located in the cerebral and pleural ganglion have a neural connection to the bag cells (Brown et al. 1989) and that these neurons relay sensory input to the bag cells to induce their discharge. Although injection of AGP-A or califin triggers egg laying of *Aplysia*, it is unclear how the atrial gland is involved in the regulation of egg laying. Because the atrial gland is located near the genital pore, it is an attractive hypothesis that copulation stimulates the secretion of peptide hormone from the gland. However, as the peptides are packaged in the secretory vesicles in the glandular cells, the peptides are normally released into the lumen of the hermaphroditic duct but not

Table 8.2 Structures of gonadotropin-releasing hormone (GnRH), adipokinetic hormone, and corazonin

Phyla	Order	Species	Common name	Structure
GnRH				
Chordata	Mammalia	*Mus musculus*	mice	pQ---HWSYGLRPGa
	Aves	*Gallus gallus*	chicken	pQ---HWSYGLQPGa
	Cyclostmata	*Petromyzon marinus*	lamprey	pQ---HWSHQWFPGa
	Ascidiacea	*Ciona intestinalis*	tunicate	pQ---HWSDYFFPGa
Mollusca	Cephalopoda	*Octopus vulgaris*	octopus	pQ-NYHFSNGWHPGa
	Gastropoda	*Aplysia californica*	sea hare	pQ-NYHFSNGWYA-a
		Lottia gigantea	limpet	pQ-HYHFSAGWLS-a
	Bivalvia	*Crossosteria gigas*	oyster	pQ-NYHFSNGWQP-a
		Patinopectin yessoensis	scallop	pQ-NFHYSNGWEP-a
Adipokinetic hormone				
Arthropoda	Insecta	*Locusta migratria*	locust	pQLNF--TFNW-GTa
Corazonin				
Arthropoda	Insecta	*Aedes gambiae*	mosquito	pQ-TFQYSRGW-TMa

Note that several gaps (indicated by hyphens) were placed in the peptide sequence for alignment

pQ pyroglutamine, *a* C-terminal amide

into the hemolymph. One possibility is that peptides in the atrial gland have pheromonal action (Susswein and Benny 1985).

Lymnaea stagnalis lays eggs in an egg mass that contains about 50–100 eggs in a gelatinous sheath. The egg laying of *Lymnaea* consists of four sequential phases: resting, shell turning, oviposition, and inspection of the egg mass (Ter Maat et al. 1989). As in the bag cells in *Aplysia*, the neurosecretory caudodorsal cell (CDC) triggers the sequential behaviors (Geraerts and Bohlken 1976). CDC located in the cerebral ganglion sends to the axon terminals on the cerebral commissure and releases several peptide hormones (Geraerts et al. 1983; Vreugdenhil et al. 1985) when the CDC is activated to initiate the afterdischarge. For more information on egg laying of *Lymnaea*, refer to the recent review by Koene (2010).

The structures of the peptide hormones released from CDC are quite similar to those released from the bag cells in *Aplysia*. For instance, caudodorsal cell hormone (CDCH) in *Lymnaea* consists of 36 amino acids, and the 16 residues are identical to those of ELH (Ebberink et al. 1985) (Fig. 8.2). Moreover, the organization of the CDCH precursor is quite similar to that of ELH (Vreugdenhil et al. 1988) (Fig. 8.3). As is the ELH precursor, CDCH is located in the C-terminal region, whereas α-, β_2-, and β_3-caudodorsal cell peptides (CDCPs), which correspond to α-, β-, and γ-BCP, respectively, are located in the middle region. Although the overall similarity of the amino acid sequences between ELH precursor and CDCH precursor is 25 %, similarities in amino acids in the peptide-coding regions are 50–70 %. CDCH and CDCP coordinate actions to induce egg laying, including auto-excitation and modulation of the excitability of the right pedal N motor neuron (RPeN) (Hermann et al. 1997).

In prosobranch gastropods, the ganglionic factor that induces egg laying was reported in the flat-top shell, *Gibbula umbilicalis* (Clare 1986). In this study, extract of the cerebral ganglion, but not of the visceral ganglion, induced egg laying in the closely related gastropod *Gibbula cineraria* when the snail was in reproductive season. Because the same batch of the brain factor failed to induce egg laying in the nonreproductive female, it appears that the brain factor does not promote the sexual maturation of females. The brain factor also does not induce sperm release in the male. Although the entity of the factor is unknown, it seems to be a certain peptide, because egg-laying activity was diminished by protease digestion but not by heating or acid treatment.

The extract of the parietal ganglion of the whelk *Busycon* also induces egg laying in this animal (Ram 1977). It is suggested that the factor is a ELH-related peptide because behavior of the factor on gel filtration chromatography is quite similar to that of *Aplysia* ELH, and injection of *Aplysia* ELH also induces egg laying (Ram et al. 1982). In *Haliotis asinina*, immunohistochemistry with anti-*Haliotis* ELH antibody demonstrated the localization of ELH-related peptide-containing neurosecretory cells in the cerebral, pleuropedal, and visceral ganglia (Saitongdee et al. 2005). The numbers of the neurosecretory cells are high in cerebral and pleuropedal ganglia, and much fewer in the visceral ganglion. The localization of the peptide, together with the fact that the extract of the cerebral and pleuropedal ganglia of *G. umbilicalis* contained the egg laying-inducing activity, suggest that, as in *Aplysia* and *Lymnaea*, ELH-related peptides derived from neurosecretory cells in the brain regulate egg laying in prosobranch gastropods. Immunostaining also demonstrated that the follicular and glandular cells in the ovary contained ELH-related peptide (Saitongdee et al. 2005). ELH in the ovary may have local actions, such as oocyte maturation and ovulation.

Recent progress in EST analysis and data mining on the genome sequences showed the structure of precursor proteins for the ELH-related peptides in several mollusks, such as the prosobranch *Lottia gigantea* (Veenstra 2010), the pearl oyster *Pinctada fucata*, and *Crassostrea gigas* (Stewart et al. 2014). In these animals, amino sequences of the predicted ELH-related peptide are similar to those of ELH and CDCH (Figs. 8.2 and 8.3). However, organizations of precursor proteins are somewhat different from those of ELH and CDCH. For instance, BCP or CDCP peptides are not found on the precursor proteins of *Pinctada*, *Crassostrea*, and *Lottia*. In *Pinctada* and *Crassostrea*, ELH-related peptides are duplicated on the respective precursors. Considering that egg-laying behaviors in *Aplysia* and *Lymnaea* are inducible through the coordination of different peptides derived from the same precursor, it is an interesting question how ELH-related peptides are involved in the reproduction of the animals.

8.6 Gonadotropin-Releasing Hormone (GnRH)

In mammals, GnRH is a key peptide hormone to assure the proper development and function of the gonad (Guillemin 1978; Schally 1978; Iversen et al. 2000). The mammalian GnRH is a 10-mer peptide with N-terminal pyroglutamine and C-terminal Pro-Gly-NH_2 structures (Baba et al. 1971; Matsuo et al. 1971). More than 20 kinds of GnRH have been found in chordates so far (Roch et al. 2011). All share the aforementioned structural features, although some differences in amino acids were found in the middle region of the peptides (Table 8.2).

In mollusks, the existence of GnRH-like peptides was initially demonstrated by immunohistochemistry with an antibody that recognizes vertebrate GnRH or by testing the actions of vertebrate GnRH in the chiton (Amano et al. 2010b), oyster and mussel (Nakamura et al. 2007; Pazos and Mathieu 1999), abalone (Amano et al. 2010a; Nuurai et al. 2014), freshwater snail (Goldberg et al. 1993; Young et al. 1999), sea hare (Zhang et al. 2000), and octopus (Di Cosmo and Di Cristo 1998). Now, structures of GnRH-related peptides are known in those animals (Table 8.2). Interestingly, invertebrate GnRHs have two amino acid insertions between the N-terminal pyroglutamine and histidine, and, except for octopus GnRH, the C-terminal glycine residue is missing. Thus, octopus GnRH is a 12-mer peptide whereas other invertebrate GnRHs are 11-mer peptides.

Immunohistochemistry demonstrated that GnRH-containing neurons were found in the cerebral ganglion of the Pacific abalone, *Haliotis discus hannai* (Amano et al. 2010a), and in the cerebral and pleuropedal ganglia of *H. asinina* (Nuurai et al. 2014). In both studies, the authors suggested the existence of several distinct GnRH-related peptides, because the anti-GnRH antibody recognized multiple peptides in the extract of the nervous system. Because immunopositive nerve fibers were not found on the gonad, they hypothesized that *Haliotis* GnRH has hormonal action on the tissue. It is noteworthy that, in the ovary of *H. asinina*, oocytes in an early stage of gonadal maturation are immunopositive to anti-lamprey GnRH antibody (Nuurai et al. 2010). It is plausible that GnRH is involved in oocyte maturation in this animal. Although it was reported that repetitive injections of salmon GnRH analogue induced maturation of the gonad in the Hawaiian limpet *Cellana* (Hua and Ako 2013), the functional relevance of GnRH to gonadal maturation in prosobranchs has not been fully understood.

In the nervous tissue of a freshwater pulmonate, *Helisoma trivolvis*, neurons immunopositive to the anti-mammalian GnRH antibody were diffusely distributed in all the circumesophageal ganglia (Young et al. 1999). In the peripheral nervous system, immunopositive nerve processes were found in the reproduction-associated organs such as penial complex, vas deferens, oviduct, and ovotestis. GnRH-containing neurons in the left cerebral ganglia appeared to be involved in the regulation of the penial complex, because retrograde filling of the fluorescent dye, Lucifer Yellow, from the cut end of the penis nerve stained those neurons (Young et al. 1999).

In *Aplysia californica*, a GnRH precursor predicted by the transcriptome data of *Aplysia* (Zhang et al. 2008) consisted of 147 amino acids, including N-terminal

signal peptide (27-mer) and a single copy of GnRH. Immunohistochemistry with a specific antibody to *Aplysia* GnRH demonstrated that GnRH-containing neurons were mainly located in the pedal and cerebral ganglia of the animal (Zhang et al. 2000; Jung et al. 2014). Immunopositive nerve processes were found in the neuropil region in the head ganglia, but not in the peripheral nerve on the reproductive organs. Hormonal action of GnRH on the reproductive organs is not likely in this animal, because repetitive injection of *Aplysia* GnRH to the sexually immature *Aplysia* failed to induce gonadal maturation but induced acute changes in behaviors such as feeding and locomotion (Tsai et al. 2010). Moreover, bath application of GnRH to isolated preparation of pedal ganglia modified the firing rate of several neurons in the ganglia (Seaman et al. 1980). Thus, the primary function of *Aplysia* GnRH seems to be a regulation of behavior rather than that of gonadal maturation (Sun and Tsai 2011).

In bivalves, the structure of GnRH was determined in *Crassostrea gigas* as pGNYHFSNGWQP-NH_2 for *C. gigas* (Bigot et al. 2012) and pGNFHYSNGWQP-NH_2 for *Patinopecten yessoensis* (Treen et al. 2012), respectively, Recently, the structure of GnRH of *Pinctada fucata* was reported by predicting the precursor cDNA through analysis of the genome DNA (Stewart et al. 2014).

When the expression of the GnRH precursor in *C. gigas* was quantified by Q-PCR, expression was high in the visceral ganglia, whereas it was negligible in other tissues tested, including gonadal tissue (Bigot et al. 2012). In fact, GnRH-containing neurons visualized by immunohistochemistry were found in the central nervous system but not in other tissues. The expression level of GnRH precursor mRNA in the visceral ganglion is not constant during the development of the gonad. There is a tendency that, in the male oyster, expression of GnRH precursor is higher in gonadal maturation, whereas it is higher in the gonadal proliferation and sexual maturation phases in the female oyster.

Identification of the GnRH receptor is successful in *C. gigas*. The GnRH receptor cloned in *C. gigas* (cg-GnRH-R) is a GPCR, sharing 20–30 % homology with vertebrate GnRH receptors (Rodet et al. 2005). The consensus sequences of mammalian GnRH receptors for ligand binding are conserved in the cg-GnRH-R. The cg-GnRH-R gene consists of six exons, and it was predicted that alternative splicing generates four cg-GnRH-R subtypes with different lengths of N-terminal and C-terminal intracellular regions and different numbers of transmembrane regions (Rodet et al. 2008).

Reverse transcription PCR (RT-PCR) confirmed the expression of three of the four subtypes in peripheral tissues including the gonad. Variety in the receptor structure, together with the wide distribution of cg-GnRH-R in nonreproductive tissues, implies that GnRH is a multifunctional peptide in this animal. Apparently, the next important issue to be confirmed is the endogenous ligands to those receptors. Using an expression system such as the *Xenopus* oocyte, determination of the affinity orders of those receptors to GnRH, corazonin, and adipokinetic hormone (AKH) is attractive (see below).

Several lines of functional assay suggest that GnRH mediates the gonadal maturation in bivalves. For instance, vertebrate GnRH increased ^{3}H-thymidine incorporation into dispersed gonadal cells of *Mytilus edulis* and *Crassostrea gigas*, suggesting that proliferation of gonadal cells is promoted (Pazos and Mathieu 1999). A GnRH antagonist effectively inhibited the mitogenic action of mammalian GnRH. GnRH likely has a hormonal action on the gonad of *M. edulis* because immunohistochemistry with anti-human GnRH antibody demonstrated GnRH-positive neurons in the cerebral and pedal ganglia, but not in the nerve endings on the gonad.

In the scallop, it was reported that peptidic extract of the cerebrapedal ganglia of the scallop promoted BrdU incorporation into cultured gonadal tissue, which was blocked by pre-incubation with anti-mammalian GnRH antibody (Nakamura et al. 2007). Immunostaining with the same antibody demonstrated immunopositive neurons in the ganglia. However, as in the oyster, no immunopositive fibers were found around the gonad. These results suggest that GnRH-related peptide promotes proliferation of gonadal cells via a hormonal action. Unfortunately, those experiments were conducted with exogenous GnRH. Now, structures of GnRHs are known in those animals. Confirmation of the mitogenic action of endogenous GnRH on the gonad will be necessary.

The sex differentiation of bivalves is rather a complicated phenomenon (Mackie 1984). For instance, *Mytilus* is a rather rigid gonochorist, and sex reversal is hardly inducible. By contrast, *Crassostrea* is a protandrous hermaphrodite, and sex reversal is naturally inducible. In *Crassostrea*, the undifferentiated gonad in nonmating season rapidly differentiates to the matured ovary or testis in a few months (Enriquez-Diaz et al. 2009). Involvement of GnRH in this dynamic remodeling of the gonad is an interesting issue to be examined.

GnRH in cephalopods was initially demonstrated by immunohistochemistry with anti-chicken GnRH antibody (Di Cosmo and Di Cristo 1998); then, it was chemically isolated from the brain of the common octopus, *Octopus vulgaris* (Iwakoshi et al. 2002). Differing from other molluscan GnRHs, octopus GnRH shares the same C-terminal structure (–Pro-Gly-NH_2) with mammals.

Sexual maturation of the female octopus is hormonally controlled by a pair of optic glands that lies on the optic tract in the vicinity of the optic lobe. The gland regulates various reproductive events such as proliferation of gonadal cells and yolk-protein synthesis (O'Dor and Wells 1973), probably by secreting the steroid hormone. Immunohistochemistry with antibodies against chicken GnRH or octopus GnRH demonstrated that octopus GnRH-containing neurons were diffusely distributed in various lobes in the octopus brain, including the subpedunculate and the posterior olfactory lobes (Iwakoshi et al. 2002; Iwakoshi-Ukena et al. 2004). Because GnRH-containing nerve processes are found in the optic tract and optic gland, it is suggested that GnRH neurons stimulate optic gland activity, which results in gonadal maturation. Di Cosmo et al. (2003) reported that the activity of the optic gland is inhibited by FMRFamide-containing neurons in the subpedunculate lobe. Accordingly, antagonistic regulation of the optic gland is suggested. These regulatory pathways via the subpedunculate lobe–optic gland axis

are quite similar to the hypothalamo–hypophysial axis in vertebrates. In addition, octopus GnRH has direct action on the gonad, which promotes steroid synthesis (Kanda et al. 2006).

The olfactory lobe, located in the vicinity of the optic gland, is a region that receives sensory information through the olfactory nerve (Budelmann 1995). In this lobe, sensory inputs such as chemoreception are integrated with the visual information to initiate reproduction with the proper timing. GnRH neurons in the olfactory lobe appeared to regulate the activity of the optic gland, because GnRH-containing nerve processes emanating from the lobe make contact with the glandular cells in the optic gland. It was reported that a glutamine receptor agonist, *N*-methyl-*D*-aspartate (NMDA), elevated the expression level of GnRH precursor mRNA in GnRH neurons in the olfactory lobe (Di Cristo et al. 2009). It is plausible that glutaminergic neural input to the GnRH neurons in the olfactory lobe promotes gonadal maturation through augmentation of the GnRH signaling system.

Recently, a novel GnRH receptor was identified in *Octopus vulgaris* (Kanda et al. 2006). The homology of the amino acid sequence between the octopus GnRH receptor and vertebrate GnRH-receptors is around 30 %, and octopus GnRH activates the GnRH receptor expressed in *Xenopus* oocytes. RT-PCR and in situ hybridization demonstrated that the octopus GnRH receptor is expressed in various brain regions including the pedunculate, olfactory, and optic lobes, as well as reproductive organs including ovary and oviduct. Thus, regulatory action of GnRH on female reproduction is suggested. Moreover, the GnRH receptor was also expressed in the digestive system and cardiovascular system. The GnRH system may regulate various physiological and behavioral responses in *Octopus*, such as memory formation, feeding, and circulation (Iwakoshi-Ukena et al. 2004).

By analyzing the similarity in amino acid sequences of neuropeptide receptors, it was postulated that corazonin receptor, AKH receptor, and AKH/corazonin-related peptide receptor in insects are evolved from the ancestral GnRH receptor, during the evolution of Protostomia that led to the divergence of Mollusca, Annelida, and Arthropoda (Hauser and Grimmelikhuijzen 2014). AKH is a peptide hormone that regulates energy metabolism (Stone et al. 1976) and corazonin regulates heartbeat (Veenstra 1989) in insects. In this theory, ligand peptides were also evolved from the ancestral GnRH precursor.

In fact, GnRH shares structural similarity with AKH, corazonin, and corazonin-related peptides (Table 8.2). Moreover, corazonin precursor genes were found in the genome sequences of several mollusks, including *Aplysia*, *Lottia*, and *Crassostrea* (Hauser and Grimmelikhuijzen 2014). In this context, it is noteworthy that anti-GnRH antibody recognizes multiple peptides in the nervous tissues of several mollusks. Apparently, elucidation of the entity of immunoreactivity to anti-GnRH antibody is the next important issue.

Recently, De Lisa et al. (2013) proposed that changes in the expression level of GnRH-precursor mRNA could be a biomarker for monitoring the effect of EDC on the rayed Mediterranean limpet, *Patella caerulea*, which is an interesting approach to elucidate how EDC affects the regulatory neuropeptide system of mollusks. However, as I have described, GnRH has a broad range of physiological functions

including promotion of gonadal development, regulation of feeding, and locomotion. Even a pheromonal action that triggers the release of gametes was reported in the chiton *Mopalia* sp. (Gorbman et al. 2003). Moreover, multiple GnRH-related peptides including AKH-related peptide could be functional in the mollusks so far examined. Accordingly, careful characterization of GnRH is essential before discussing the relevance between the GnRH system and EDC. For further information on molluscan GnRH, readers can refer to recent reviews (Minakata et al. 2009; Sun et al. 2012; Di Cristo 2013; Osada and Treen 2013).

