STELLINGEN

behorende bij het proefschrift "Imposex in the common whelk, *Buccinum undatum*",
door B.P. Mensink te verdedigen op 7 december 1999.

1. Blootstelling aan tributyltin (TBT) leidt tot een schijnbare masculinisatie van zich ontwikkelende wulken.
   (dit proefschrift)

2. Volwassen vrouwelijke wulken zijn minder gevoelig voor de masculiniserende effekten van tributyltin dan juveniele wulken.
   (dit proefschrift)

3. Het is prematuur te spreken over imposex als voorbeeld van 'endocrine disruption' bij de wulk, zolang niet is aangetoond dat in niet-gonade weefsel van juveniele wulken steroid hormonen worden geproduceerd.
   (dit proefschrift)

4. Boomkorvisserij heeft direkt een groter effekt (gehad) op populaties van wulken in de zuidelijke Noordzee dan de waargenomen imposex.
   (dit proefschrift)

5. Monitoring van TBT via imposex in ontwikkelende wulken heeft meer zin dan volwassen wulken te bestuderen.

6. Bij de recent meerdere malen geuitte twijfel omtrent de mogelijk hormoonverstorende potentie van enkele (toegelaten) middelen onderschat men de kwaliteit van bestaande testen voor reproductie-effekten.
   (A.H. Piersma voordracht op symposium 'Endocrine disrupting compounds : Wildlife and Human Health Risks', 27 oktober 1998, Den Haag).

7. Organohalogeen verbindingen vormen een natuurlijk probleem. Onderscheid dient gemaakt te kunnen worden tussen natuurlijke en anthropogene bronnen alvorens over verontreiniging kan worden gesproken.

8. Bij het bestuderen van effekten op reproductie mag onderzoek naar de eraan ten grondslag liggende mechanismen niet ontbreken.

9. Het 'Eco' van Ecotoxicologie heeft meer betrekking op ecosystemen dan op ecologie.

10. De maatschappij is gebaat bij uitgebreid en gedegen onderzoek naar genetisch gemodificeerde organismen (GMO's). Met het vernietigen en verstoren van experimenten wordt de maatschappij dan ook geen dienst bewezen.

11. Communicatie en regulatie zijn nauw aan elkaar gecorreleerd.

12. De schadelijke werking van TBT op de geslachtsontwikkeling bij mariene gastropoden komt goed 'uit de verf'.

IMPOSEX IN THE COMMON WHELK,

BUCCINUM UNDATUM

Berend Mensink

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IMPOSEX IN THE COMMON WHELK,

BUCCINUM UNDATUM

Bernhard Peter Mensink

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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"...Road of life is rocky,
and you may stumble too,
while you point your fingers
someone else is judging you..."

(Bob Marley, Could you be loved)

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The research described in this thesis comprises laboratory and field studies into development and occurrence of imposex in the common whelk, *Buccinum undatum* L., under chronic exposure to tributyltin (TBT). Emphasis has been put on the occurrence of male sexual characteristics in females, i.e. numbers and shapes, and the physiological consequences. Special attention has been given to juvenile whelks, since their development included differentiation of the sexual organs. This project was carried out at the Department of Marine Biogeochemistry and Toxicology of the Netherlands Institute for Sea Research (NIOZ) and was supported by the Ministry of Transport, Public Works and Water Management, National Institute of Coastal and Marine Management (Rijkswaterstaat-RIKZ) and the Ministry of Housing, Physical Planning and the Environment, Directorate-General for Environmental Protection (VROM-DGM).

In this *Chapter* the context of the research, information on the common whelk, on tributyltin, an introduction to imposex, the objectives and the approach of this thesis will be given.

