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

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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.

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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 $\fallingdotseq$ 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
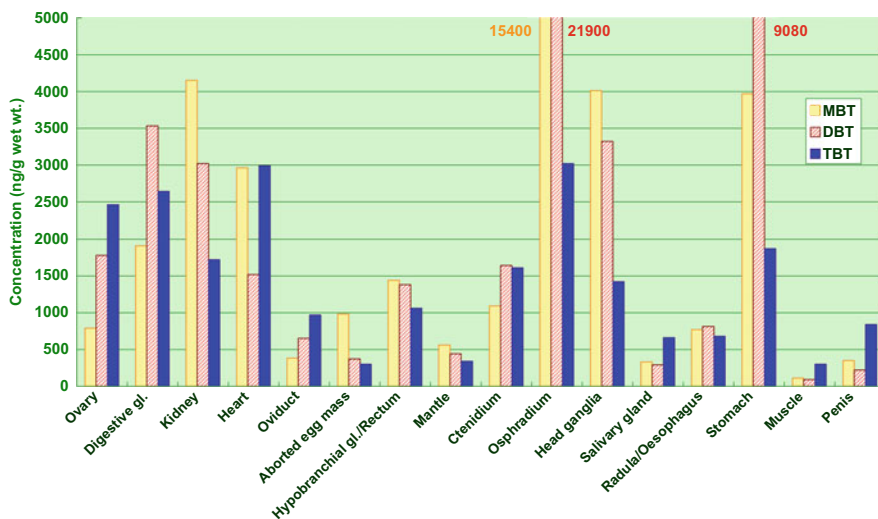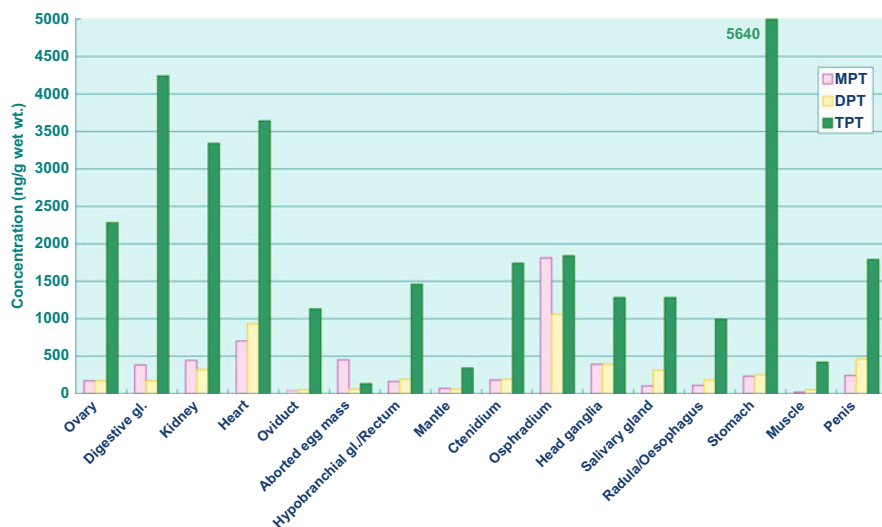

Fig. 4.1 Tissue distribution of butyltin compounds in imposex-exhibiting female rock shells (*Thais clavigera*) (Horiguchi et al. 2012)