8.7 Perspectives

In the past four decades, many peptides have been identified in mollusks. Now, in the post-genome era, identification of neuropeptides is accelerated through the combination of the prediction of neuropeptide precursors by the annotation of genome sequences and the structural analysis of tissue extracts with mass spectrometry. Accordingly, the importance of the functional analysis of identified peptides is expanding more and more. However, except for a few gastropods such as *Aplysia* and *Lymnaea*, functional analysis of neuropeptides is hampered by the fact that the nervous system consists of small-sized neurons and the soft body is covered with a hard shell. Moreover, considering the length of the reproductive cycle and long larval stages, including several steps of metamorphosis, it may not be easy to obtain gene-manipulated mollusks.

One of the practical approaches is to select the appropriate target animal, clarify its genetic background to predict a precursor gene for neuropeptides and peptide hormones, and then conduct the analysis of peptide structure by mass spectrometry on nervous tissue or reproduction-related organs. Novel approaches with techniques such as microanalysis of trace amounts of peptides in hemolymph and the expression of the hybrid gene of the 5′-upstream region of the neuropeptide precursor gene and appropriate reporter genes such as luciferase on the cultured cell, which are compatible with standard biochemical, molecular biological, and physiological techniques, are encouraged to clarify the physiological functions of identified peptides. Noninvasive observation of the gonad by magnetic resonance imaging is an interesting approach to investigate the changes in the structure of the gonad during reversible sex reversal (Davenel et al. 2006).

The receptor systems for peptide ligands are another important issue to be examined. Besides the neuropeptide receptors discussed in this review, several neuropeptide receptors including for FMRFamide (Linguelia et al. 1995), vasopressin/oxytocin-related peptides (van Kesteren et al. 1995), and *Achatina* cardioexcitatory peptide (ACEP)-1-like peptide (Tensen et al. 1998) have been cloned in mollusks. However, considering the diversity of neuropeptides and peptide hormones, many receptors still remain to be identified. For the identification of the neuropeptide receptors, molecular cloning of the orphan GPCR is one of the well-accepted approaches. In addition, recent progress in annotation of the

genome sequences, together with the massive sequence data obtained by transcriptome analyses, may be helpful in predicting the nucleotide sequence of mRNA for neuropeptide receptors. Characterization of cloned receptors is possible on an appropriate expression system such as *Xenopus* oocytes.

Now, nuclear receptor RXR is recognized to be the target molecule of organotin (Nishikawa et al. 2004; Castro et al. 2007). Because RXR regulates gene expression, directly or indirectly, it is possible to assume that EDC modifies expression of precursor genes of neuropeptides and their receptors in mollusks. In this context, analysis of the 5′-upsteam region of those genes on the genome sequences is also important to understand when and how the expression of the precursor gene is regulated.

Because EDC generally disturbs reproduction, it reduces the population of mollusks in the field and has profound effects on ecological balance. EDC also causes damage to the fishery cultivation industry by augmenting the costs of cultivation, because, under the influence of EDC, released juveniles of commercially valuable mollusks, such as abalone and clam, do not reproduce, even after they are fully grown-up. If EDCs have more acute and drastic effects, such that the compounds kill animals or induce apparent deformity in the animals, people will be more careful about using and disposing such chemicals. In reality, the actions of EDC are silent and creeping; hence, we underestimate their threat.

Unfortunately, at present, we cannot give a satisfactory explanation for the influence of EDC on regulatory neuropeptide and peptide hormone systems. If we could see, however, the large picture of the neuropeptide–peptide hormone systems of mollusks in the near future, we would be able to predict how a particular chemical affects the regulatory peptide systems, which will consequently minimize the impact of EDC on both ecological balance and the fishery industry. Because mollusks hold an important place in both the environment and the fishery industry, research on molluscan neuropeptides will satisfy not only our scientific interest in the peptide biology, but also our appetite.

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Chapter 9
Mode of Action of Organotins to Induce the Development of Imposex in Gastropods, Focusing on Steroid and the Retinoid X Receptor Activation Hypotheses

Toshihiro Horiguchi

Abstract Basic knowledge of endocrinology or reproductive physiology of prosobranch gastropods is reviewed, focusing on vertebrate-type steroids as possible sex hormones in gastropods. Major points of the view for criticism are steroid-producing cells, enzymes to synthesize and/or metabolize steroids, and functional receptors for steroids. Mechanism of induction and promotion of the development of imposex is also reviewed, regarding six hypotheses proposed as the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods: (1) an increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase; (2) an increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase; (3) TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels; (4) TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia; (5) an increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT; and (6) activation of the retinoid X receptor (RXR). The latest information about nuclear receptors other than RXR in gastropods, namely, retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR), is also described.

Keywords Aromatase • Alanine-proline-glycine-tryptophan amide (APGWamide) • Enzymes to synthesize steroids • Functional receptors for steroids • Retinoid X receptor (RXR) • Sex hormones • Steroid-producing cells • Tributyltin (TBT) • Triphenyltin (TPhT) • Vertebrate-type steroids

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Abbreviations

PPAR	peroxisome proliferator-activated receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
TBT	tributyltin
TPhT	triphenyltin

9.1 Introduction

In the former part of this chapter, basic knowledge of endocrinology or reproductive physiology of prosobranch gastropods is reviewed, in terms of vertebrate-type steroids. Because neuropeptides of mollusks, including gastropods, are reviewed in the previous chapter (Chap. 8), I will critically review existed knowledge of vertebrate-type steroids detected in gastropods, focusing on the possibility of vertebrate-type steroids to be sex hormones in gastropods. Major points of the view for criticism are steroid-producing cells, enzymes to synthesize and/or metabolize steroids, and functional receptors for steroids. The latest information about genes coding vertebrate-type steroid hormone receptors in gastropods is also described.

In the latter part of this chapter, mechanism of induction and promotion of the development of imposex is reviewed: regarding the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods, six hypotheses have been proposed:

1. An increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al. 1996)
2. An increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006)
3. TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason 1996)
4. TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983)
5. An increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000)
6. Activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004)

Although scientific debate is still continuing, there are several papers in which a hypothesis of activation of RXR is supported (Castro et al. 2007; Horiguchi et al. 2007, 2008a, 2010a, b; Sternberg et al. 2008; Urushitani et al. 2011). The latest information about nuclear receptors other than RXR in gastropods, namely, retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR), is also described (see below).

9.2 A Critical Review on Steroid Hormones in Gastropods

Because sex steroid hormones, such as testosterone and 17β-estradiol, play physiologically important roles in the development of sex organs and the maturation of gonads (i.e., oogenesis and spermatogenesis) in vertebrates, it has been hypothesized that similar sex steroid hormones might also regulate the reproduction of invertebrates, such as gastropods (LeBlanc et al. 1999). After the removal of the hermaphroditic organ, oogenesis and spermatogenesis were observed, respectively, in the gonads of 17β-estradiol-treated females and testosterone-treated males of the slug *Limax marginatus*; egg laying was also induced by 17β-estradiol in female slugs, implying the existence of vertebrate-type sex steroid hormones in this species (Takeda 1979, 1983). The in vitro metabolism of androstenedione and the identification of endogenous steroids (androsterone, dehydroepiandrosterone, androstenedione, 3α-androstanediol, estrone, 17β-estradiol, and estriol) by gas chromatography with mass spectrometry (GC-MS) were reported for *Helix aspersa* (Le Guellec et al. 1987). Several vertebrate-type sex steroids (androsterone, estrone, 17β-estradiol, and testosterone) and the synthetic estrogen (ethynyl estradiol) were also identified by high-resolution GC-MS in the gonads of *Thais clavigera* and *Babylonia japonica*. The detection of the synthetic estrogen, ethynyl estradiol, in the gonads, presumably represents environmental rather than endogenous origins—indicating that contamination of the habitat of *B. japonica* had occurred (Lu et al. 2001). It is therefore likely that the presence of other vertebrate-type sex steroids in *T. clavigera* and *B. japonica* may have been due to environmental exposure as opposed to synthesis in vivo.

Scott (2012) reviewed the evidence for the presence, biosynthesis, and uptake of steroids in mollusks and concluded that there was no convincing evidence for biosynthesis of vertebrate steroids by mollusks. Furthermore, Scott (2012) also pointed out in his review that the "mollusk" genome does not contain the genes for key enzymes that are necessary to transform cholesterol in progressive steps into vertebrate-type steroids. To the best of our knowledge, there has been no scientific report on steroid-producing cells in mollusks. On the other hand, there is strong evidence that mollusks are able to absorb vertebrate steroids from the environment and are able to store some of them (by conjugating them to fatty acids) for weeks to months (Scott 2012). We should also remember that the three steroids that have been proposed as functional hormones in mollusks (i.e., progesterone, testosterone, and 17β-estradiol) are the same as those of humans. Since humans (and indeed all vertebrates) continuously excrete steroids not just via urine and feces, but via their body surface (and, in fish, via the gills), it is impossible to rule out contamination as the sole reason for the presence of vertebrate steroids in mollusks (even in animals kept under supposedly "clean laboratory conditions"). Essentially, the presence of vertebrate steroids in mollusks cannot be taken as reliable evidence either of endogenous biosynthesis or of an endocrine role (Scott 2012).

Meanwhile, the biotransformation of testosterone has been characterized in the mud snail (*Ilyanassa obsoleta*) (Gooding and LeBlanc 2001). However, as there has been no scientific verification on the presence of an androgen receptor (AR) in gastropods (see below), we should perhaps interpret the biological significance of the transformation of testosterone in the *I. obsoleta* exposed at a relatively high dose (1.0 μM (150,000 DPM) [^{14}C] testosterone), with caution (Gooding and LeBlanc 2001). It is also possible that such an apparent biotransformation of high doses of steroids might be a kind of metabolism for xenobiotics in mollusks.

Aromatase-like activity has been measured and reported in several gastropod species (Morcillo and Porte 1999; Santos et al. 2002); however, the measured aromatase-like activity does not necessarily confirm the existence of vertebrate-type aromatase in gastropods. To the best of our knowledge, there has been no scientific report that has elucidated the successful isolation of aromatase protein from invertebrates. Further evidence of steroid-producing cells as well as synthetic/metabolic enzymes for steroid biosynthesis also needs to be obtained to clarify the existence of vertebrate-type sex steroid hormones in gastropods.

Although an estrogen receptor (ER)-like cDNA has been isolated from *Aplysia californica* (Gastropoda: Opisthobranchia) and the protein it encodes functions as a constitutively activated transcription factor, estrogen cannot bind this protein (Thornton et al. 2003). Similarly, an ER-like protein has also been isolated from *T. clavigera* though this too is not bound by estrogen (Kajiwara et al. 2006; Iguchi et al. 2007). This *T. clavigera* protein is also a constitutively activated transcription factor (Iguchi et al. 2007). To the best of our knowledge, no scientific report has described the successful cloning of AR from the tissues of invertebrates, including gastropods. In the absence of direct evidence for ER and AR, their physiological role in mollusks remains in doubt, even if estrogens and androgens are detected in tissues. Based on a study of fully sequenced invertebrate genomes, homologues of ER and AR have yet to be found in invertebrates (Escriva et al. 1997). Actually, recent findings of nuclear receptors in mollusks (*Crassostrea gigas*, *Biomphalaria glabrata*, and *Lottia gigantea*) also revealed that no functional nuclear receptors, such as AR and ER, have been confirmed in bivalves and gastropods, although homologues of ER and the estrogen-related receptor (ERR) were identified in them (Vogeler et al. 2014; Nordberg et al. 2014; Kaur et al. 2015). Therefore, the mollusk genome does not seem to contain genes for functioning classical nuclear steroid receptors (Scott 2012; Simakov et al. 2013; Nordberg et al. 2014). Thus, it is doubtful whether gastropods have vertebrate-type steroids as sex hormones. The absence of a molluscan AR and the constitutive expression of the ER in vitro suggest alternative pathways may exist for spermatogenesis/oogenesis in mollusks (Kaur et al. 2015). Further studies are necessary to identify steroid receptors and clarify their functions in gastropods.

On the other hand, in reviewing the evidence as to whether vertebrate sex steroids (e.g., testosterone, estradiol, progesterone) have hormonal actions in mollusks, Scott (2013) has criticized almost all related papers, in terms of their experimental designs (i.e., tested compounds or mixtures that were only presumed

to behave as steroids (or modulators of steroids) on the basis of their effects in vertebrates and pointed out neither "blinding" procedures (implying the possibility of "operator bias") nor evaluation of results (i.e., no statistical analysis)).

9.3 Involvement of the Retinoid X Receptor (RXR) and Other Nuclear Receptors in the Development of Imposex in Gastropods

Regarding the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods, as described before, six hypotheses have been proposed:

1. An increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al. 1996)
2. An increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006)
3. TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason 1996)
4. TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983)
5. An increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000)
6. Activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004)

Experimental evidence, however, is weak for five hypotheses other than the hypothesis of (6) activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004). Although it is doubtful whether gastropods have vertebrate-type steroids as sex hormones, as mentioned above in this chapter, there is also a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (Bettin et al. 1996; Spooner et al. 1991). Regarding the hypotheses (1), (2), and (3), Spooner et al. (1991) reported that testosterone levels were significantly elevated in TBT-exposed dogwhelks (*Nucella lapillus*) on days 28 and 42 when compared to the control, although the penis length of female *Nucella lapillus* started to increase on day 14. In another study, a combination of the aromatase inhibitor fadrozole (5 μg/g wet wt) and testosterone (0.1 μg/g wet wt) had little effect on the induction and/or promotion of imposex in *T. clavigera*, as indicated by the incidence of imposex and penis growth (Iguchi et al. 2007). Consequently, there seems uncertain about the mechanism by which organotins induce imposex in gastropods, assuming that vertebrate-type steroid hormones are involved. Meanwhile, there is a possibility that the results given in support of the "inhibition of testosterone excretion" hypothesis (Ronis and Mason 1996) may reflect a phenomenon that is at least partly short term and/or

associated with acutely toxic TBT concentrations (Matthiessen and Gibbs 1998). On the other hand, regarding the hypothesis of (2), an increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006), we should interpret the biological significance of the transformation of testosterone in gastropods, with caution, as mentioned above. It is also possible that such an apparent biotransformation of high doses of steroids might be a kind of metabolism for xenobiotics in mollusks (Scott 2012).

It is unknown whether aromatase-like activity is actually inhibited by TBT concentrations in tissues of gastropods collected at natural sites slightly contaminated by TBT. There is also contradictory evidence of the relationship between reduced aromatase-like activity and advance imposex symptoms in the gastropod *Bolinus brandaris* (Morcillo and Porte 1999).

Santos et al. (2005) suggested the involvement of AR, besides aromatase inhibition, in the development of imposex in *N. lapillus*. If gastropods also have AR similar to vertebrates, it may be profitable to consider the possible activation of androgen receptor-mediated responses caused by TBT or TPhT in gastropods, as the enhancements of androgen-dependent transcription and cell proliferation by TBT and TPhT have been reported in human prostate cancer cells (Yamabe et al. 2000). However, gastropods may not inherently have AR (Escriva et al. 1997; Scott 2012; Simakov et al. 2013; Vogeler et al. 2014; Nordberg et al. 2014; Kaur et al. 2015).

Several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *Lymnaea stagnalis*) act as ovulation, egg-laying, or egg-releasing hormones (Chiu et al. 1979; Ebberink et al. 1985). Féral and Le Gall (1983) suggested that TBT-induced imposex in *O. erinacea* might be related to the release of neural morphogenetic controlling factors. Their study used in vitro tissue cultures derived from a presumed penis-forming area of the immature slipper limpet, *Crepidula fornicata*, and the isolated nervous systems of male or female *O. erinacea* in the presence/absence of TBT (0.2 μg/L) (Féral and Le Gall 1983). The accumulation of TBT or TPhT in the central nervous systems of *H. gigantea* (Horiguchi et al. 2002), *N. lapillus* (Bryan et al. 1993), and *T. clavigera* (Horiguchi et al. 2012) indicates the potential for the toxic effects of TBT and TPhT on neuroendocrine systems. Oberdörster and McClellan-Green (2000) reported that APGWamide, a neuropeptide released from the cerebral ganglia of gastropods such as *L. stagnalis*, markedly induced the development of imposex in female *I. obsoleta*. The effect of APGWamide in the induction and/or promotion of the development of imposex, however, appears weak based on the experimental results of the incidences of imposex and penis growth (Oberdörster and McClellan 2000; 2002), because the incidences of imposex and penis growth were higher and much longer in gastropods exposed to TBT and/or TPhT in the laboratory, respectively (Horiguchi 2006).

Thus, at present, five hypotheses other than the hypothesis of (6) activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004) regarding the induction mechanism of imposex in gastropods cannot be fully supported.

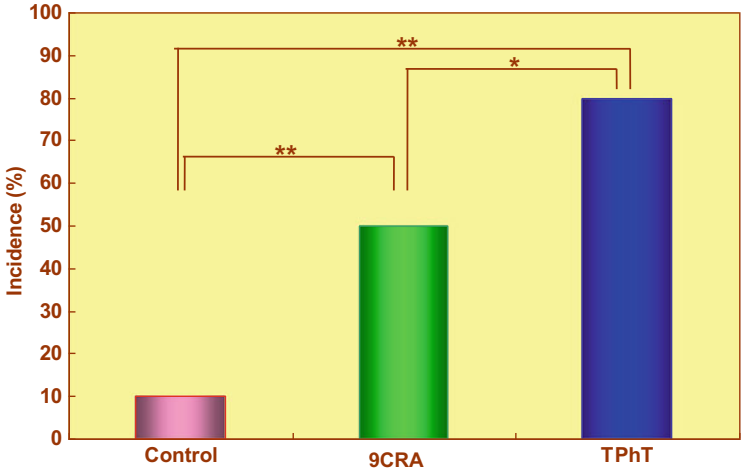

Fig. 9.1 Incidences of imposex in female rock shells (*Thais clavigera*) 1 month after treatment with fetal bovine serum (control), 9-*cis*-retinoic acid (9CRA) at 1 μg/g (wet wt.), or triphenyltin chloride (TPhT) at 1 μg/g (wet wt.). $^{*}P < 0.05$; $^{**}P < 0.01$ (Nishikawa et al. 2004)

Nishikawa et al. (2004) proposed a unique mechanism of action of TBT or TPhT on the development of imposex in gastropods, which was completely different from other hypotheses already proposed as the imposex induction mechanism. Nishikawa et al. (2004) showed that organotins (both TBT and TPhT) bound to the human retinoid X receptors (hRXRs) with high affinity and a single injection of 9-*cis* retinoic acid (9CRA), the natural ligand of hRXRs, into female rock shells (*T. clavigera*) induced the development of imposex (Figs. 9.1 and 9.2). The cloning of an RXR homologue from *T. clavigera* revealed that the ligand-binding domain of the rock shell RXR was very similar to that of the vertebrate RXR and bound to both 9CRA and organotins (Nishikawa et al. 2004). Horiguchi et al. (2008a) treated female rock shells (*Thais clavigera*) with a single injection of 3 different concentrations (0.1, 1, or 5 μg/g wet wt) of 9CRA or with a single concentration (1 μg/g wet wt) of TBT, TPhT (as positive controls), or fetal bovine serum (as a negative control) to confirm the effectiveness of 9CRA in inducing the development of imposex in *T. clavigera*. 9CRA induced imposex in a dose-dependent manner (Fig. 9.3); imposex incidence was significantly higher in the rock shells that received 1 μg ($P < 0.05$) or 5 μg ($P < 0.001$) 9CRA than in the controls. After 1 month, the rock shells treated with 5-μg 9CRA exhibited substantial growth of the penis-like structure. The length of the structure differed between the 0.1 and 5 μg 9CRA treatment groups ($P < 0.05$) but not between the 1 and 5 μg 9CRA treatment groups ($P > 0.05$). Compared with the control, the vas deferens sequence (VDS) index increased significantly in the 1 μg ($P < 0.05$) and 5 μg ($P < 0.001$) 9CRA groups. A light microscopic histological observation revealed that the penis-like structures behind the right tentacle in female rock shells treated with 5 μg 9CRA