Context of research

Imposex is defined as the process of the development of male sexual characteristics in female gastropods. For more than a decade, the occurrence of imposex in gastropods was thought to be restricted to areas in the immediate vicinity of the coast close to marinas and harbours, and although first observations were reported in Europe (Blaber 1970), it was quickly found to be a world-wide phenomenon. At least 72 species belonging to 49 genera of mesogastropods and neogastropods have been found to be affected by this phenomenon, but the development and sensitivity show differences between species (Fioroni *et al.*, 1991; Gibbs *et al.*, 1997; Swennen *et al.*, 1997).

In 1991 and 1992 the first findings of *B. undatum* suffering from imposex in the open North Sea were reported and the imposex frequencies correlated well with shipping traffic intensities (ten Hallers-Tjabbes *et al.*, 1994). Furthermore, an increasing number of publications indicated a decline of populations of *B. undatum* over the last decades in some coastal areas around the North Sea, and human activities such as pollution and fisheries were suspected to be the cause (de Vooijs *et al.*, 1991; ten Hallers-Tjabbes *et al.*, 1996a; Cadée *et al.*, 1995; Michaelis, 1996; Rumohr *et al.*, 1998; Nicholson & Evans, 1997; Philippart, 1998).

Studies described in this thesis were undertaken to investigate the cause-effect relationships for these findings in *B. undatum*.

Ecology of the common whelk, *Buccinum undatum* L.

The common whelk, *Buccinum undatum* L., is a benthic predatory snail species, that lives subtidally at the seabed. It preys predominantly on live benthic molluscs, polychaetes, urchins and moribund animals (Himmelman & Hamel, 1993; Dakin, 1912). Its occurrence ranges from the area north of the Bay of Biscay (France) up to the eastern coast of Canada and Arctic. Whelks may live for over 10 years and grow over 11 cm in length (Fahy *et al.*, 1995).

Table 1.1 Classification

Phylum	Mollusca
Class	Gastropoda
Subclass	Prosobranchia
Order	Monotocardia
Suborder	Stenoglossa (Neogastropoda)
Superfamily	Buccinacea
Family	Buccinidae
Genus	Buccinum
Species	undatum

In north-western Europe, whelks generally start reproducing in autumn/winter (September-December), when water temperature decreases. The female produces an egg-mass, consisting of many egg-capsules, which in turn contain hundreds of eggs. When fertilised, only few embryos will develop per egg-capsule while the other eggs are used as nutritional supply by the developing embryos. Depending on temperature and food availability, juveniles will hatch in late winter-spring (February-May) and emerge by eating their way out of the capsule (Hancock, 1967). Juveniles are not dispersed with currents, but stay confined to the area of egg disposition (Thomas & Himmelman, 1988). Some larger fish species like cod eat the smaller whelks, while large North Sea crabs and lobsters attack even adult whelks. They chip and peel the shell until they reach the withdrawn soft parts of the animal (Thomas & Himmelman, 1988). The shell of living animals is a hard substrate for hydroids, sponges and sea-anemones, whereas the empty shell provides housing and protection for adult hermit crabs, *Pagurus bernhardus* (own observations).

Whelk populations are under the influence of human activities. Organotin pollution of the marine environment is the subject of this thesis. In addition, in many countries whelks are consumed as a delicacy and recently whelk fisheries in Ireland and the United Kingdom have increased due to market opportunities in the far East (Fahy *et al.*, 1995; Nicholson & Evans, 1997). Also, long-term flatfish (bottom) fisheries has adversely affected the whelk and other non-target benthic invertebrates (Philippart, 1998). On board of trawlers a great number of damaged shells of non-target animals can be recorded and most of them are discarded (own observations).

Tributyltin and its impact on the environment

Since the early 1970s the biocide tributyltin (TBT) has been in use in marine anti-fouling paints world-wide. These paints proved effective and durable as self-polishing paint systems, from which TBT leached with a constant rate. They kept the hulls of ships free of algae, weeds and banacles for a period up to five years, which would reduce the operational speed and manoeuvrability of the ship a.o. leading to an increase of fuel consumption.