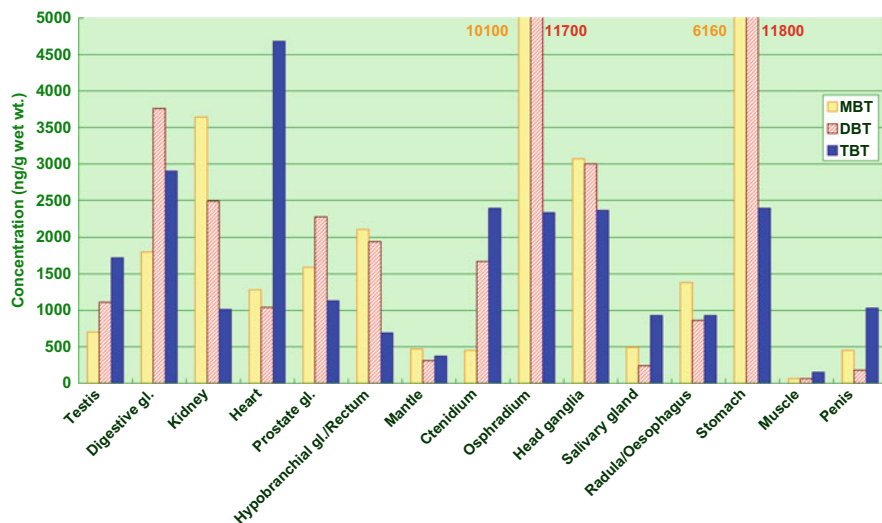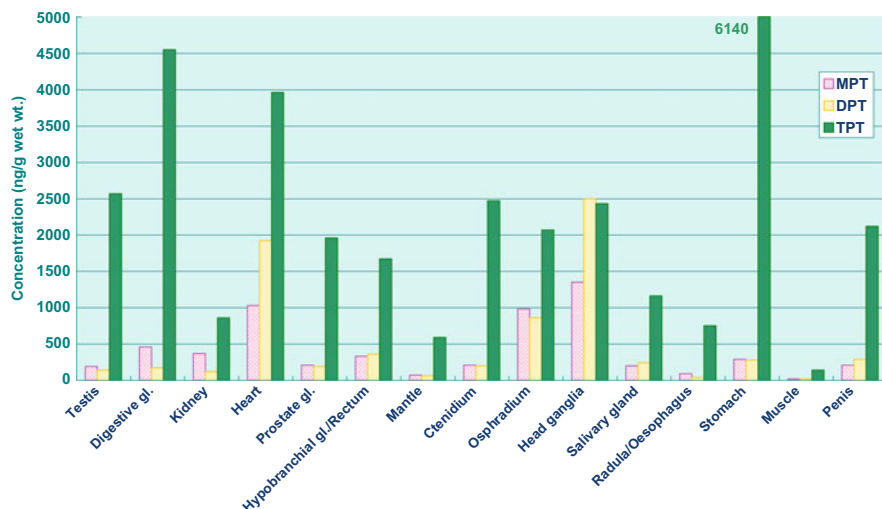
Fig. 4.2 Tissue distribution of butyltin compounds in male rock shells (*Thais clavigera*) (Horiguchi et al. 2012)

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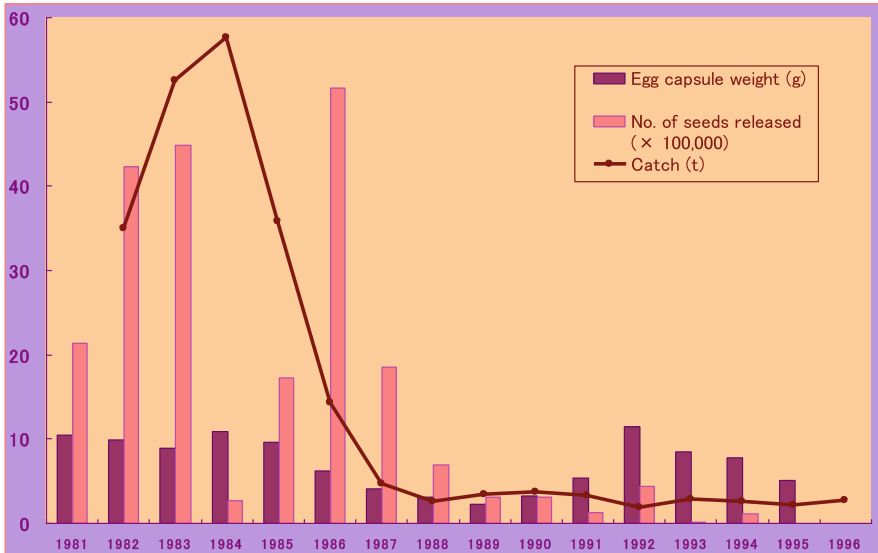
Fig. 4.5 Temporal trends in average weight of egg capsules spawned by an adult ivory shell, *Babylonia japonica*, at the Tottori hatchery, as well as numbers of seeds/juveniles released into the sea and the total catch off Tottori Prefecture, Japan

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