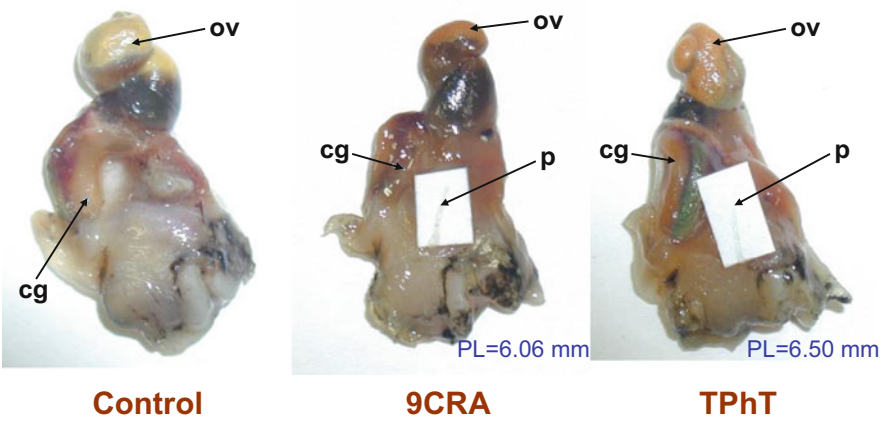

Fig. 9.2 Substantial penis growth observed in female rock shells (*Thais clavigera*) after a month of 9-*cis* retinoic acid (9CRA) injections. *cg* capsule gland, *ov* ovary, *p* penis, *PL* penis length (*Left*). Neither penis nor vas deferens was observed in the control female (after shell removal). (*Center*) Substantial penis growth as well as vas deferens development in a female that received 9CRA injection at 1 μg/g (wet wt.) (after shell removal; penis length: 6.06 mm). (*Right*) Substantial penis growth as well as vas deferens development in a positive control female that received TPhT injection at 1 μg/g (wet wt.) (after shell removal; penis length: 6.50 mm). Signs of imposex symptoms, based on penis length and the vas deferens sequence (VDS) index of females that received 9CRA injections, were clearly promoted and were similar to those in females receiving TPhT injections (Nishikawa et al. 2004)

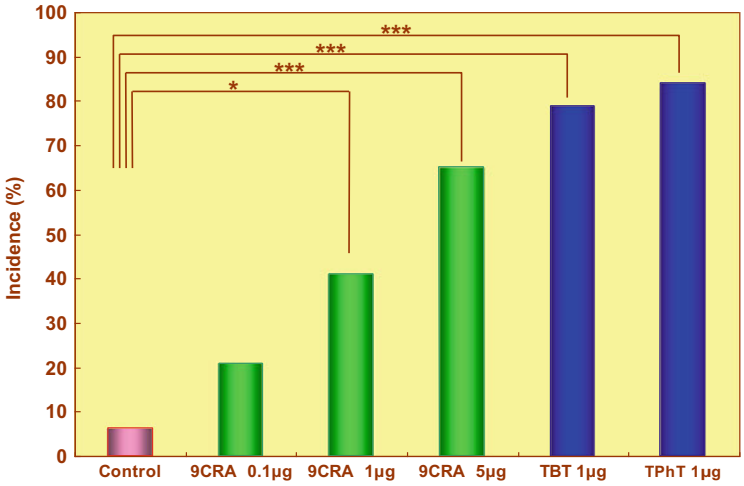

Fig. 9.3 Incidence of imposex in female rock shells (*T. clavigera*) 1 month after treatment with fetal bovine serum (Control), three different concentrations of 9-*cis*-retinoic acid (9CRA), tributyltin (TBT) chloride, or triphenyltin (TPhT) chloride. $^{*}P < 0.05$; $^{***}P < 0.001$ (Horiguchi et al. 2008a)

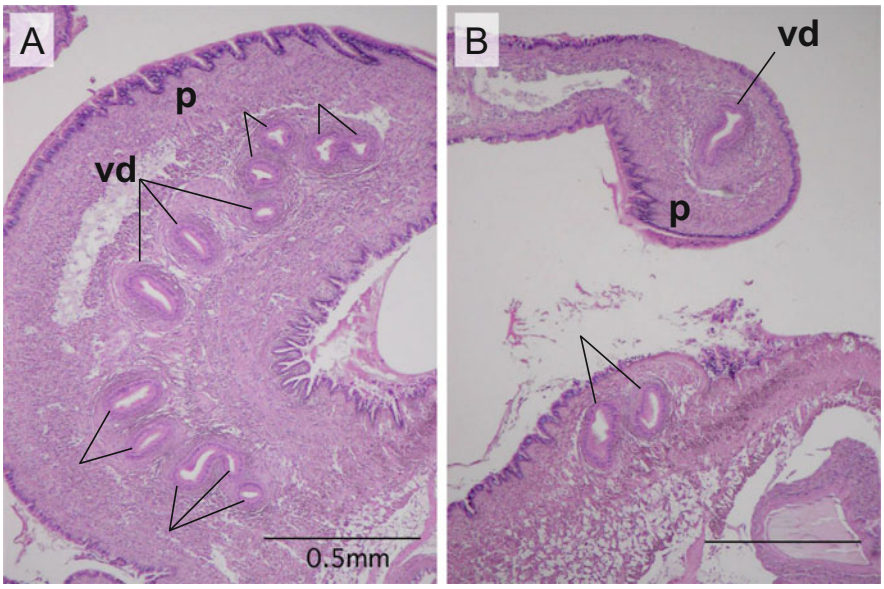

Fig. 9.4 Histology of the penis-like structure (7.00 mm long) that developed behind the right tentacle of a female rock shell (*T. clavigera*) 1 month after treatment with 9CRA at 5 μg/g (wet wt.). The sections in A and B were stained with hematoxylin and eosin. *Scale bars* represent 0.5 mm. *p* penis, *vd* vas deferens (Horiguchi et al. 2008a)

were essentially the same as the penises and vasa deferentia of normal males and of TBT-treated or TPhT-treated imposexed females (Fig. 9.4; Horiguchi et al. 2008a).

Horiguchi et al. (2007) investigated RXR gene expression and measured the RXR protein content in various tissues of wild male and female rock shells (*T. clavigera*) to further elucidate the role of RXR in the development of organotin-induced imposex in gastropod mollusks. By using the methods of quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry with a commercial antibody against human RXR α, they revealed that RXR gene expression was significantly higher in the penises of males ($P < 0.01$) and in imposexed females ($P < 0.05$) than in the penis-forming areas of normal females (Fig. 9.5). Western blotting demonstrated that the antibody could detect rock shell RXR and showed that the male penis had the highest RXR protein content among the analyzed tissues of males and morphologically normal females. Moreover, immunohistochemical staining revealed nuclear localization of RXR protein in the epithelial and smooth muscle cells of the vas deferens and in the interstitial or connective tissues and epidermis of the penis in males and in imposexed females. Same results were also obtained, using the specific antibody for *T. clavigera* RXR (Horiguchi et al. 2010b). Based on the results of these studies, RXR could be involved in organotin-mediated induction of male-type genitalia (penis and vas deferens) in female rock shells (Horiguchi et al. 2007).

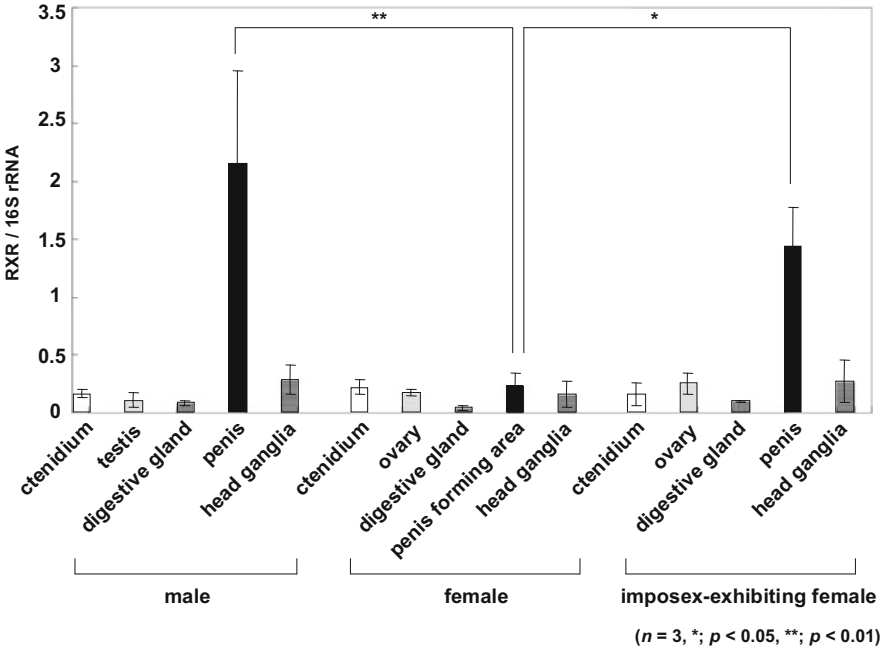

Fig. 9.5 RXR gene expression in various tissues of male, normal female, and imposex-exhibiting female rock shells (*T. clavigera*) (Horiguchi et al. 2007)

To further examine the role of RXR in the development of imposex in gastropods, Horiguchi et al. (2010a) investigated the time course of expression of the RXR gene in various tissues (ctenidium, ovary or testis, digestive gland, penis-forming area or penis, and head ganglia) of female and male *T. clavigera* exposed to TPhT in a flow-through exposure system for 3 months. Accumulation of TPhT in the tissues was clearly observed in exposed individuals, whereas no accumulation of TPhT was observed in the control groups. In females, a 3-month exposure to TPhT resulted in the development of imposex, and penis lengths in imposex-exhibiting females were significantly longer in small females (shell height $<$20 mm) than in large females (shell height $\geq$20 mm). RXR gene expression in the ovary, penis-forming area or penis, and head ganglia of females exposed for 3 months was significantly higher than the expression in control females; the highest RXR gene expression was found in the penis-forming area or penis (Fig. 9.6). Moreover, RXR gene expression in the penis-forming area or penis of each female exposed to TPhT seemed to be associated with penis length (Fig. 9.7). In males, the ratio of penis length to shell height was significantly larger in the exposed groups than in the controls. Although RXR gene expression in males exposed for 3 months was not significantly higher than expression in control males in any tissues, the highest gene expression was observed in the penises of exposed males. These results further suggest that RXR plays an important role in

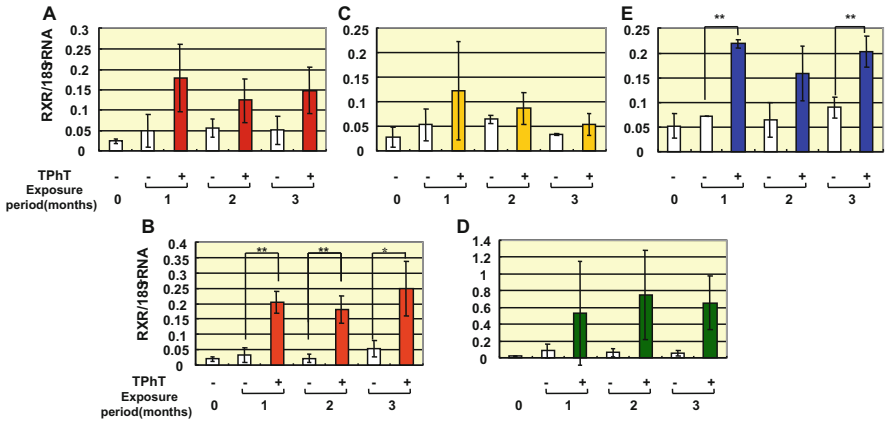

Fig. 9.6 Normalized RXR gene expression in (**A**) ctenidium, (**B**) ovary, (**C**) digestive gland, (**D**) penis-forming area or penis, and (**E**) head ganglia of female rock shells exposed to TPhT chloride at 500 ng/L for 3 months in a flow-through system. *TPhT* triphenyltin; − control females; + TPhT-exposed females; $*P < 0.05$; $**P < 0.01$ (Horiguchi et al. 2010a)

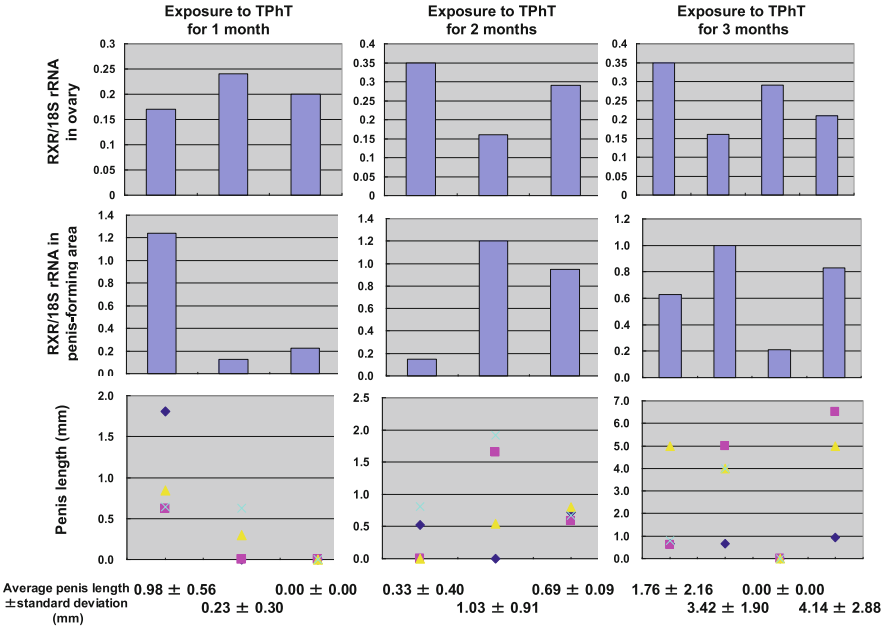

Fig. 9.7 Relationships between average penis length and RXR gene expression in penis-forming area/penis and ovary of female rock shells exposed to TPhT chloride at 500 ng/L for 3 months in a flow-through system. TPhT, triphenyltin. *Bars* in the *top* and *middle rows* represent normalized RXR gene expression in the ovary (*top row*) and penis-forming area/penis (*middle row*) of females exposed to TPhT. Dots or symbols in the *bottom row* represent measured values of penis length of each female in the respective composite samples. RXR gene expression in the penis-forming area or penis of each female exposed to TPhT seemed to be associated with an increase in penis length. However, that in the ovary did not (Horiguchi et al. 2010a)

the development of male genitalia (i.e., penis and vas deferens) in gastropods, although RXR might also have other physiological functions.

Oehlmann et al. (2007) reviewed endocrine disruption in prosobranch gastropods, indicating and discussing its evidence and ecological relevance; they also described the results of their laboratory experiments using 9CRA. Although they injected 9CRA into female *N. lapillus*, no development of imposex was observed in specimens of *N. lapillus* examined during the experimental period (56 days), even in the highest dose (2.5 μg/g wet wt.) group. On the other hand, Castro et al. (2007) demonstrated that injections of 9CRA at 1 μg/g wet wt. in female *N. lapillus* induced the development of imposex to the same degree as did TBT (1 μg/g). In considering why contradictory results were obtained by Oehlmann et al. (2007) and Castro et al. (2007), we need to carefully examine the experimental methodologies used by each scientific group. For example, injection of 9CRA in snails should be done under shaded conditions because 9CRA is easily photodegraded.

Although Castro et al. (2007) reported that imposex in *N. lapillus* could be mediated by RXR, their experimental data showed that the level of expression of the RXR gene was highest in the gonads, unlike in the results in *T. clavigera* reported by Horiguchi et al. (2007). Castro et al. (2007) discussed the mechanism of induction of imposex by organotins in gastropods on the basis of a scenario that integrated the interaction between three cascades (retinoic, neuroendocrine, and steroid), although the physiological role of AR as well as ER in mollusks remains in doubt because no scientific report has described the successful cloning of an AR and a functional ER from the tissues of gastropods.

A recent study has provided further evidence of RXR involvement, through the cloning of RXR in the mud snail *I. obsoleta* (Sternberg et al. 2008). In light of this cloning, Sternberg et al. (2010) reviewed the mechanism of induction of imposex in prosobranch gastropods and suggested that there was environmental–endocrine control of reproductive maturation in gastropods. However, Sternberg et al. (2008) did not measure RXR gene expression in the presumptive penis-forming area or penis in females; instead, they measured it in the "the gonad-viscera complex"—probably a combination of gonadal and digestive gland tissues—although the development of imposex essentially or primarily involves the differentiation and growth of male genitalia (e.g., the penis and vas deferens). Sternberg et al. (2008) also discussed synchronized expression of RXR mRNA with recrudescence of the reproductive tract (primarily the gonad, but their samples might also have included the digestive gland) in *I. obsoleta*. Thus, their discussion seems to have confused the development of imposex (principally the differentiation and growth of the penis and vas deferens) with events of the male reproductive cycle, such as gonadal maturation and regression.

Urushitani et al. (2011) reported two isoforms of RXR cDNAs, RXR isoform 1 (*TcRXR-1*) and RXR isoform 2 (*TcRXR-2*), in the rock shell *Thais clavigera*. The deduced amino acid sequences of *TcRXR-1* and *TcRXR-2* are highly homologous with those of other gastropods. These TcRXR isoforms displayed 9CRA-dependent activation of transcription in a reporter gene assay using COS-1 cells. The transcriptional activity of TcRXR-2, the encoded protein of which has

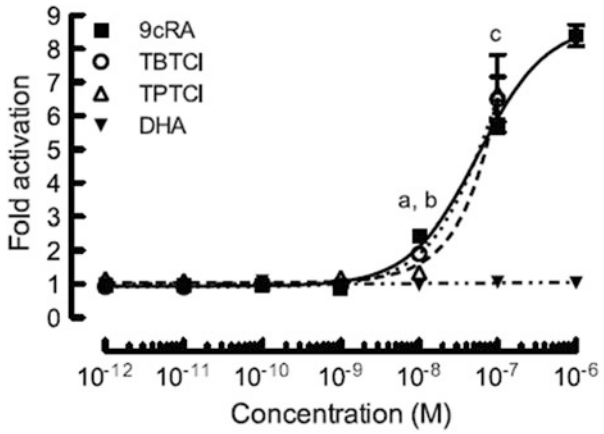

Fig. 9.8 Dose–response profiles of the activities of TcRXR-1 following exposure to various chemicals. COS-1 cells were incubated for 40–42 h with or without increasing concentrations of various ligands. *9cRA* 9-*cis* retinoic acid, *DHA* *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid, *TBTCl* tributyltin chloride, *TPTCl* triphenyltin chloride. Each point represents the mean of triplicate experiments. *Vertical bars* present ± S.D. [a]Significant difference between 9cRA and vehicle-treated control, [b]significant difference between TBTCl and vehicle-treated control, [c]significant difference between TPTCl and vehicle-treated control; $P < 0.01$ by ANOVA with Dunnett's post hoc test (Urushitani et al. 2011)

five additional amino acids in the T-box of the C domain, was significantly lower than that of TcRXR-1. The EC_{50} values were 1.1×10^{-7} M (95 % confidence intervals: 7.7×10^{-8} M to 1.6×10^{-7} M) in human RXR α, 6.4×10^{-8} M (5.3 to 7.9×10^{-8} M) in TcRXR-1, and 1.2×10^{-7} M (6.5×10^{-8} M to 2.2×10^{-7} M) in TcRXR-2. The induction of transcriptional activity of TcRXR-1 by 9CRA, docosahexaenoic acid (DHA), and the chemicals TBTCl and TPhTCl was analyzed, and the activity was induced by 10^{-8} M 9CRA, 10^{-8} M TBTCl, and 10^{-7} M TPhTCl but was unchanged by DHA (Fig. 9.8) (Urushitani et al. 2011). Induction of transcriptional activity of TcRXR-1 is caused by 9CRA, TBTCl, and TPhTCl, but not by DHA. It has been reported that 9CRA is a specific ligand of human RXR; however, 9CRA has not been detected in mammalian tissues (Ulven et al. 2001; Werner and DeLuca 2001), and in fiddler crabs and mollusks, its concentration is lower than those of other retinols (Dmetrichuk et al. 2008; Hopkins et al. 2008). DHA is a natural ligand in mammals, although it activates RXRs at much higher concentrations than 9CRA (de Urquiza et al. 2000). Urushitani et al. (2011) used a concentration of DHA (10^{-6} M) that was lower than that required for the induction of transcriptional activity of RXR in the mollusk *Biomphalaria glabrata* (Bouton et al. 2005), but they could not measure the transcriptional activity of TcRXR-1 induced by 10^{-4} M DHA because of toxicity. Similar results have been reported using the daphnid RXR, where DHA was also shown not to be a ligand in a reporter assay (Wang and LeBlanc 2009).

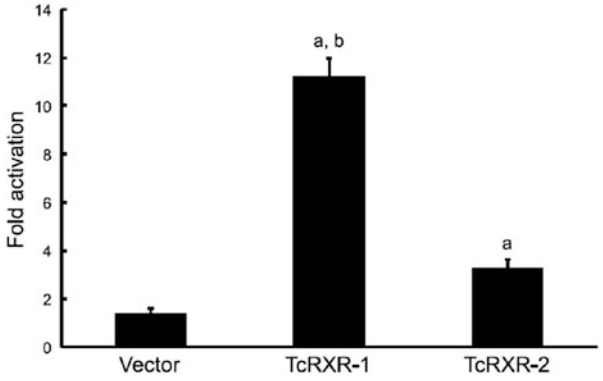

Fig. 9.9 Analysis of transcriptional activity of TcRXR isoforms. COS-1 cells were seeded into 12-well plates and then transiently transfected with the retinoid X responsible element (RXRE)-reporter vector and the *TcRXR* isoforms–fused expression vectors. COS-1 cells were incubated with or without 10^{-6} M of 9-*cis* retinoic acid (9cRA) for 40–42 h. Transcriptional activities of empty expression vector (Vector), expression vector containing *TcRXR-1* (TcRXR-1), and expression vector containing *TcRXR-2* (TcRXR-2). Data represent means ± S.D. ($n = 3$). [a]Significantly different from control, [b]significantly different from TcRXR-2; both $P < 0.01$ by Student's *t*-test (Urushitani et al. 2011)

Decreases of the transcriptional activity by TcRXR-1 were observed when more than equal amount of *TcRXR-2* fused expression vector was existed in a co-transfection assay. Overexpression of TcRXR-2 led to lower transcriptional activity than did overexpression of TcRXR-1 (Fig. 9.9). The *TcRXR* isoforms were co-transfected to investigate the effect of TcRXR-2 on the transcriptional activity of TcRXR-1 (Fig. 9.10). In these assays, significant decreases in the transcriptional activity of TcRXR-1 were observed in the presence of 0.1 or 0.5 μg *TcRXR-2*-fused expression vector (Fig. 9.10). It may imply that this difference has a functional basis in the regulation of the molluscan endocrine system (Urushitani et al. 2011). Urushitani et al. (2011) found that co-transfection of more than equal amounts of *TcRXR-2*-fused expression vector resulted in a decrease in the transcriptional activity of TcRXR-1. In mature males of *N. lapillus*, *T. clavigera*, and *I. obsoleta*, penis length varies seasonally at locations in the field that are lightly contaminated by organotins (Galante-Oliveira et al. 2010; Horiguchi et al. 2008b; Oberdörster et al. 2005). Expression of RXR mRNAs also changes seasonally in males of *T. clavigera* from lightly contaminated sites, although expression of *TcRXR-1* and *TcRXR-2* was not measured separately (Horiguchi et al. 2008b). In *I. obsoleta*, seasonal changes in RXR mRNA levels have also been reported (Sternberg et al. 2008). It was also previously reported that male penis length in *T. clavigera* decreased in the laboratory after the spawning season (Horiguchi et al. 2010a). From these findings, the interaction between TcRXR-1 and TcRXR-2 could contribute to the seasonal change in penis length in males of *T. clavigera*. Specifically, it appears that differences in the expression of *TcRXR-2* contribute to seasonal differences in penis length of male rock shells

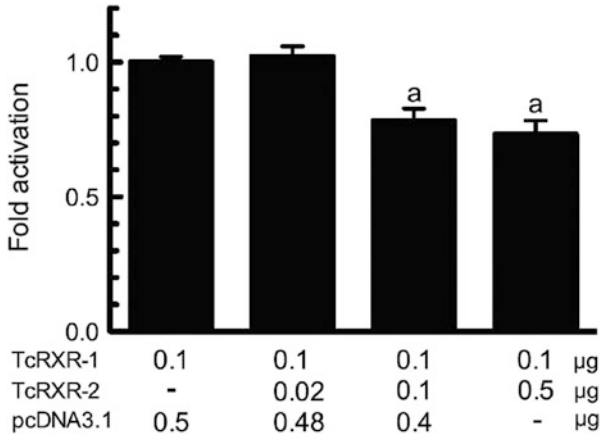

Fig. 9.10 Alterations of TcRXR isoforms' transcriptional activities. Decreases of the transcriptional activity by TcRXR-1 were observed when the amount of *TcRXR-2* was increased by more than 0.1 μg. The fold transactivation was compared with the activity of each vehicle-treated control and then normalized by the mean of the transcriptional activity for *TcRXR-1* (0.1 μg) and the pcDNA3.1 expression vector (0.5 μg). Results of triplicate luciferase assays are shown as means ± S.D. Statistically significant differences compared with the transcriptional activity of *TcRXR-1* (0.1 μg) and pcDNA3.1 expression vector (0.5 μg) in transfected cells were determined by ANOVA with Dunnett's post hoc test ([a], $P < 0.05$) (Urushitani et al. 2011)

together with possible changes in endogenous retinoids or natural ligands of TcRXRs.

These findings suggest that retinoic acids could play an important role in the development of male genitalia and their components and that RXR isoforms might underlie a novel mechanism regulating genes in gastropods. Actually, orthologs of enzymes for retinoic acid synthesis, as well as RAR sequences, have been reported in the mollusk *Lottia gigantea* (Albalat and Cañestro 2009). In vertebrates, retinoic acids and derivatives are involved in cell proliferation and differentiation, organ homeostasis, and regeneration of tissues and organs (see reviews: Albalat 2009; Chambon 1996; De Luca 1991; Kastner and Chan 2001; Kastner et al. 1995; Mark et al. 2009). All-*trans*- and 9-*cis*-RA have been detected in the mollusk *L. stagnalis* (Dmetrichuk et al. 2008). Urushitani et al. (2013) isolated a retinoic acid receptor (RAR)-like cDNA (*TcRAR*) in the rock shell, *T. clavigera*, as a candidate partner for RXR, and examined the transcriptional activity of the TcRAR protein by using all-*trans* retinoic acid (ATRA). However, no ligand-dependent transactivation by this protein was observed. Urushitani et al. (2013) also examined the transcriptional activity of the TcRAR-ligand-binding domain fused with the GAL4-DNA-binding domain by using retinoic acids, retinol, and organotins and again saw no noteworthy transcriptional induction by these chemicals. The use of a mammalian two-hybrid assay to assess the interaction of the TcRAR protein with the TcRXR isoforms suggested that TcRAR might form a heterodimer with the RXR isoforms.

The transcriptional activity of domain-swapped TcRAR chimeric proteins (the A/B domain of TcRAR combined with the D–F domain of human RARα) was also examined and found to be ATRA dependent (Urushitani et al. 2013). These results suggest that TcRAR is not activated by retinoic acids but can form a heterodimer with TcRXR isoforms.

Although 9CRA is a natural ligand for RXRs in vertebrates (Heyman et al. 1992; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1992; Levin et al. 1992), whether the same is true for RXRs in *T. clavigera* or other gastropods is not clear because 9CRA is difficult to detect in vivo (Horton and Maden 1995). The natural ligand for gastropod RXR may be some compound other than 9CRA. Identification of the natural ligand for gastropod RXR is required for further analysis of the mechanism of imposex induction by organotins. Dmetrichuk et al. (2008) recently detected ATRA and 9CRA in the central nervous systems of adults of the pulmonate gastropod *L. stagnalis* by high-performance liquid chromatography–mass spectrometry. Because ATRA and 9CRA were detected in the tissues of *L. stagnalis*, this species likely also has metabolic enzymes for synthesizing or transforming retinoic acids (RAs). Whether gastropods can synthesize 9CRA, ATRA, or both from β carotene must also be determined, in terms of the enzymes involved in the synthesis and metabolism of RAs (e.g., Raldh2, Cyp26). Because DHA also acts as a ligand for RXR in the brain of the fetal mouse (Urquiza et al. 2000), the possibility that DHA is a natural ligand for gastropod RXR should be examined, despite of observations of no transcriptional activity of TcRXR-1 induced by DHA (Urushitani et al. 2011).

In addition to identifying the natural ligand for gastropod RXR, we must also examine the binding and activation properties of the ligand with respect to RXR and determine whether RXR forms homodimers, homotetramers, or heterodimers with other nuclear receptors. Activation of RXR–peroxisome proliferator-activated receptor (PPAR) heterodimers by organotin compounds promotes adipocyte differentiation (Grün and Blumberg 2006; Grün et al. 2006; Kanayama et al. 2005), and the binding and activation properties of various organotins with RXR–PPAR heterodimers have been analyzed by le Maire et al. (2009). Although gastropod imposex can be induced by very low concentrations (~1 ng/L) of TBT and/or TPhT (Bryan et al. 1988; Gibbs et al. 1987; Horiguchi et al. 1994, 1995, 1997), the mechanism of how such nanomolar levels of TBT and/or TPhT can activate gastropod RXR remains to be clarified. Meanwhile, Pascoal et al. (2013) suggested additional involvement of putative PPAR pathways. Application of rosiglitazone, a well-known vertebrate PPAR γ ligand, to dogwhelks (*N. lapillus*) induced imposex in the absence of TBT. Therefore, Pascoal et al. (2013) has pointed out that it is likely also to be driven by PPAR-responsive pathways, while TBT-induced imposex is linked to the induction of many genes and has a complex phenotype.

Overall, anyway, these findings suggest that RXR is involved in the induction of male-type genitalia (penis and vas deferens) in normal male and organotin-exposed female gastropods.

We also should note that there are several steps in the development of imposex induced by certain organotin compounds, such as TBT and TPhT, in gastropods. At the initial stage of imposex development, differentiation and growth of male-type genitalia (i.e., penis and vas deferens) occur. This leads to ovarian spermatogenesis at the severely affected stage, involving oviduct blockage due to the proliferation of epidermal tissues surrounding the vas deferens (Gibbs and Bryan 1986; Gibbs et al. 1988, 1990, 1991; Horiguchi 2000; Horiguchi and Shimizu 1992; Horiguchi et al. 1994, 2000, 2002, 2005, 2006; Oehlmann et al. 1996; Schulte-Oehlmann et al. 1997). The author considers that the true mechanism of action of TBT or TPhT in the development of imposex in gastropods must encompass an explanation of each of the characteristics mentioned above (Horiguchi 2000).

It appears that the physiological regulatory system of reproduction may differ between gastropods and vertebrates. Further studies involving histological, immunohistochemical, biochemical, and molecular biological techniques are needed to elucidate the basic endocrinology and complete mechanism of action of TBT or TPhT in the development of imposex in gastropods. This may involve the identification of a natural ligand and target gene(s) of the gastropod RXR as well as the determination of when and how the differentiation and proliferation of stem cells of the penis and vas deferens in female snails are initiated and promoted, thus leading to epidermal differentiation and proliferation and the development of these organs. Morphogenetic factors could be involved in the formation of the curved penis and vas deferens. It is also possible that other factors, such as certain neuropeptides induced in the head ganglia by exposure to organotins, are associated with the RXR gene-mediated development of imposex if these factors are induced downstream of the RXR cascade (Morishita et al. 2006).

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Chapter 10
Effects of Organotins in Mollusk's Lipids

Denise Fernandes and Cinta Porte

Abstract Organotin compounds have been shown to alter lipid homeostasis and trigger adipocyte differentiation and a predisposition to obesity in vertebrates by binding to nuclear receptors (e.g., the retionid X receptor, RXR). RXR is highly conserved in evolution, and RXR homologues with high ligand affinity for tributyltin and triphenyltin have been cloned from gastropods. Thus, significant alteration of lipids as a consequence of exposure to organotin compounds is likely to occur also in mollusks. This chapter reviews the still fragmentary knowledge on the induction of lipid disturbance and membrane toxicity by organotin compounds and the potential link between those lipid alterations and the occurrence of imposex and/or altered levels of esterified steroids. Finally, the chapter emphasizes the need to characterize the richness of mollusk's lipids (e.g., cardiolipin, plasmalogens, and many others) with the new available technologies to better understand the toxicity of organotin compounds but also other pollutants/stressors, in this very diverse animal group.

Keywords Mollusks • Lipids • Obesogens • Organotin compounds • Endocrine alteration

10.1 Lipids in Aquatic Organisms

Lipids are essential materials for the formation of cell and tissue membranes; they also store metabolic energy and function as bioactive molecules. Moreover, they are extremely important in the physiology and reproductive processes of aquatic organisms and reflect the special biochemical and ecological conditions of the aquatic environment (Abad et al. 1995; Pazos et al. 1997). Mollusks produce a variety of lipids that contain essential fatty acids and sterols, among them triacylglycerols (TAG), and wax esters play an important role as a reserve of fatty acids that are destined either to oxidation to provide energy (ATP) or to be

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incorporated into phospholipids, which are the building blocks for the membrane lipid bilayer. Fatty acids provide the hydrophobic interior of all cell membranes, forming an impermeable barrier to water and polar molecules and separating the cell contents from the extracellular medium. The physical properties of the membrane are determined by the fatty acid components of individual lipids and their interaction with sterols and proteins (Bergé and Barnathan 2005). In addition to the major polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6 n-3) acids, a great number of various fatty acids occur in marine organisms, e.g. saturated, mono- and di-unsaturated, branched, halogenated, hydroxylated, methoxylated, and non-methylene-interrupted dienoic fatty acids (NMID).

NMIDs occur in significant amounts in the membrane of marine bivalves. They are PUFAs with a distinctive structure where the pairs of double bonds in the fatty acid are separated by a number of methylene –CH2– groups, rather than a single –CH2– as in regular PUFAs. Because of this structure, they are less susceptible to lipid peroxidation than other PUFAs. Another feature of the membrane lipid composition of marine bivalves is that they have significant amounts of plasmalogens (Kraffe et al. 2004). These membrane lipids differ from regular phospholipids in the carbon chain in the sn-1 position that is linked to the glycerol backbone by a vinyl ether linkage instead of the regular ester linkage. Plasmalogens are reported to act as endogenous membrane antioxidants (Hulbert et al. 2014).

Lipids have been reported to play a key role in mollusk reproduction and to support energy requirements for priority functions (i.e., basal metabolism, growth) in periods of food scarcity when carbohydrate levels (the main energetic reserve in mollusks) are depleted (Abad et al. 1995). They are usually mobilized for gametogenesis and constitute a very important energy reserve in the oocytes, which assures future viability of the larvae (Gallager et al. 1986). EPA and DHA have been reported to be essential for optimal growth in some species of juvenile bivalves. Correlations between the reproductive state of individuals and the lipid content of the gonads have been reported for several bivalve and prosobranch species (Morais et al. 2003). Notwithstanding, lipids not only provide energy, they also facilitate the absorption of fat-soluble vitamins (vitamins A, D, E, and K) and play an important role in the production and regulation of eicosanoids (Bergé and Barnathan 2005).

Therefore, an interference of pollutants with the synthesis, metabolism, and mobilization of lipids may consequently alter energy storage and reproduction in mollusks, with potential consequences at higher biological organization levels (e.g., population).

10.2 Organotin Compounds as "Obesogens"

Recent findings have highlighted that exposure to organotin compounds and other environmental chemicals at rather low concentrations alter lipid homeostasis and trigger adipocyte differentiation and a predisposition to obesity in vertebrates (Grün

and Blumberg 2009; Grün 2014). Thus, tributyltin (TBT) and triphenyltin (TPT) promote adipogenesis by binding to the retinoid X receptor (RXRα) and peroxisome proliferator-activated receptor (PPARγ) and consequently induce the differentiation of murine preadipocyte cells (3 T3-L1) to adipocytes (Inadera and Shimomura 2005; Kanayama et al. 2005). Furthermore, in utero exposure to TBT led to liver steatosis, increased lipid accumulation and maturation of adipocytes in mouse models, and induced ectopic adipocyte formation in *Xenopus laevis* (Grün et al. 2006; Iguchi et al. 2007).

TBT and TPT bind to human RXR with similar affinity as 9-*cis* retinoic acid (9-*cis* RA; $EC_{50} = 3$ to 10 nM) and to PPARγ with higher affinity than the synthetic ligand troglitazone ($EC_{50} = 20$ nM vs. 1000 nM) (Grün et al. 2006). RXR, which are highly conserved in evolution, form heterodimers with orphan nuclear receptors (whose endogenous ligands are unknown) such as peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), and pregnane X receptor (PXR) (Szanto et al. 2004). These orphan receptors are lipid sensors as they get activated by lipid molecules and play an important role in lipid homeostasis. Thus, knocking out RXR in mice disturbed lipid metabolism functions controlled by PPARα, PPARγ, LXRα, PXR, and FXR (Szanto et al. 2004), showing the importance of RXR in lipid homeostasis.

RXR homologues have been cloned from the gastropods *Thais clavigera* (Nishikawa et al. 2004), *Nucella lapillus* (Castro et al. 2007), and *Biomphalaria glabrata* (Bouton et al. 2005). All three RXR homologues showed high similarity with vertebrate RXR, and 9-*cis* RA was a high affinity ligand, an indication that retinoid signaling pathways may exist in these gastropod species. Moreover, the injection of 9-*cis* RA into females of *T. clavigera* and *N. lapillus* induced the development of imposex, leading to an increase in penis length and vas deferens similar to that induced by TBT and/or TPT in these species (Nishikawa et al. 2004; Castro et al. 2007). Receptors such as PPARs appear to have emerged later in the evolution of the nuclear receptor family (Thornton 2003) and until recently have only been identified in vertebrates. However, Vogeler et al. (2014) reported the sequence of a PPAR orthologue in the mollusk *Crassostrea gigas*. Thus, a lipid regulation mechanism possibly mediated by RXR or RXR/PPAR heterodimer in gastropods deserves further investigation.

Actually, an increasing number of studies have reported the ability of TBT to induce lipid accumulation in aquatic vertebrates. Thus, an increase in whole-body lipid content along with a raise of lipid-related plasma parameters (triacylglycerols, cholesterol and lipase) was observed in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) exposed to TBT (Meador et al. 2011). TBT is also reported to induce lipid accumulation in zebrafish larvae (Tingaud-Sequeira et al. 2011; Riu et al. 2014). However, despite of these evidences, studies investigating the ability of organotin compounds to disrupt lipid metabolism in mollusks, which are often exposed to significant concentrations of these compounds, are still scarce.