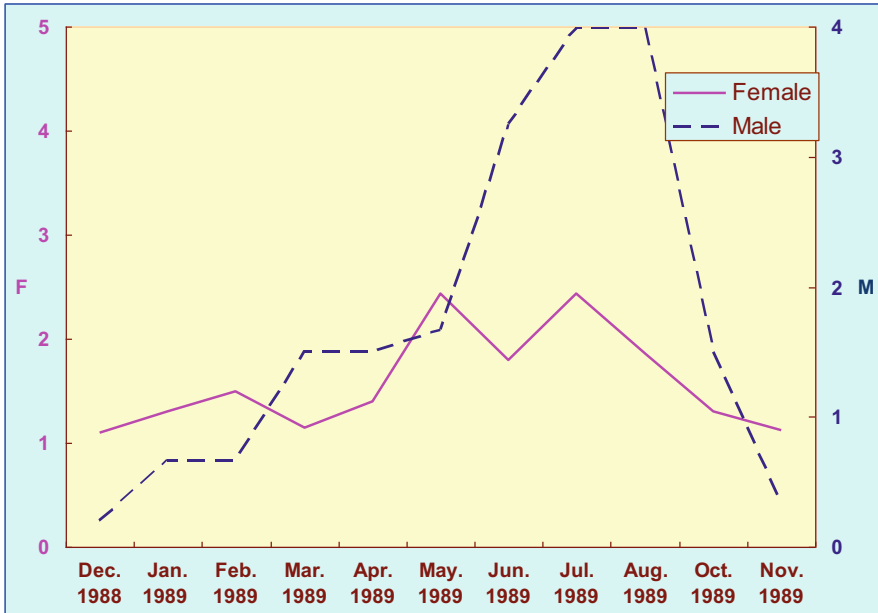
Fig. 4.6 Reproductive cycle of the ivory shell (*Babylonia japonica*) in 1989, represented by population reproductive development scores. Female (*F*) reproductive cells were scored on the basis of five categories, and those of males (*M*) were based on four categories. The female curve includes imposex-exhibiting 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)
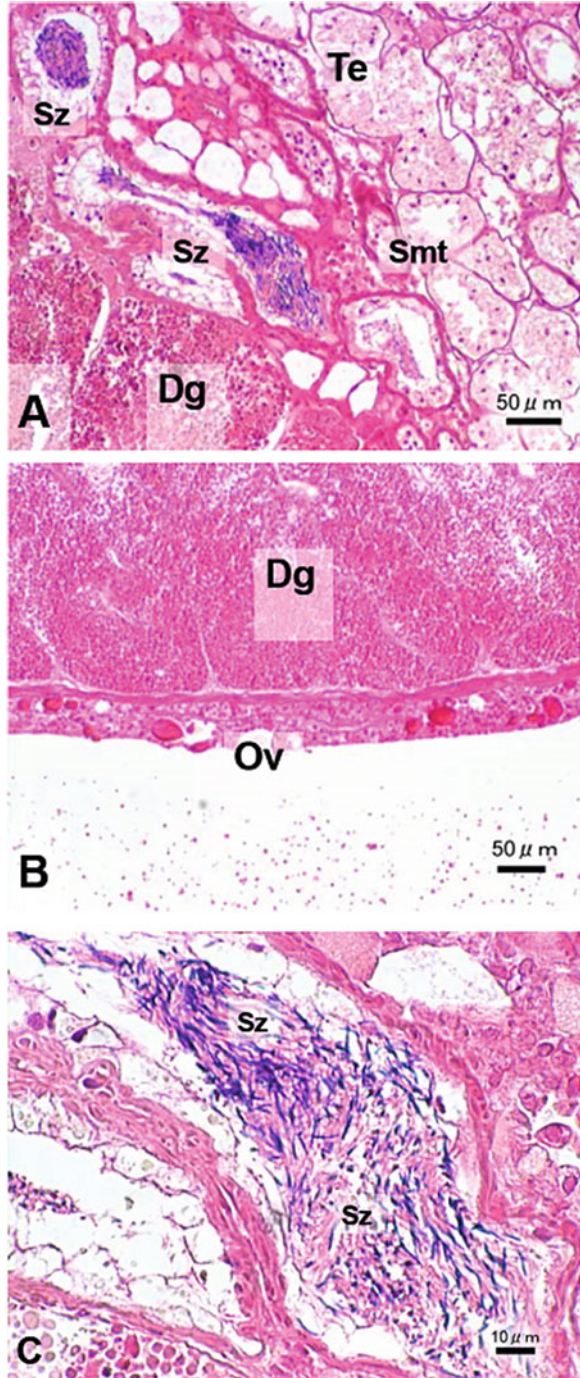
apparently normal female *B. japonica* without any penis or vas deferens formation (1 of 6, 16.7 %) may imply that the development of male-type genital