10.3 Evidences of Lipid Disturbance in Mollusks Following Organotin Exposure

Several exposure experiments have evidenced changes in mollusk's lipids following organotin exposure. Thus, 1 week exposure to TPT had a significant effect on lipid content, fatty acid content, and fatty acid metabolism in the digestive gland/gonad complex of females of *Marisa cornuarietis* exposed to concentrations of 30–500 ng/L TPT as Sn for 7 days, whereas males demonstrated very few significant alterations. Percentage of lipids, total fatty acid content, carbon chain length, and unsaturation degree of fatty acids all decreased significantly in TPT exposed females. In parallel, the activity of peroxisomal acyl-CoA oxidase (AOX), the first and rate-limiting enzyme of β-oxidation responsible for the breakdown of C14–C18 and $C > 20$ fatty acids, was significantly induced, which supported the observed decrease in fatty acid content (Lyssimachou et al. 2009). The observed decrease in fatty acid unsaturation degree was indicative of oxidative damage caused by TBT exposure, as the higher the unsaturation index of a fatty acid molecule the higher its susceptibility to peroxidation (Hulbert et al. 2007). Moreover, the higher sensitivity of females to lipid disruption in comparison to males requires further research as well as the potential link between lipid disruption and imposex development. In other words, one may wonder whether lipid changes are among the first molecular responses in a cascade of events that finally lead to the development of imposex. The fact that total lipid content and almost all fatty acid groups decreased in such a short exposure period in exposed females is of special concern, given the multifunctional role of fatty acids in cell structure and function, energy metabolism and storage, bioactive signaling, and synthesis of various compounds involved in physiological regulation (e.g., steroids, eicosanoids, etc.) (Benatti et al. 2004).

Additionally, a relative increase of arachidonic acid was observed after 7 days exposure to 125 and 500 ng/L TPT-Sn in females of *Marisa cornuarietis*, but not in males (Lyssimachou et al. 2009). Arachidonic acid is required in cell signaling and as a substrate for eicosanoids synthesis but also acts as a regulator of lipogenesis in vertebrates, where it inhibits de novo lipogenesis in the liver and changes the hepatic fatty acid profile via reduced desaturases activity (Lamaziere et al. 2013). Thus, a link between the observed increase of arachidonic acid in females exposed to 125 and 500 ng/L TPT-Sn and the decrease in the content of lipids, fatty acids, and unsaturation degree is suggested.

On the other hand, long-term exposure (100 days) of the gastropod *M. cornuarietis* to TBT leads to a significant increase in the percentage of total lipids and total fatty acid content (two- to three fold) in the digestive gland/gonad complex of females exposed to 500 ng/L TBT-Sn. No such lipogenic effect of TBT was observed after exposure to lower TBT concentrations nor in males (Janer et al. 2007). One may see a certain parallelism in the response to TBT of both mollusks and vertebrates (Grün 2014); however, the lack of knowledge on the physiological role of RXR in mollusks and the fact that the effect was only observed at rather high concentrations of TBT are a clear limitation to extrapolate vertebrate to invertebrate

data. Additionally, the reason behind the higher sensitivity of females to TBT (or TPT) exposure in comparison to males is a key issue that requires further research.

Besides the effects on total lipid and fatty acid levels, long-term exposure to TBT altered fatty acid profiles in *M. cornuarietis* exposed to 125 and 500 ng TBT-Sn/L; the overall change occurred both in terms of unsaturation degree and carbon chain length and was similar to the one detected after 1 week exposure to TPT (Lyssimachou et al. 2009). Thus, females exposed to TBT showed a relative increase of monoenic fatty acids and a relative decrease of polyunsaturated fatty acids. Similar changes were observed in males; however, the magnitude of the response to TBT was rather low, and most of the observed changes were not statistically significant (Janer et al. 2007).

Similarly, TBT disturbed lipid homeostasis in the filamentous fungus *Cunninghamella elegans*; it led to a decrease of phosphatidylethanolamine and phosphatidylserine but increased the levels of phosphatidic acid, phosphatidylinositol, and phosphatidylcholine (Bernat et al. 2014). These changes were observed together with a decrease in the overall unsaturation of phospholipids. However, a detailed study on the disturbance of different lipid classes by TBT or other contaminants (e.g., phosphatidylethanolamines, phosphatidylserines, phosphatidylcholines, plasmalogens, di- and triacylglycerols, among others) has not been carried out in mollusks so far.

Recently, Titley-O'Neal et al. (2013) by applying gene set enrichment analysis revealed that transcripts involved in the biological processes of general metabolism, immune system, lipid metabolism, and stress were affected in gonads of the gastropod *Strombus gigas* collected in areas with high boating activity in the British Virgin Islands. Lipid-related processes, such as lipid and fatty acid biosynthetic process, arachidonic acid metabolism, and prostaglandins/thromboxane synthesis, were identified by gene set enrichment analysis as processes affected at TBT-polluted sites, although the authors could not directly link changes at the transcriptomic level to high TBT levels. The analysis also revealed a decrease in expression of genes related to reproduction in organisms from one of the TBT-polluted sites. Certainly, ecotoxicogenomics applied to laboratory exposure experiments (acute and chronic) and to field studies might be a powerful tool to investigate and characterize the mode of action of organotin compounds (and other pollutants) in aquatic organisms and particularly in mollusks.

10.4 Endocrine Alteration

In mollusks, the esterification of steroids with fatty acids appears to be an important regulation mechanism of endogenous steroid levels (Gooding and LeBlanc 2001; Janer et al. 2005). Esterification of steroids occurs upon acyl-CoA moieties, whose activation depends on the concentration of the corresponding fatty acids (Hochberg 1998). Estradiol esters formation was achieved using the fatty acid moieties C16:0,

C16:1, C18:0, C18:1, C18:2, and C20:4 in the oyster *Crassostrea virginica* (Janer et al. 2004), whereas exposure of mussels *Mytilus edulis* to estradiol resulted in the formation of estradiol esters with C16:0, C16:1, and C16:2 fatty acid moieties (Labadie et al. 2007). Most of the endogenous estradiol and testosterone detected in the digestive gland/gonad complex of *M. cornuarietis* are in the esterified form (Janer et al. 2006). Interestingly, 1 week exposure to TPT resulted in a significant increase in esterified testosterone levels (60–85 %) and a concomitant decrease in esterified estradiol (50–84 %) in females (Lyssimachou et al. 2008). In contrast, fatty acid conjugates of both, testosterone and of estradiol, were considerably reduced in female of *M. cornuarietis* exposed to TBT for 100 days, but no effect was observed in exposed males (Janer et al. 2006). Higher susceptibility of females than males to lipid alterations following organotin exposure was detected in both studies. Thus, the hypothesis that changes in fatty acid availability might trigger alterations in endogenous steroid levels is a challenging one.

So far, the connection between the reported changes in lipid levels and the induction of imposex can only be speculated, as both TBT and methyltestosterone (MT) induced imposex in *M. cornuarietis* after 150 days exposure (Janer et al. 2006). However, exposure to MT did not induce significant effects on lipid levels nor on the fatty acid profile.

10.5 Membrane Toxicity

Organotin access to biomembranes is facilitated by the organic moiety lipophilicity, and at high concentrations (mM range), they disturb membrane structure and alter membrane permeability and fluidity and cell signaling (Ortiz et al. 2005; Bonarska-Kujawa et al. 2012). Organotins, especially those with bulky alkyl or aryl groups, change the degree of hydration of lipid phosphate and carbonyl groups, alter lipid packing and fluidity, and disrupt protein organization within phospholipid membranes. They promote lipid peroxidation by formation of phospholipids complexes through dative Sn-O-P bonds, which damage cell membranes and stimulate lipid signaling pathways (for a review, see Grün 2014). Thus, exposure of ovaries of the tunicate *Ciona intestinalis* to 10^{-5} M TBT for 5 h caused a reduction in total lipids and triglycerides but an increase in phospholipids and PUFAs, including highly unsaturated fatty acids (HUFAs) and arachidonic acid (Puccia et al. 2005). Phospholipids and PUFAs are involved in maintaining membrane fluidity, and the authors suggested that this increase is an adaptive mechanism to TBT toxicity. Earlier on Masia et al. (1998) reported an increased resistance to the toxic action of TBT of *Saccharomyces cerevisiae* when the culture medium was supplemented with PUFAs.

In contrast, a decrease in the total and n-3 PUFAs, especially 22:6 n-3 (DHA), and an activation of the oligomycin-sensitive Mg-ATPase were detected in gill mitochondrial membranes from cultivated mussels *Mytilus galloprovincialis* exposed to 1.0 μg/L TBT for 120 h (Fiorini et al. 2012). A decrease in mitochondrial membrane polarity was also observed at both 0.5 and 1.0 μg/L TBT (Fiorini

et al. 2012). Fatty acid composition of mussel cardiolipin, the phospholipid class specifically located in mitochondrial membranes, is dominated by DHA fatty acid, which exceeds 70 % of the total fatty acids (Kraffe et al. 2008). The lower content of DHA in TBT-exposed mussel gills may favor the catalytic activity of oligomycin-sensitive Mg-ATPase (Fiorini et al. 2012).

10.6 Future Perspectives

Lipids are recognized as extremely diversified molecules, and currently, nearly 10,000 different structures of lipids have been stored in the most comprehensive lipid structure database (LIPID MAPS, http://www.lipidmaps.org). Given the large amount of lipid classes and their complex structures, it is an enormous challenge to fully analyze them. Thin-layer chromatography (TLC) and gas chromatography (GC) coupled to mass spectrometry were some of the most frequently used analytical methods to determine mollusk's lipids. However, liquid chromatography coupled to a combination of different mass analyzers and, particularly, the development of high-resolution mass spectrometry, together with the extensive use of electrospray ionization (ESI), have made possible to resolve complex lipidomes and to identify and quantify hundreds of molecular lipid species (Ivanova et al. 2009). Indeed, polar lipids, such as phospholipids and sphingolipids, and even nonpolar lipids, such as diacylglycerols (DAGs) and TAGs, have been successfully measured (Wenk 2005). Although lipid analysis is still full of challenges, lipidomic studies have strongly emerged in the last 10 years in the area of biochemical research for disease biomarker discovery, drug development, and drug safety assessment, among others.

This is an interdisciplinary field, which needs expertise on analytical chemistry, data handling and data preprocessing, data mining, statistical analysis, biomarker identification, and interpretation of biochemical pathways and that, in the near future, will bring a new perspective to the study of the effects of pollutants/stressors at the cellular and individual level and certainly on the effects of organotin compounds in mollusks.

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Chapter 11
Reproductive Organ Development in the Ivory Shell *Babylonia japonica* and the Rock Shell *Thais clavigera*

Toshihiro Horiguchi

Abstract Results of histological examination of normal differentiation and development of the genital tract and gonad in the ivory shell *Babylonia japonica* (Buccinidae) are described. The formation of male-type genitalia (penis and vas deferens) in imposex-exhibiting females seems to mimic the normal development of male genitalia in prosobranch gastropods, on the basis of observations using a wild-caught 2-year-old specimen and laboratory-reared juveniles aged 0–24 months. Gonad differentiation was unclear before age 14 months but progressed after 16 months. Both sexes had a complete genital tract and mature gonads at 20 months. However, differentiation and development occurred earlier in females than in males. Development of the genital tract preceded gonad differentiation. Vas deferens morphogenesis in males resembled that in imposex-exhibiting females. Histological examination of the development of male genitalia in imposex-exhibiting female rock shells, *Thais clavigera* (Muricidae), using specimens from a wild population and tributyltin (TBT)-exposed females in the laboratory, allowed observation of a variety of vas deferens morphogenesis patterns. Taking into consideration observed results both from wild female specimens and from TBT-exposed females in the laboratory, the vas deferens sequence (VDS) index for *T. clavigera* has been proposed as VDS 1–6, which is a little different from that for *Nucella lapillus*. Comparison of the differentiation and development of male genitalia in normal males and imposex-exhibiting females among gastropod species implies it does not seem to be strictly regulated: relatively large variation in the differentiation and development of genitalia could occur among individuals, as well as among species of prosobranch gastropods.

Keywords Imposex • Ivory shell (*Babylonia japonica*) • Rock shell (*Thais clavigera*) • Vas deferens • Penis • Gonad • Differentiation • Morphogenesis • Tributyltin (TBT) • Triphenyltin (TPhT) • Retinoid X receptor (RXR)

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Abbreviations

AFS Convention	the International Convention on the Control of Harmful Anti-fouling Systems on Ships
DMSO	dimethyl sulfoxide
EDCs	endocrine-disrupting chemicals
H&E	hematoxylin and eosin
IMO	International Maritime Organization
OECD	Organization for Economic Co-operation and Development
9cRA	9-*cis* retinoic acid
RXR	retinoid X receptor
TBT	tributyltin
TBTCl	tributyltin chloride
TPhT	triphenyltin
VDS index	vas deferens sequence index

11.1 Introduction

Masculinized female gastropod molluscs were first reported by Blaber (1970), who described a penis-like outgrowth behind the right tentacle in spent females of the dog whelk, *Nucella lapillus*, around Plymouth, UK. The term "imposex," however, meaning "imposed sexual organs," was defined by Smith (1971) to describe the syndrome of superimposition of male genitalia, such as a penis and vas deferens, on female prosobranch gastropods. Currently, imposex is thought to be an irreversible syndrome (Bryan et al. 1986). In severe cases of imposex, reproductive failure may occur, resulting in population decline or mass extinction (Gibbs and Bryan 1986, 1996). Imposex in many species is induced by tributyltin (TBT) and triphenyltin (TPhT) released from antifouling paints on ships and fishing nets (Bryan et al. 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1995, 1997). The use of TBT- or TPhT-based antifouling paints was addressed by the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention), which was adopted by the International Maritime Organization (IMO) on 5 October 2001 (IMO 2001). According to the AFS Convention, all ships are prohibited from applying or reapplying organotin compounds as antifouling biocides after 1 January 2003, and by 1 January 2008 ships either will not use organotin compounds as antifouling biocides or such antifouling systems will be covered with a coating that prevents leaching into the environment. It took longer than expected for the AFS Convention to be ratified by member states, but it finally came into effect on 17 September 2008 (http://www.imo.org/). Continued monitoring is needed to protect marine and aquatic ecosystems from organotin pollution and to allow the systems to recover from its impacts.

On the other hand, it is necessary to establish useful testing methods to properly evaluate and strictly regulate harmful chemical substances, such as

endocrine-disrupting chemicals (EDCs), to protect and conserve ecosystems. OECD has established several test guidelines for wildlife (i.e., fish and crustaceans), but not yet for mollusks (OECD 2016). Although fundamental knowledge of the biology, including reproductive physiology and endocrinology, of mollusks is essential to develop and establish a useful test method to properly evaluate harmful chemical substances, such as EDCs, for valid legislation, less is known about molluscan basic biology (see Chaps. 8 and 9). For example, it is doubtful whether gastropod mollusks have vertebrate-type steroids as sex hormones (see Chap. 9). Also, the differentiation of gonads and accessory sex organs (i.e., genital tracts) is still obscure in mollusks. The histological development of the vas deferens and penis in *N. lapillus* exhibiting imposex was reported by Gibbs and Bryan (1986) and Gibbs et al. (1987). Less is known about imposex development, in terms of the general development of the vas deferens and penis, in other species, although there are a few reports describing the general scheme of imposex development in prosobranch gastropods (Stroben et al. 1995). In the case of *Thais clavigera*, this process remained unclear until recently, because extensive contamination by organotins (TBT and TPhT) throughout Japan (Horiguchi et al. 1994) meant that only specimens in the severe stages of imposex had been observed there. Horiguchi et al. (2012a), by histological analysis of less severely exposed specimens from a wild population and females exposed to TBT in the laboratory, finally revealed the steps in the development of the vas deferens and penis in the imposex-exhibiting female rock shell *T. clavigera*. They observed a variety of patterns of vas deferens morphogenesis in wild females of *T. clavigera*. The immature vas deferens, however, was observed only beneath or behind the penis, and no vas deferens was observed close to the vaginal opening (i.e., vulva) of the capsule gland in TBT-exposed female *T. clavigera*. This observation differed from the vas deferens formation observed in wild female *T. clavigera*, as well as in female *N. lapillus* (Gibbs et al. 1987). Considering the observations of both wild and TBT-exposed females of *T. clavigera* in the laboratory, the vas deferens sequence (VDS) index for *T. clavigera* was proposed as VDS 1–6 (Horiguchi et al. 2012a). This VDS index differs from that of *N. lapillus* (Horiguchi et al. 2012a), especially in the initial developmental stages of imposex. Thus, it is possible that the processes responsible for development of the vas deferens and penis in imposex-exhibiting female gastropods differ among gastropod species.

In the ivory shell *Babylonia japonica*, histopathological and analytical chemical studies strongly suggest that reproductive failure in adult females accompanying imposex, possibly induced by TBT and TPhT, could have caused a marked decline in *B. japonica* populations; this decline might be one factor behind the decrease in the total catch of *B. japonica* in Japan since the 1970s (Horiguchi et al. 2006). Here, I discuss female and male *B. japonica* to discern normal differentiation and development of the genital tract and gonad (Horiguchi et al. 2014); this will be useful in determining whether the formation of male-type genitalia in imposex-exhibiting females mimics the normal development of male genitalia in prosobranch gastropods.

11.2 Development of Genitalia in *Babylonia japonica*

A 2-year-old male *Babylonia japonica* was used as a specimen from the wild population, which had been produced as seed at the hatchery of the Tottori Prefectural Sea Farming Association (TPSFA) and then released; it was captured in trawling nets in Miho Bay, Japan (35°27′50.37″ N, 133°21′01.84″ E) in July 2010. Laboratory-reared *B. japonica* juveniles were also used for the study. Adult *B. japonica* were collected in Miho Bay and landed at Yodoe, Tottori, Japan, as part of the commercial fishery, and then reared at the TPSFA hatchery for seed production in tanks with flow-through ambient-temperature natural seawater from the Sea of Japan. Egg capsules deposited by adults were rinsed with distilled water and then moved to other tanks with flow-through seawater. Larvae hatched after approximately 3 weeks. Veliger larvae settled toward the bottom of the tanks within several days of hatching. Settled juveniles were fed minced Antarctic krill (*Euphausia superba*) every day. Juvenile *B. japonica* were reared at TPSFA for 6 months (i.e., from age 0 to age 6 months). Juveniles older than 6 months were reared at the National Institute for Environmental Studies (NIES) in acrylic aquaria (length, 90 cm; width, 45 cm; height, 45 cm) containing a magnetic-drive circulating pump with the filtration media composed of coral sand and pieces. The aquaria were filled with deep seawater collected from a depth of about 400 m in Suruga Bay, Japan. Water temperature was maintained at 23 ± 1 °C by a water temperature controller. These juveniles were also fed Antarctic krill (*E. superba*) every day. Concentrations of TBT and TPhT in both seawater systems were below the detection limit. Laboratory-reared juveniles were kept in tanks at TPSFA or aquaria at NIES from July 2009 to August 2011. *B. japonica* juvenile samples were collected at 6, 12, 14, 16, 18, 20, and 24 months after hatching. The number of juveniles sampled was 6, 10, 10, 17, 15, 12, and 10, respectively (Horiguchi et al. 2014). After removal of the shell, whole soft tissues were fixed in Gendre's fluid (Horiguchi et al. 2002). Tissues, including the presumptive penis-forming area behind the right tentacle and the gonads, were removed and embedded in paraffin. Serial sections were prepared using a rotary microtome, stained with hematoxylin and eosin (H&E), and observed under a light microscope for histological examination of *B. japonica* genitalia and gonads (Horiguchi et al. 2014).