organs (penis and vas deferens) and ovarian spermatogenesis in females exposed to TBT or TPhT might be controlled through different physiological pathways. 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
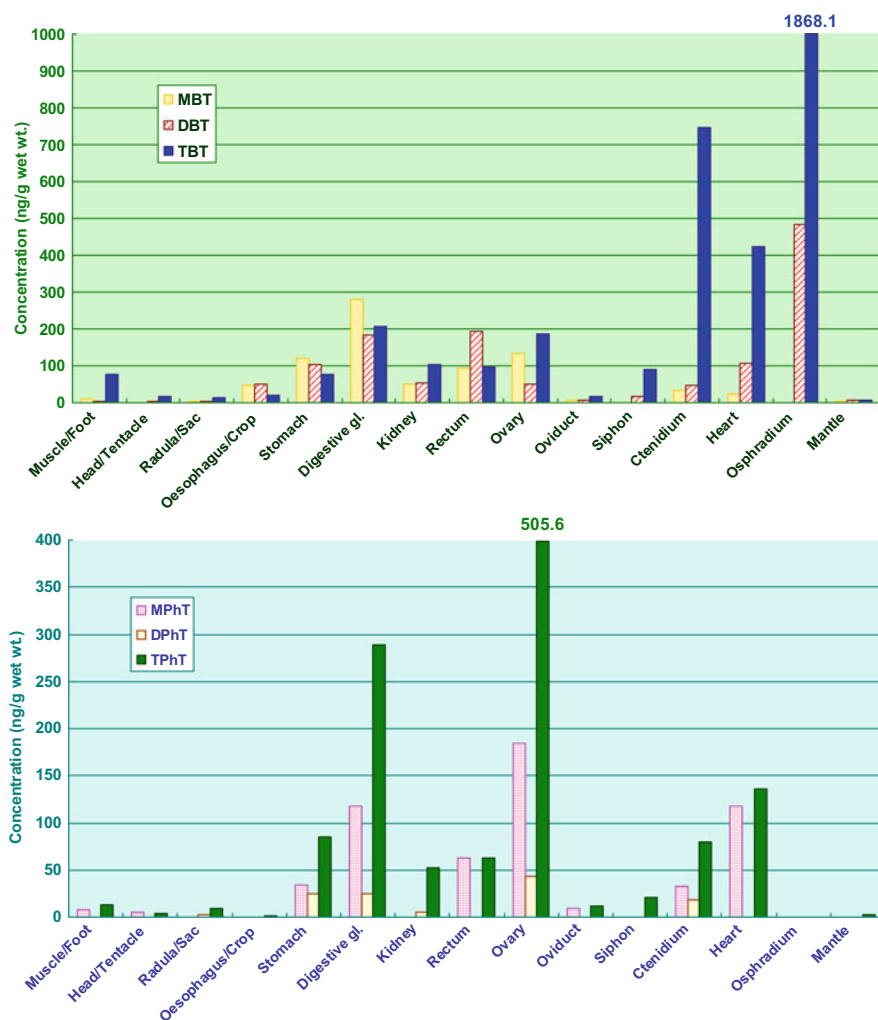

Fig. 4.8 Tissue distributions of organotin compounds in ivory shell (*Babylonia japonica*) from Yodoe, Tottori, Japan (June 1991): *top*, butyltins in females (including imposex individuals); *bottom*, phenyltins in females (including imposex individuals) (Horiguchi et al. 2006)

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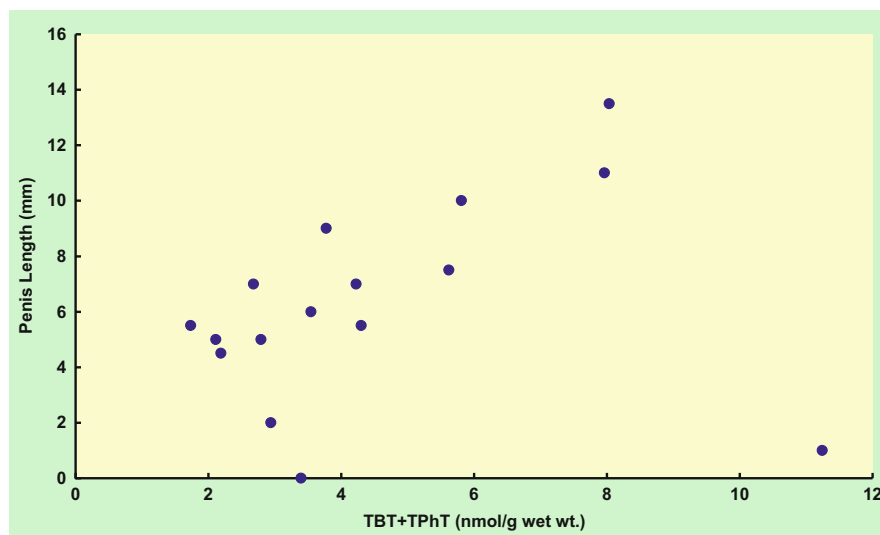
Fig. 4.9 Relationship between triorganotin (sum of TBT and TPhT) concentrations in gonads and penis length in female *Babylonia japonica* (Horiguchi et al. 2006)

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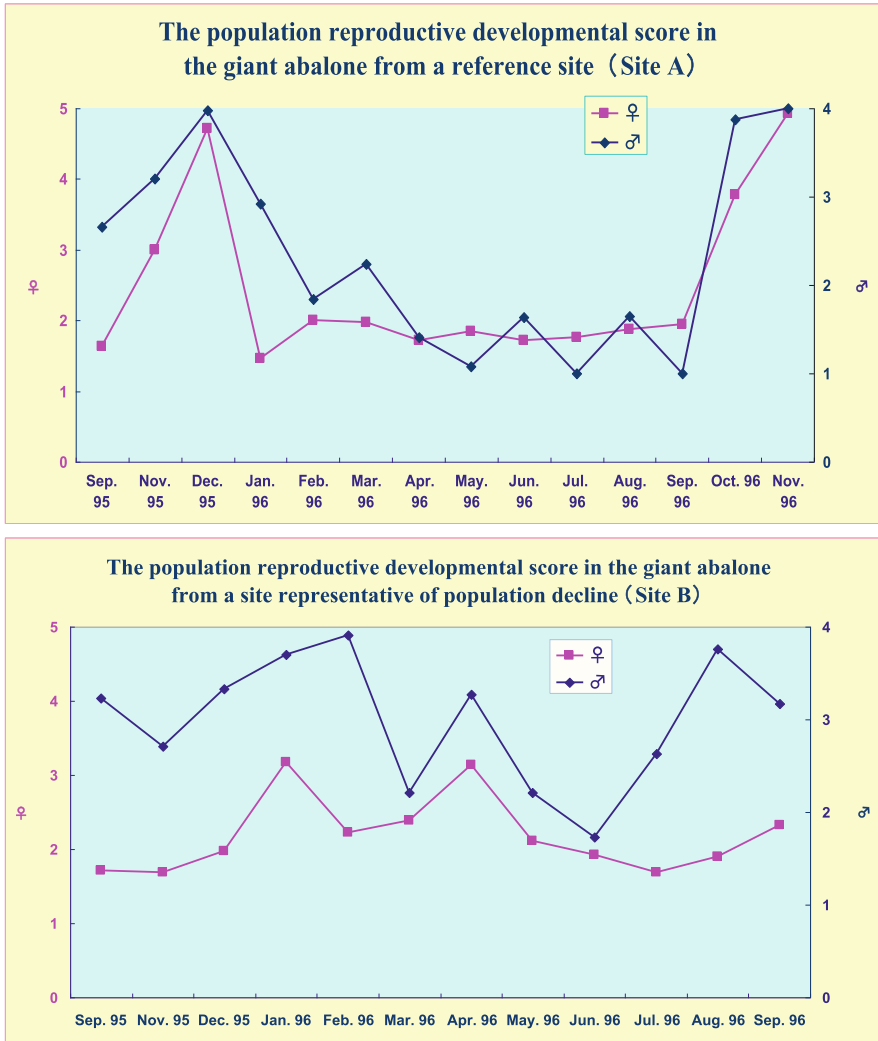
Fig. 4.12 Reproductive developmental scores of the two different populations of giant abalone (*Haliotis madaka*), from Tsushima (*top*) and Jogashima (*bottom*) (Horiguchi et al. 2000)

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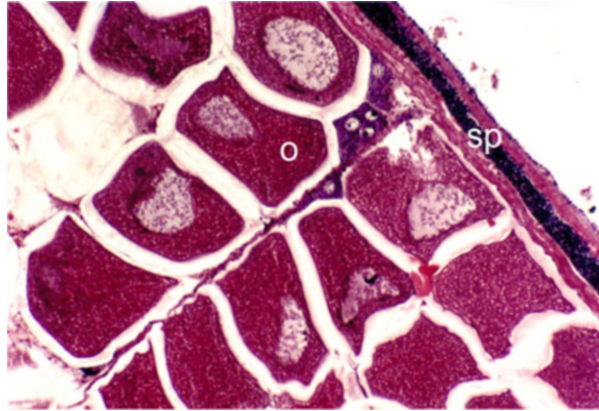