Table 11.1 summarizes the development of reproductive organs in the ivory shell *B. japonica* (Horiguchi et al. 2014).

In the 2-year-old wild-caught male specimen from Miho Bay, the germinal epithelium was composed mainly of spermatogonia and spermatocytes (Fig. 11.1B). It had a small penis behind the right tentacle, where the vas deferens and its opening were evident (Fig. 11.1A) (Horiguchi et al. 2014).

Regarding laboratory-reared juveniles, in females aged 6 months, the area of ovarian tissue was very narrow and immature, seeming to consist mainly of germinal cords. Several germinal cords were scattered under the epidermis near the digestive gland; these were different in length and not connected with each other. One of the germinal cords had a tubular form and was connected to the

Table 11.1 Summary of reproductive development in the ivory shell *Babylonia japonica*

Males	Age to initiate	Age to complete
Differentiation of testis	16 months	20 months
Vas deferens formation	14 months	20 months
Penis formation	16 months	20 months
Copulation for fertilization	N.C. (>24 months)	–
Females		
Differentiation of ovary	16 months	20 months
Development of vagina, bursa copulatrix, and capsule gland	14 months	20 months
Development of albumen and sperm-ingesting glands	16 months	20 months
Spawning of egg capsules	18 months	–

Horiguchi et al. (2014)
N.C. not confirmed

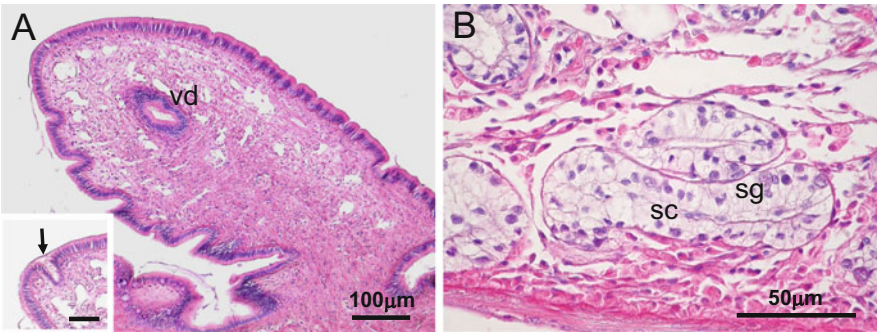

Fig. 11.1 Reproductive organs of a wild-caught male *Babylonia japonica*, age 2 years (Horiguchi et al. 2014). (**A**) Vas deferens (*vd*) in the penis. *Inset* (*lower left*) shows open vas deferens (*arrow*). *Bar* 50 μm. (**B**) Part of the testis containing spermatogonia (*sg*) and spermatocysts (*sc*) in the seminiferous tubules

immature oviduct. A few oogonia were observed (Fig. 11.2A). The vagina and capsule gland were not yet separated. Both vagina and capsule gland had single-layer cuboidal epithelium. Whether the vagina opened or not differed among individual specimens. In males aged 6 months, the testicular tissue area was very narrow and immature. It seemed to consist basically of germinal cords, some of which had partially formed into tubular shapes. A few spermatogonia were observed (Fig. 11.3A). Spermatogonia morphologically resembled oogonia, with polyhedral nuclei. No differentiation of the vas deferens was observed. The testicular duct consisted of transitional epithelium with scant and pale cytoplasm. The testicular duct morphologically resembled the oviduct (Fig. 11.4A). The terminus of the testicular duct, which was lined with a single layer of cuboidal cells, became a closed end near a shallow invagination of the epidermis near the kidney (Fig. 11.4B) (Horiguchi et al. 2014).

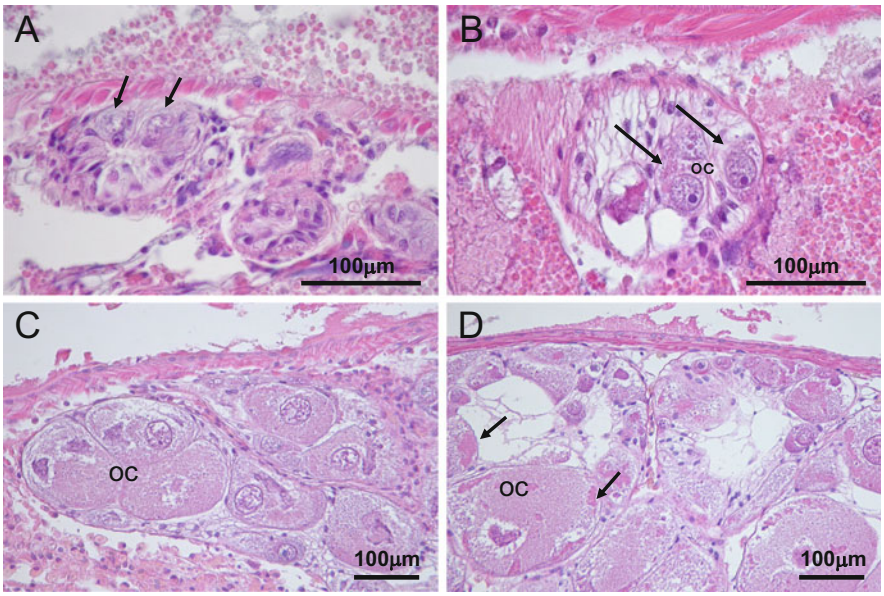

Fig. 11.2 Ovaries in female *Babylonia japonica*, age 6–16 months (Horiguchi et al. 2014). (**A**) Oogonia (*arrows*) in a 6-month-old specimen. (**B**) Oocytes at the early stage (*oc*) in a 12-month-old specimen (*arrows*). (**C**) Oocytes at the late stage in a 14-month-old specimen. (**D**) Mature oocytes containing eosinophilic granules (*arrows*) in a 16-month-old specimen

In females aged 12 months, the characteristics of the ovarian tissue were similar to those in 6-month-old females. The ovarian tissue area was narrow and immature close to the digestive gland. Many specimens showed only oogonia; rarely, immature oocytes as well as oogonia were observed (Fig. 11.2B). Germinal cords were lengthening and gradually connecting to each other. There was still no separation between the vagina and capsule gland (Fig. 11.5B). The vaginal opening still differed among individual specimens (Fig. 11.5A). One of the germinal cords formed a tube that was connected to the immature oviduct. Both vagina and capsule gland had single-layer cuboidal epithelium, but pseudo-stratification was observed in parts of the vagina and capsule gland. The oviduct had transitional epithelium with scant and pale cytoplasm (Fig. 11.5B). In males aged 12 months, the testicular tissue area was expanding compared with that in 6-month-old specimens. Germinal cords were tubular in form and were lengthening and thickening, although only spermatogonia were observed (Fig. 11.3B). The morphological difference between ovarian and testicular tissues was becoming clear. No differentiation of penis and vas deferens was observed. The testicular duct was similar to that in 6-month-old specimens (Horiguchi et al. 2014).

In females aged 14 months, the ovarian tissue was developing in a narrow area on the side opposite the digestive gland. Oocytes were observed in all specimens (Fig. 11.2C). One specimen contained oocytes that were almost mature, in which the ovarian tissue was developing well beyond the digestive gland. The vagina,

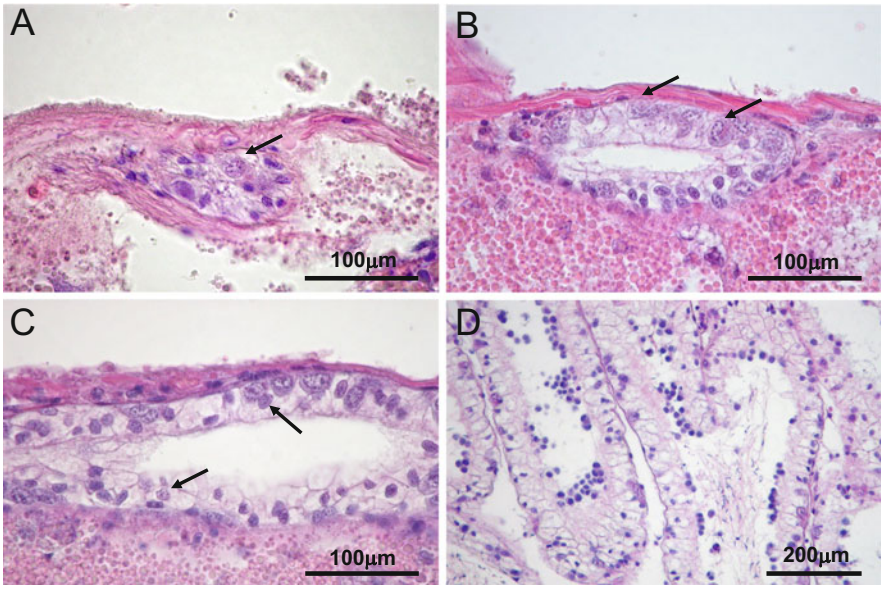

Fig. 11.3 Testes in male *Babylonia japonica*, age 6–16 months (Horiguchi et al. 2014). (**A**) Spermatogonium (*arrow*) in a 6-month-old specimen. (**B**) Multiple spermatogonia in a 12-month-old specimen (*arrows*). (**C**) Spermatocytes in a 14-month-old specimen (*arrows*). (**D**) All stages of spermatogenesis in a 16-month-old specimen

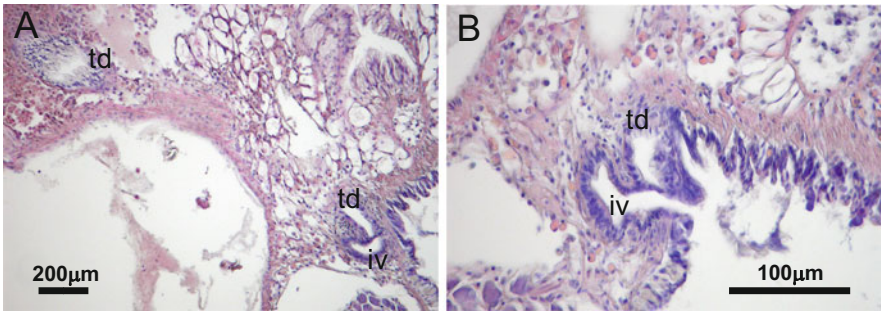

Fig. 11.4 Testicular duct near junction with the vas deferens in a male *Babylonia japonica*, age 6 months (Horiguchi et al. 2014). (**A**) Testicular duct (*td*) and invagination (*iv*) of epidermis near the kidney. (**B**) High-magnification view of invagination of the epidermis (*iv*)

bursa copulatrix, and capsule gland were also developing (Fig. 11.6.2). Differentiation of the sperm-ingesting gland as well as the albumen gland was recognized only in one female specimen (Fig. 11.6.1). The oviduct was connected to the lower genital duct. The vagina consisted of single-layer ciliated epithelium (Fig. 11.6.2A). An unknown secretory gland in part of the vagina consisted of single-layer columnar epithelium with eosinophilic cytoplasm (Fig. 11.6.2C). We preliminarily refer to this as the "vaginal gland." Simple glands were arranged in

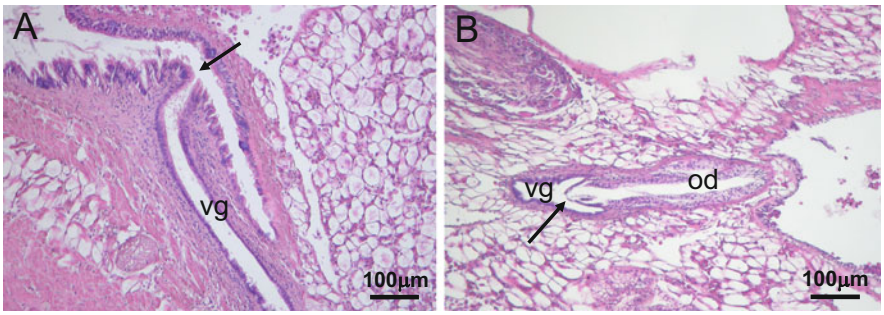

Fig. 11.5 Undifferentiated vagina and capsule gland in a female *Babylonia japonica*, age 12 months (Horiguchi et al. 2014). (**A**) Vagina/capsule gland (*vg*) and its opening (*arrow*). (**B**) Junction of vagina/capsule gland (*vg*) and oviduct (*od*)

cords forming the thick wall of the capsule gland (Fig. 11.6.2D). The superficial layer of the gland was composed of ciliated cells (Fig. 11.6.2D). Secretory cells contained many eosinophilic granules. The bursa copulatrix consisted of single- or double-layer cuboidal epithelium (Fig. 11.6.2B). In males aged 14 months, the testicular tissue area, where spermatocytes were observed, was developing in a narrow area close to the digestive gland (Fig. 11.3C). Despite the lack of penis formation, the vas deferens was gradually forming through invagination of the epidermis (Fig. 11.7B). The vas deferens, showing as a small depression, was incomplete and formed into a groove. Part of the groove of the discontinuous vas deferens became tubular (Fig. 11.7C). The vas deferens opened outside the mantle cavity near the kidney (Fig. 11.7D) (Horiguchi et al. 2014).

In females aged 16 months, the ovarian tissue area was developing widely, beyond the digestive gland. Mature or almost mature oocytes containing eosinophilic granules were observed in the ovary (Fig. 11.2D). The vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely differentiated (Fig. 11.8). The oviduct was directly connected to the albumen gland (Fig. 11.8A, C). The capsule gland was thickening. The albumen gland consisted of branching folded epithelium, which consisted of columnar epithelium cells with basophilic granules in their cytoplasm (Fig.11.8A–C). The sperm-ingesting gland consisted of single-layer epithelium, but its morphological features varied in part of the gland, where branching folded epithelium had formed. The epithelial cells of the sperm-ingesting gland were generally cuboidal, but columnar epithelium was present in the branching folded areas. The epithelial cells in the area connecting the sperm-ingesting gland and the capsule gland were ciliated (Fig. 11.8D). The oviduct close to the albumen gland was thick and consisted of ciliated epithelium, with branching folds. In contrast, the epithelial cells in the rest of the oviduct were columnar. Although the oviduct was connected to the albumen gland, we also observed a branch of the oviduct that opened into the mantle cavity leading outside the soft body. This part of the oviduct gradually thinned and consisted of transitional epithelium (Fig. 11.8B). In males aged

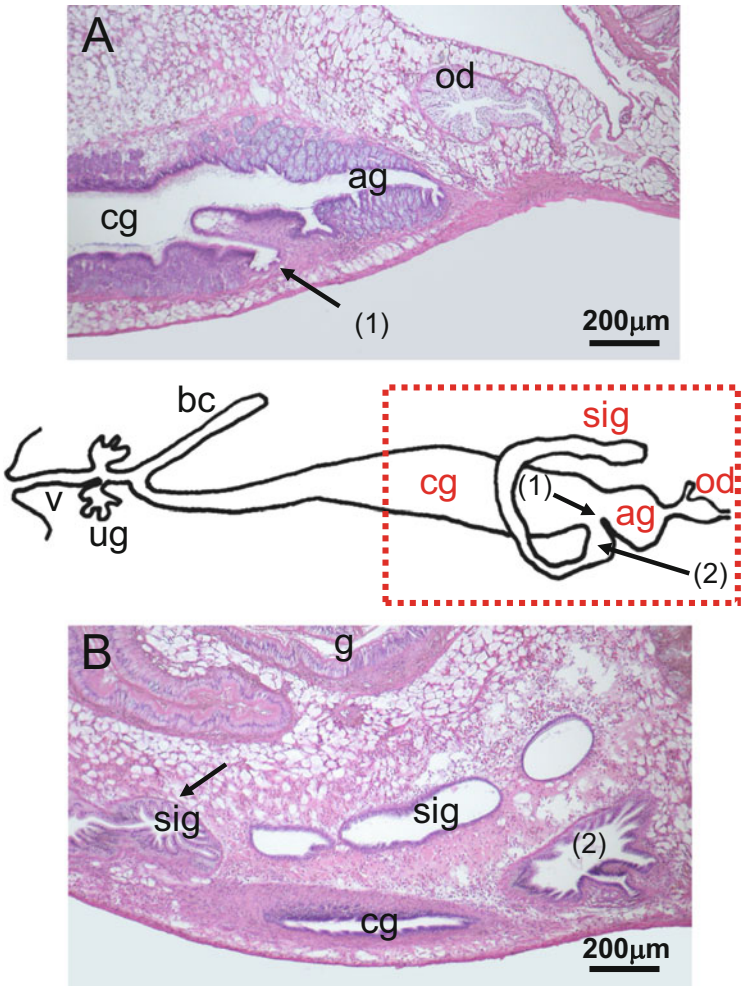

Fig. 11.6.1 Upper half of the genital tract of a female *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). (**A**) Junction of oviduct (*od*), capsule gland (*cg*), and albumin gland (*ag*). Albumin gland consists of secretory cells with basophilic mucus. Sperm-ingesting gland opens to capsule gland (*arrow 1*). (**B**) Gut (*g*), capsule gland, and sperm-ingesting gland (*sig*). Portions of the sperm-ingesting gland are lined with branching fold epithelium (*arrow* and *"2"*)

16 months, the testicular tissue area was developing and spreading beyond the digestive gland. Spermatocytes and spermatids were generally observed in the testis of male specimens. Spermatozoa were observed in the testis of only one male, whereas the other one male had only spermatogonia in a narrowly developing testis (Fig. 11.3D). A protuberance for penis formation was observed behind the right tentacle in almost all male specimens, but invagination of the vas deferens into the penis was observed in only one male (Fig. 11.9B). Although the vas deferens was observed in the upper area (i.e., close to the prostate gland) in all male specimens,

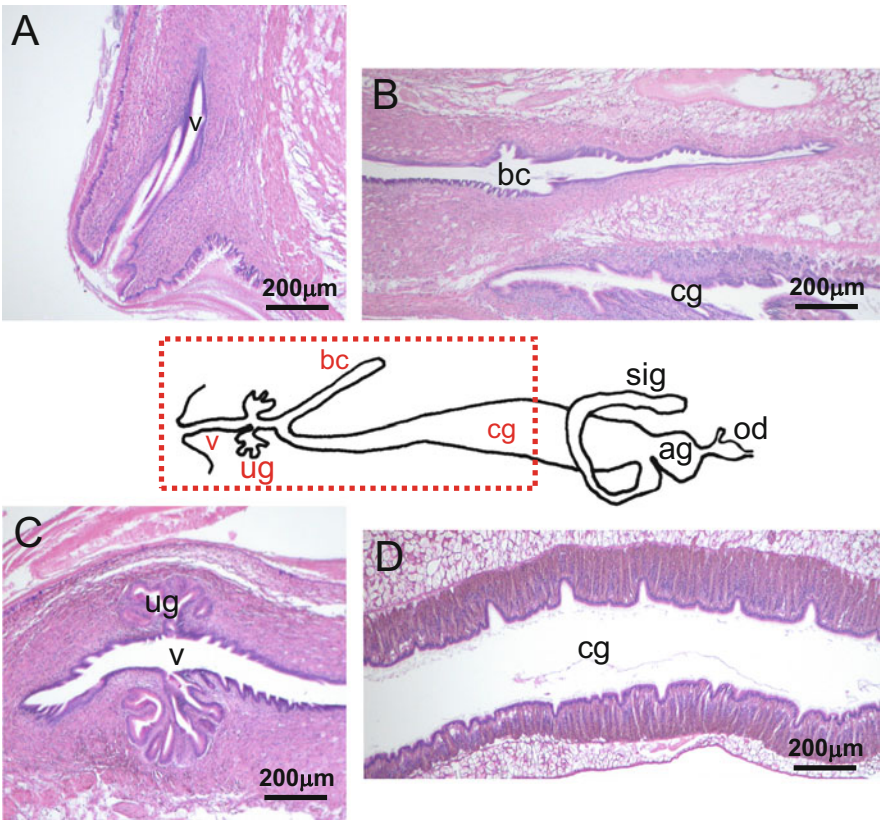

Fig. 11.6.2 Lower half of the genital tract of a female *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). (**A**) Vagina (*v*) and vaginal orifice. (**B**) Bursa copulatrix (*bc*) and lower part of capsule gland (*cg*). (**C**) Unknown gland (*ug*) opening into the vaginal lumen. (**D**) Capsule gland consisting of a wall with simple glands

the vas deferens was not continuous in all specimens (Fig. 11.9). The epithelium of the vas deferens was lined with ciliated cells (Fig. 11.9C). The connection between the vas deferens and testicular duct varied considerably among specimens (Fig. 11.9D). It seems that the upper part of the vas deferens was formed through the invagination of epithelial cells and connected to the testicular duct close to the kidney (Fig. 11.9D). The duct then seemed to extend toward the penis behind the right tentacle, parallel to the rectum, to form the lower genital duct (i.e., vas deferens). Expansion of the duct seemed to result from the invagination of epithelial cells or fusion of the epithelial groove to form the duct structure; this differed from duct (i.e., vas deferens) formation from the opposite side (i.e., the area close to the penis behind the right tentacle). The testicular duct opened to the uppermost area of the vas deferens invagination at almost the same time as when the uppermost area of the vas deferens closed. In contrast, the vas deferens close to the penis seemed to form through invagination of the epithelium (Horiguchi et al. 2014).

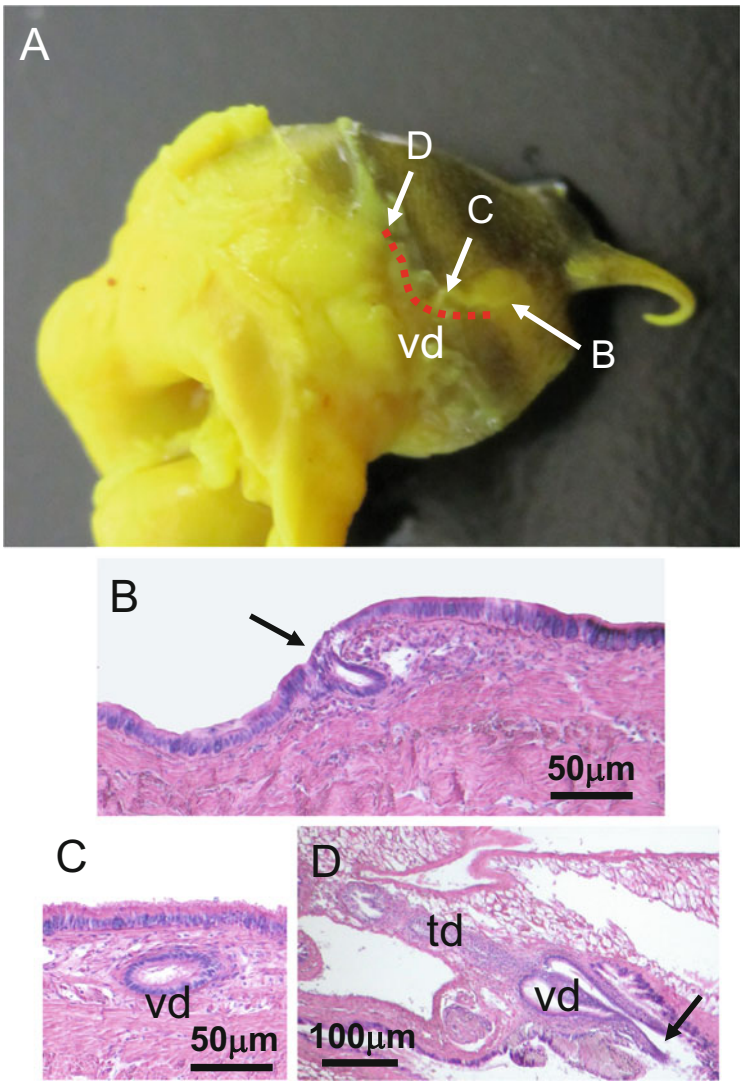

Fig. 11.7 Formation of the reproductive tract in a male *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). (**A**) Appearance of soft body removed from the shell. *Red dashed line* indicates discontinuous vas deferens. (**B**) Invagination of vas deferens in penis-forming area (*arrow*). (**C**) Discontinuous vas deferens (*vd*) in cross section. (**D**) Junction area of vas deferens (*vd*) and testicular duct (*td*), showing opening to mantle cavity

In females aged 18 months, the ovarian tissue area was developing far beyond the digestive gland and had matured more than in 16-month-old specimens. The ovary contained many mature oocytes, which contained eosinophilic granules. Vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely differentiated. The oviduct was directly connected to the albumen gland. Other

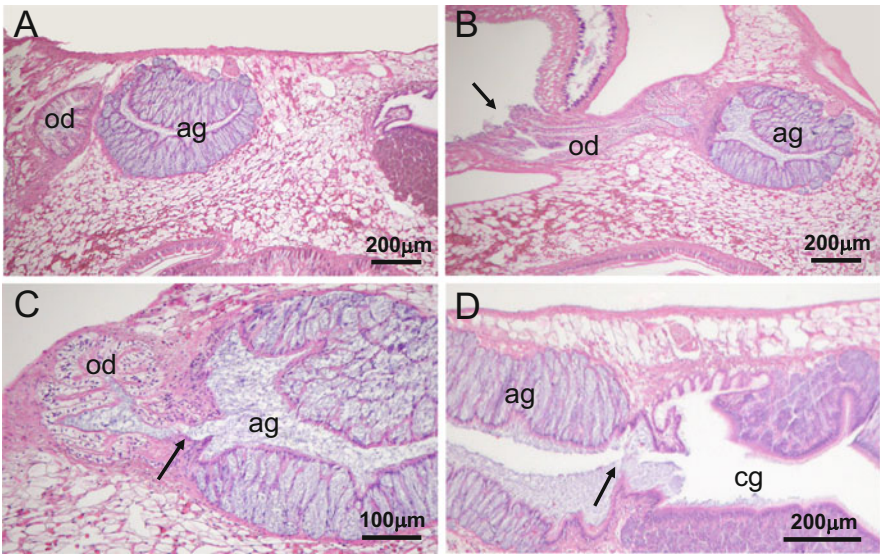

Fig. 11.8 Upper genital tract of a female *Babylonia japonica*, age 16 months (Horiguchi et al. 2014). (**A**) Oviduct (*od*) close to albumen gland (*ag*), which is lined with branching fold epithelium. (**B**) Branch of oviduct opening into the mantle cavity leading outside the body (*arrow*). (**C**) Junction of oviduct (*od*) and albumen gland (*ag*) (*arrow*). (**D**) Junction of albumen gland (*ag*) and capsule gland (*cg*) (*arrow*)

characteristics of the ovary and female accessory sex organs (i.e., genital tract) were the same as those observed in 16-month-old specimens. Females at age 18 months were observed to spawn and lay eggs (unfertilized) in aquaria at the NIES laboratory. In males aged 18 months, testicular tissue was more developed than in 16-month-old specimens, and half the specimens had spermatozoa in the testis. There were, however, large differences in testicular maturation among the male specimens. In mature males with spermatozoa in their testis, formation of the vas deferens was completed from the closed to open condition during development of the penis protuberance. In contrast, in male specimens with incomplete maturation of the testis, formation of the penis and vas deferens was incomplete and discontinuous (Horiguchi et al. 2014).

In females aged 20 months, the ovarian tissue was mature. The genital tract was completely developed: the vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely connected to each other. Histological features of the ovary and female genital tract were the same as those in 16-month-old specimens. In males aged 20 months, the testicular tissue was mature. The genital tract was completely developed: the testicular duct, vas deferens, and penis were completely connected to each other. However, spermatozoa were not observed in the vas deferens. The histological features of the testis and male genital tract were the same as those in 16-month-old specimens (Fig. 11.10) (Horiguchi et al. 2014).

In females aged 24 months, the ovarian tissue was mature. The genital tract was completely developed: the vagina, bursa copulatrix, capsule gland, sperm-ingesting

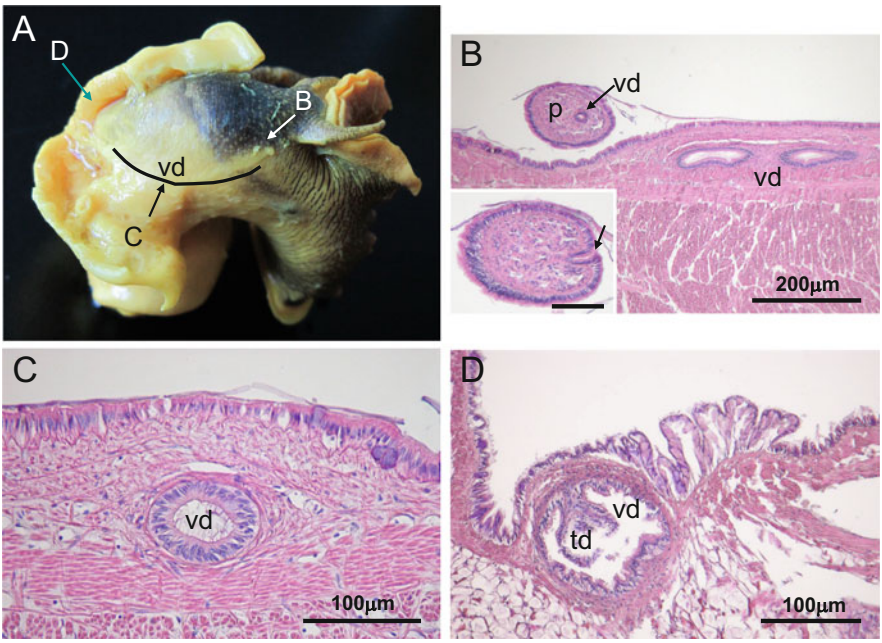

Fig. 11.9 Formation of the reproductive tract in a male *Babylonia japonica*, age 16 months (Horiguchi et al. 2014). (**A**) Appearance of soft body removed from the shell. *Black solid line* indicates the continuous (i.e., completely formed) vas deferens. (**B**) Vas deferens (*vd*) and adjacent penis (*p*). Inset (*lower left*) shows orifice of vas deferens in the penis of the same specimen (*arrow*). *Bar* 100 μm. (**C**) Vas deferens with ciliated epithelium in the body. (**D**) Junction of testicular duct (*td*) and vas deferens. Testicular duct is open into the vas deferens

gland, and albumen gland were completely connected to each other. Histological features of the ovary and female genital tract were the same as those in 16-month-old specimens. In males aged 24 months, the testicular tissue was mature. A few males appeared to have released sperm, judging from the histological features of their testis. The genital tract was completely developed. The testicular duct, vas deferens, and penis were completely connected to each other, but no spermatozoa were observed in the vas deferens in any of the male specimens. Although the vas deferens was open, penis size was still small (average penis length, 0.55 mm) (Horiguchi et al. 2014).

To understand the induction of imposex in prosobranch gastropods by organotin compounds, it is necessary to examine and understand in detail the normal processes of the genital tract and gonad differentiation and development. Because the planktonic stage of *B. japonica* is estimated to last approximately 4 to 5 days (Hamada et al. 1988, 1989), it would be easy to maintain and raise veliger larvae in the laboratory. Moreover, the methodology for hatchery production of *B. japonica* seed had been established since the 1980s (Kajikawa et al. 1983). Therefore, *B. japonica* is useful as a target species for research on differentiation and development of the genital tract and gonad (Horiguchi et al. 2014).

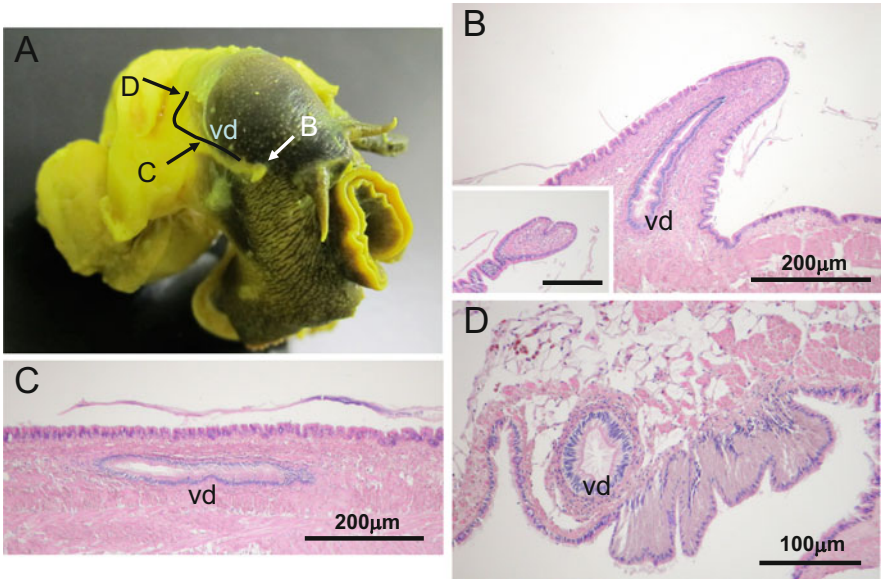

Fig. 11.10 Formation of the reproductive tract in a male *Babylonia japonica*, age 20 months (Horiguchi et al. 2014). (**A**) Appearance of soft body removed from the shell. *Black solid line* represents the completed vas deferens. (**B**) Vas deferens (*vd*) in the penis. Inset (*lower left*) shows the orifice of the vas deferens in the tip of penis of the same specimen. *Bar* 100 μm. (**C**) Vas deferens (*vd*) in the body. (**D**) Vas deferens (*vd*) near the junction with the testicular duct

As described here and summarised in Table 11.1, the development of the *B. japonica* genital tract precedes differentiation of the gonad: this is the opposite of the sequence in vertebrates such as mammals (Gilbert 2006; Jost et al. 1973). This observation suggests that the regulatory mechanisms of endocrinological or reproductive organs and their functions differ between gastropods and vertebrates. In this regard, recent critical reviews of the presence of functional receptors for steroids and of enzymes for steroid synthesis or metabolism (Horiguchi 2009; Scott 2012, 2013, as well as Chap. 9), have pointed out that it is doubtful whether gastropod mollusks inherently have vertebrate-type steroids as sex hormones (Horiguchi et al. 2014).

Observations of a 2-year-old wild-caught male suggest that it takes about 2 years for complete development of the genital tract (i.e., testicular duct, vas deferens, and penis) and the mature testis. This finding does not contradict observations that laboratory-reared males at age 20 months and much older had a complete genital tract (i.e., testicular duct, vas deferens, and penis) and a mature testis (Horiguchi et al. 2014).

Differentiation and subsequent development of the genital tract and gonad seem to occur earlier in females than in males, an observation supported by the finding that 18-month-old females spawned and deposited eggs (unfertilized) in aquaria at the NIES laboratory. Males at the same age seem unable to copulate and fertilize eggs because of small penis size, incomplete vas deferens, and immature testis (Horiguchi et al. 2014).

The retinoid X receptor (RXR) could be mediating molecular mechanisms of the differentiation, proliferation, and morphogenesis of male genitalia in male and imposex-exhibiting female prosobranch gastropods (Nishikawa et al. 2004; Castro et al. 2007; Horiguchi et al. 2007, 2008, 2010a, b; Sternberg et al. 2008; Urushitani et al. 2011). Thus, development of a specific antibody for *B. japonica* RXR could provide useful information about when and where RXR expression is observed in the tissues of juvenile *B. japonica* under normal development. Laboratory experiments exposing *B. japonica* to TBT or TPhT over approximately 2 years, and using molecular, biochemical, and immunohistochemical techniques, could provide detailed information about the expression of mRNA for RXR and the presence of RXR protein during development under organotin exposure. The results of such studies should help clarify the mechanism of imposex induction by TBT and TPhT (Horiguchi et al. 2014).

11.3 Comparison of *Thais clavigera* and *Babylonia japonica*: The Formation of Male-Type Genitalia in Imposex-Exhibiting Females Mimics the Normal Development of Male Genitalia, with Difference Among Species

Various histological characteristics indicative of the initial stages of imposex were observed in females from a wild *Thais clavigera* population in Hiraiso, Japan (Horiguchi et al. 2012a) (Figs. 11.11 and 11.13). Unidentified aggregated cells, which may have been differentiating into a penis, and invagination of the epidermal tissue toward the formation of the vas deferens were observed in the presumptive penis-forming area of female *T. clavigera* (Horiguchi et al. 2012a) (Fig. 11.11A, B). In a female with a tiny penis, the epidermal tissue behind the penis was making an invagination, which was elongating into the penis to form an initial stage of the vas deferens (Fig. 11.12). However, this was a blind duct without any opening into the penis (Fig. 11.12A). Moreover, a variety of morphogenesis patterns of the vas deferens were observed in female *T. clavigera* specimens from a wild population in Hiraiso (Fig. 11.13). They are summarised as follows: (1) the invagination of the epidermal tissue toward the formation of the vas deferens occurs at almost the same time as a protuberance is formed in the presumptive penis-forming area behind the right tentacle of female *T. clavigera*; (2) the initial vas deferens is formed by the invagination of the epidermal tissue, followed by the extension and connection of the blind duct; (3) the invagination of the epidermal tissue toward the formation of the vas deferens occurs at several locations between the vaginal opening (i.e., vulva) of the capsule gland and penis, and then the vas deferens beneath the penis extends toward the tip of penis; and (4) the penis is differentiated and formed by unidentified aggregated cells in the epidermal tissue (Horiguchi et al. 2012a).