Fig. 4.14 Spermatogenesis in the ovary of in situ exposed female abalone, near a shipyard in Jogashima for 7 months (Horiguchi et al. 2000)


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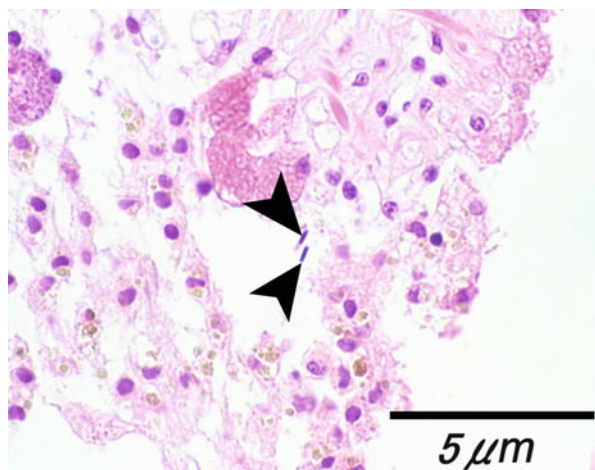
TPhT than in ovaries of controls (Horiguchi et al. 2002). No significant histological changes were observed in testis of exposed males (Horiguchi et al. 2002). This ovarian spermatogenesis caused by TBT and TPhT exposure seems similar to the masculinization of muricidae and buccinidae gastropods, such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