The five female rock shells that were removed from each group of flow-through exposure experiments, using TBT (exposure to TBTCl and a control group with

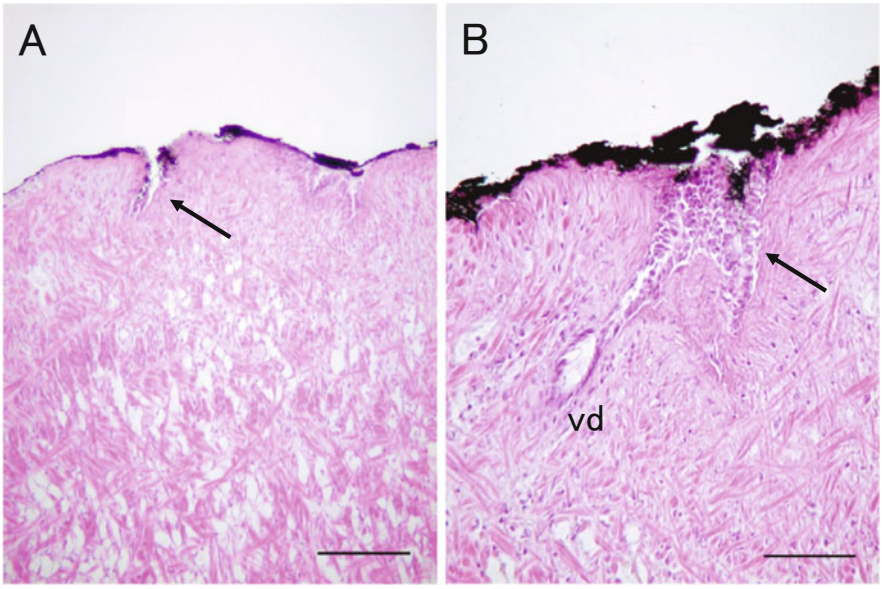

Fig. 11.11 Presumptive penis-forming area behind the right tentacle of a wild female *Thais clavigera* (Horiguchi et al. 2012a). *vd* vas deferens. The epidermis of the penis-forming area was marked with India ink after fixation. Note an invagination of the epidermal tissue (*arrow*), which will lead toward vas deferens formation (**A**), and unidentified aggregated cells (*arrow*), which are possibly differentiating into a penis (**B**). *Bar* 50 μm

acetone/DMSO) after each of 5, 7, 12, and 24 days of the experiment were histologically examined under a light microscope to elucidate the processes of development of the vas deferens and penis during the initial stages of imposex in *T. clavigera*. After 5 days of TBT exposure, the five selected female specimens consisted of four imposex-exhibiting females and an apparently normal female. One female had an immature vas deferens (Fig. 11.14A) despite having no protuberance in the presumptive penis-forming area behind the right tentacle. In the other four females, however, no vas deferens (i.e., invagination of the epidermal tissue) was observed. After 7 days of TBT exposure, the five selected female specimens consisted of three imposex-exhibiting females and two apparently normal females. One female had an immature vas deferens as well as a protuberance in the presumptive penis-forming area behind the right tentacle (Fig. 11.14B). The vas deferens observed was not close to the vaginal opening of the capsule gland, but was behind a protuberance considered to be an initial stage of penis formation behind the right tentacle (Fig. 11.14B). No other females, however, displayed any invagination of the epidermal tissue, which would indicate immature vas deferens formation, after 7 days of TBT exposure. After 12 days of TBT exposure, the five selected female specimens consisted of two imposex-exhibiting females and three apparently normal females. Histological examination showed that the three normal-looking specimens had neither a penis nor vas deferens and that the two remaining

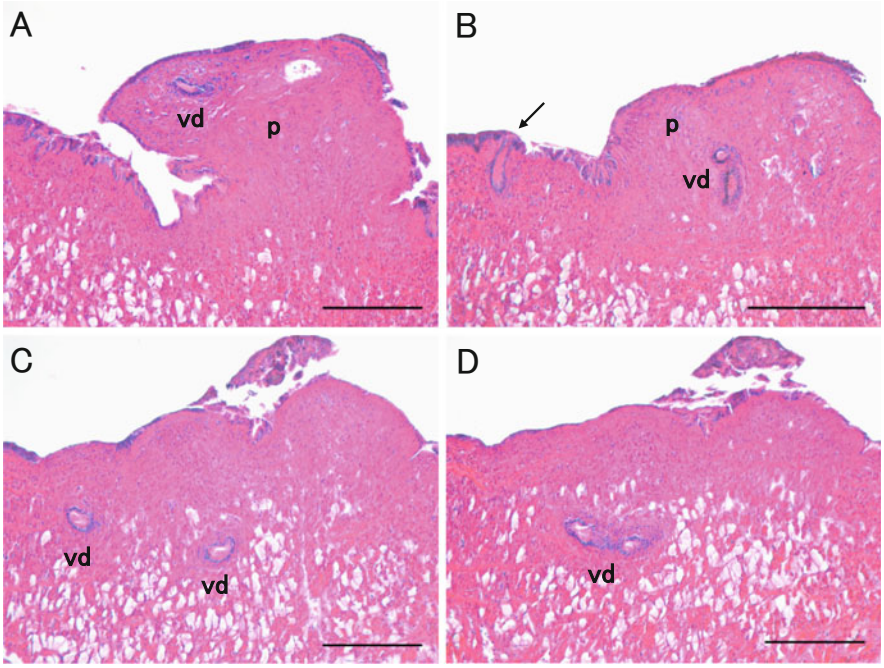

Fig. 11.12 Formation of the vas deferens behind the tiny penis of a wild female *Thais clavigera* (Horiguchi et al. 2012a). *p* penis, *vd* vas deferens. An invagination of the epidermal tissue (*arrow*) is visible behind the penis, forming the vas deferens (**B**). The vas deferens elongates into the penis (**C** and **D**), but it is a blind duct without any opening (**A**). *Bar* 100 μm

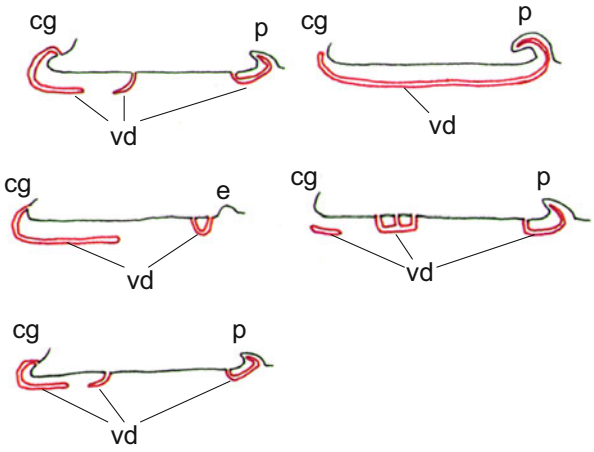

Fig. 11.13 A variety of patterns of vas deferens morphogenesis observed in wild females of *Thais clavigera* (Horiguchi et al. 2012a). *cg* capsule gland, *e* elliptical protuberance, *p* penis, *vd* vas deferens

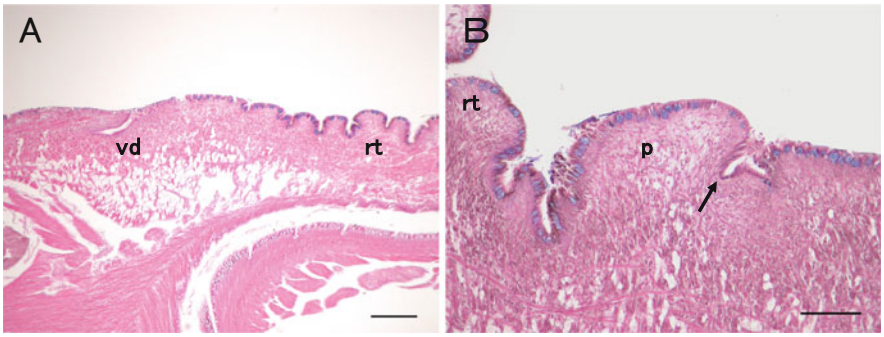

Fig. 11.14 Formation of immature vas deferens with or without a protuberance in the presumptive penis-forming area behind the right tentacle of female *Thais clavigera* exposed to tributyltin (TBT) in a flow-through exposure experiment (Horiguchi et al. 2012a). *p* protuberance as an initial stage of penis formation, *rt* base of the right tentacle, *vd* immature vas deferens. Invagination of epidermal tissue recognised as an immature vas deferens (*vd*), without any protuberance, in the presumptive penis-forming area behind the right tentacle (*rt*) of a female, after 5 days of exposure (**A**). Invagination of epidermal tissue recognised as an immature vas deferens (*arrow*) behind the protuberance as an initial stage of penis formation (*p*) in the presumptive penis-forming area behind the right tentacle of a female after 7 days of exposure (**B**). *Bars* (**A**) 200 μm; (**B**) 100 μm

specimens had both a penis and vas deferens. However, regarding these two imposex-exhibiting females, a vas deferens was only observed beneath the penis, and no vas deferens was observed close to the vaginal opening of the capsule gland, which is different from the characteristics of vas deferens formation observed in females of a wild *Thais clavigera* population in Hiraiso. One had a vas deferens that opened at the tip and base of the penis, and the other had a vas deferens that was a blind duct. After 24 days of TBT exposure, the five selected female specimens consisted of three imposex-exhibiting females and two apparently normal females. One female had an immature vas deferens as well as a protuberance in the presumptive penis-forming area behind the right tentacle. The vas deferens observed was not close to the vaginal opening of the capsule gland, but it was beneath the penis-like protuberance behind the right tentacle. No vas deferens (i.e., invagination of the epidermal tissue) was observed in the other female specimens after 24 days of TBT exposure. In no control female specimen was the development of a vas deferens observed (Horiguchi et al. 2012a).

Based on the findings from histological observations of specimens from a wild *T. clavigera* population and laboratory flow-through exposure experiments, Horiguchi et al. (2012a) concluded that the invagination of the epidermal tissue in the presumptive penis-forming area behind the right tentacle leading to the formation of the vas deferens would follow on, or occur at almost the same time as, formation of the protuberance in the presumptive penis-forming area of female *T. clavigera*. Rarely, invagination of the epidermal tissue for vas deferens formation may precede the formation of the protuberance in the presumptive penis-forming area. However, invagination of the epidermal tissue close to the vaginal opening of the capsule gland would subsequently occur, leading to the formation of the vas

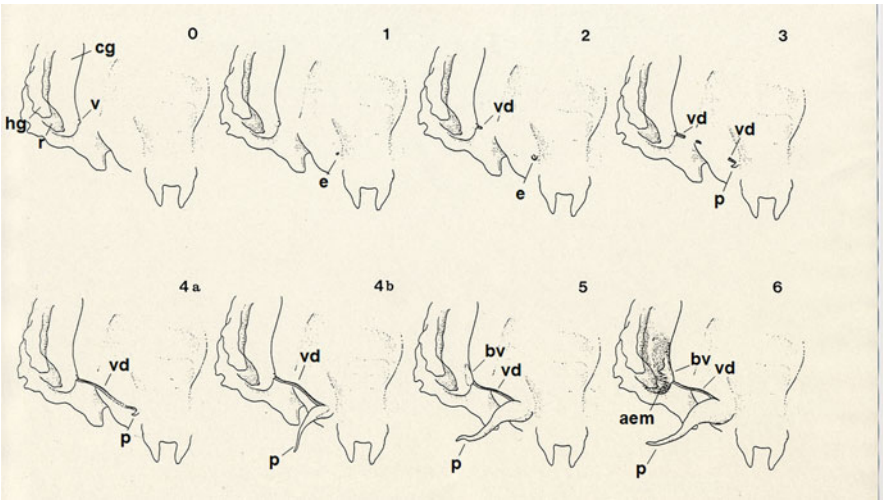

Fig. 11.15 Vas deferens sequence (VDS) index for *Thais clavigera* (Horiguchi et al. 2012a). *aem* aborted egg mass, *bv* blocked vulva, *cg* capsule gland, *e* elliptical protuberance, *hg* hypobranchial gland, *p* penis, *r* rectum, *v* vulva, *vd* vas deferens. VDS 0: Neither penis nor vas deferens is observed (a normal female). VDS 1: A protuberance is observed in the presumptive penis-forming area behind the right tentacle. Invagination of the epidermal tissue in the presumptive penis-forming area is observed, but no invagination of the epidermal tissue is observed close to the vaginal opening (i.e., vulva) of the capsule gland. VDS 2: A protuberance is clearly observed and recognised as an ellipse or an oval in the presumptive penis-forming area behind the right tentacle. Invagination of the epidermal tissue is observed close to the vaginal opening (i.e., vulva) of the capsule gland as well as in the presumptive penis-forming area. VDS 3: The protuberance is apparently/morphologically found to be a tiny penis. Invagination of the epidermal tissue occurs at several locations between the vaginal opening (i.e., vulva) of the capsule gland and the penis, leading to the formation of the vas deferens. The invaginated epidermal tissues extend from several locations and connect to each other to form the duct of the vas deferens. VDS 4: The vas deferens is completed as a duct, and subsequently, the penis grows. VDS 5: The proliferation of the epidermal tissue surrounding the vas deferens covers and blocks the vaginal opening (i.e., vulva) of the capsule gland, resulting in sterility. No aborted egg capsule mass is observed in the capsule gland. VDS 6: In addition to the symptoms seen for VDS 5, an aborted egg capsule mass, darkened and compressed, is observed in the capsule gland

deferens. The number of locations of the epidermal tissue where invagination occurs is not likely fixed, and it may sometimes occur at several locations between the vaginal opening of the capsule gland and the penis; then, the vas deferens beneath the penis would extend toward the tip of the penis. The penis may be differentiated and formed by unidentified aggregated cells in the epidermal tissue (Horiguchi et al. 2012a).

Thus, based on the findings already mentioned, the VDS index for *Thais clavigera* was proposed as follows (Fig. 11.15). VDS 0: Neither the penis nor the vas deferens is observed even in histological preparation; therefore, it is recognised as a normal female. VDS 1: A protuberance is observed in the presumptive penis-forming area behind the right tentacle, and an invagination of the epidermal tissue in the

presumptive penis-forming area could also be observed if a histological examination is conducted, but no invagination of the epidermal tissue is observed close to the vaginal opening of the capsule gland. VDS 2: A protuberance is clearly observed and recognised as an ellipse or an oval in the presumptive penis-forming area behind the right tentacle, and an invagination of the epidermal tissue in the presumptive penis-forming area is also observed if a histological examination is conducted. An invagination of the epidermal tissue is also observed close to the vaginal opening of the capsule gland. VDS 3: The protuberance is apparently/morphologically found to be a tiny penis, and the invagination of the epidermal tissue at several locations between the vaginal opening of the capsule gland and the penis, leading to the formation of the vas deferens. The invaginated epidermal tissues extend from several locations and connect to each other to form the duct of the vas deferens. VDS 4: The vas deferens is completed as a duct, and subsequently, the penis grows. VDS 5: The proliferation of the epidermal tissue surrounding the vas deferens covers and blocks the vaginal opening of the capsule gland; therefore, the release of egg capsules is obstructed. This female is considered to be sterile, but no aborted egg capsule mass is observed in the capsule gland. VDS 6: In addition to the symptoms seen for VDS 5, an aborted egg capsule mass, darkened and compressed, is observed in the capsule gland (Fig. 11.15) (Horiguchi et al. 2012a). Thus, the VDS index for *Thais clavigera* is a little different from that for *Nucella lapillus*, as defined by Gibbs et al. (1987).

As referred to in Chap. 9, the hypothesis that the activation of RXR (Nishikawa et al. 2004) is the mechanism by which TBT and TPhT induce imposex in gastropods seems to be the most likely of the six proposed hypotheses. Interaction between the organotins (i.e., TBT or TPhT) and RXR may occur in the presumptive penis-forming area behind the right tentacle or in the head ganglia, which is the central nervous system of gastropods, soon after exposure to TBT or TPhT, leading to an accumulation of TBT or TPhT in tissues (Horiguchi et al. 2012b). Specific genes and protein expressions could be involved, although the details remain unknown. The downstream physiological pathways may include the processes of differentiation, proliferation, and morphogenesis of the male genitalia (i.e., penis and vas deferens) in both male and imposex-exhibiting female gastropods.

We should also remember that both penis and vas deferens were already observed in males and imposex-exhibiting females from wild populations even at an estimated age of several months, just after settlement, in *T. clavigera* (Fig. 11.16) (Horiguchi et al., unpublished data). This finding is rather different from the age of *Babylonia japonica* completing the development of a vas deferens and penis, as described earlier in this chapter (Table 11.1). In male *B. japonica*, the onset of development of genital organs such as the vas deferens and penis seems to differ from that in imposex-exhibiting *N. lapillus* and *T. clavigera* females. First, it seems to take from 20 to 24 months for male *B. japonica* to develop a complete genital tract (i.e., testicular duct, vas deferens, and penis) and mature testis. On the other hand, the order of formation, with vas deferens formation preceding penis formation in male *B. japonica*, is similar to that in imposex-exhibiting female *N. lapillus*, although it differs from that in imposex-exhibiting female *T. clavigera*, wherein development of the vas deferens does not precede penis development.

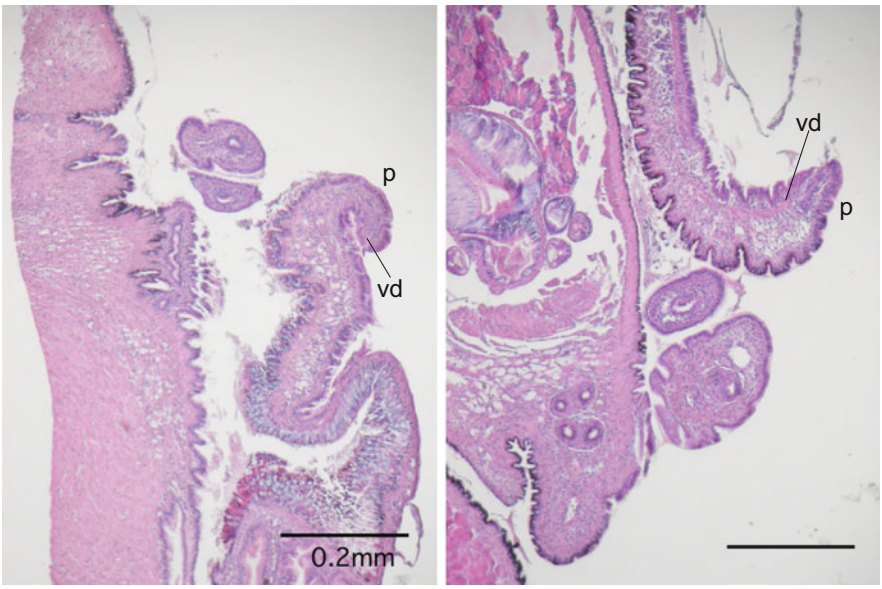

Fig. 11.16 Tiny penis and immature vas deferens observed in wild juvenile male and imposex-exhibiting female *Thais clavigera* at an estimated age of several months, just after settlement (Horiguchi et al., unpublished data). *Left*: male. *Right*: imposex-exhibiting female (shell height approximately 6–7 mm), collected at Jogashima, Japan, on January 10, 2004. *p* penis, *vd* vas deferens. *Bar* 0.2 mm

Stroben et al. (1995) described a general scheme of imposex development in prosobranch gastropods and illustrated various patterns for the process of development of the vas deferens and penis in imposex-exhibiting females. This scheme suggests that there are various, slightly different, developmental patterns of the vas deferens and penis among prosobranch gastropod species exhibiting imposex. The early process of development of the vas deferens, however, was similar in male *B. japonica* and imposex-exhibiting female *T. clavigera*, in both of which it occurred as an epidermal invagination. These results suggest that the differentiation and development of male-type genitalia in imposex-exhibiting female prosobranch gastropods generally mimic those in male prosobranch gastropods, except for the age at onset and the time to completion. We should also be aware that it does not seem to be strictly fixed or regulated: relatively large variation in the differentiation and development of genitalia could occur among individuals, as well as among species of prosobranch gastropods. It also may imply that, in mollusks, the physiological mechanisms of the differentiation and development of male-type genitalia are less strictly regulated than in vertebrates.

Although the natural ligand of the rock shell RXR and other gastropod RXRs is currently unknown (Horiguchi et al. 2007, 2008, 2010a, b; Urushitani et al. 2011), 9-*cis* retinoic acid (9cRA) is known to be the natural ligand for mammalian RXRs (Heyman et al. 1992; Levin et al. 1992; Mangelsdorf et al. 1992; Mangelsdorf and

Evans 1995). Therefore, the retinoic acids, such as 9cRA, may be important in inducing and promoting the development of the male genitalia in both male and imposex-exhibiting female gastropods (see Chap. 9). Many variations in the development of the vas deferens, the external morphology of the penis, and the modes of blocking the vaginal opening have been observed in imposex-exhibiting female *T. clavigera* (Horiguchi 1993) as well as other gastropod species, such as *Nucella lapillus*, *Ocenebra erinacea*, and *Ilyanassa obsoleta* (Bryan et al. 1986; Gibbs and Bryan 1986; Gibbs et al. 1988, 1990). Although little is known about the physiological functions of retinoic acids in invertebrates, retinoic acids are known to have key roles in embryo patterning and organogenesis in vertebrates (Morris-Kay 1997; Redfern 1997). The ventral/external split of the capsule gland in *T. clavigera* (Horiguchi et al., unpublished data), which is similar to *O. erinacea* (Gibbs et al. 1990), may also be caused by the involvement of RXR. Whether ovarian spermatogenesis (i.e., the sex change by testicular tissue formation in the ovary) in *T. clavigera* and other gastropod species (Gibbs et al. 1988; Horiguchi and Shimizu 1992) is also induced by the involvement of RXR remains unclear.

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