However, the leaching of TBT from anti-fouling paints caused a world-wide contamination, with high concentrations in sediments of ports and marinas and also detectable levels in seawater (Hashimoto *et al.*, 1998; Yamada *et al.*, 1997). TBT residues have been detected in large marine mammals and seabirds (Tanabe *et al.*, 1998; Guruge *et al.*, 1997), indicating a transport right to the top of the food chain even in the depths of the oceans. This indicates that TBT is much more resistant to environmental degradation processes than previously anticipated.

Although the finding of traces of man-made chemicals does not have to be alarming, TBT is toxic already at very low concentrations (ng/l range). Imposex development in gastropods, shell malformations and reduced growth in bivalves are the most sensitive examples of such adverse effects on non-target organisms. At higher TBT concentrations, adverse effects on the immune system are observed in flatfish. Fent (1996) has reviewed the ecotoxicology of organotins for a wide range of organisms.

Environmental policies and TBT regulation

In France, declining oyster cultures and shell malformations in Arcachon Bay (Alzieu, 1991) related to TBT contamination resulted already in 1982 in a ban on the use of TBT on vessels of less than 25m in length. Furthermore, imposex and the decline of dog-whelk populations in the United Kingdom led to a ban of TBT on small vessels in the UK in 1987, where an environmental quality standard for TBT of 2 ng/l for sea water was set. Directive 89/677/EC had similar content and many countries followed the ban. A maximum release rate of 4 μg TBT/cm^2/day (USA) and a minimum size (20 litre) of supply containers (EU) should restrict the use of TBT on larger vessels. At that time, no further measures were considered to be necessary by the Marine Environmental Protection Committee (MEPC) of the International Maritime Organisation (IMO), since it was assumed that concentrations of TBT in open sea were too low to affect marine life. At present this point of view has changed. The 'Esbjerg Declaration (1995)' on the protection of the North Sea and findings of imposex correlated to distance to shipping lanes in south-east Asian waters (Swennen *et al.*, 1997) supported a further restriction of TBT, which had already occurred in Japan (ten Hallers-Tjabbes, 1997). The MEPC has now reached unanimous agreement to impose a global ban on the application of TBT by 1st January 2003 and on its presence by 1st January 2008 (Meeting MEPC 42, 1998).

After the limitations on the use in 1990, the Board for the Authorization of Pesticides (CTB) in The Netherlands concluded unacceptable effects for the environment due to the use of anti-fouling paints based on TBT in 1995 (CTB document C-44.3.3, 7/2/1996). At present, due to the international notification procedures and a change in Dutch legislation, anti-fouling paints based on TBT are allowed for use until 1/1/2003, under the conditions put into the new legislation concerning non-agricultural biocides. This is a transitional provision of the law, which can not act contrary to the resolutions or recommendations of the IMO on this aspect (Staatscourant, 23/1/1998).

Physiology and classification of imposex

The irreversible development of at least a penis and/or a vas deferens (sperm duct) in female gastropods is a world-wide phenomenon, including many species belonging to the orders of mesogastropods and neogastropods. Most of the research was performed with coastal species, because the degree of TBT pollution was relatively high in coastal areas and the accessibility of many of these intertidal species was good. Often they could be easily collected during low-tides in great numbers. In virtually all laboratory experiments only adults have been considered. *Nucella lapillus* (the dogwhelk) is by far the most intensively studied species (e.g. Smith, 1981; Bryan *et al.*, 1986; Gibbs *et al.*, 1988; Stroben, 1994; Oehlmann, 1994). The maintenance of these snails in captivity is not very laborious.

There is a considerable body of literature describing the observed masculine sexual characteristics in female gastropods. Classification for imposex in *N. lapillus* in terms of severity was established by Gibbs *et al.*, (1987) and adjusted by Fioroni *et al.*, (1991) for general use, on the basis of penis size (Relative Penis Size Index = RPSI) and sperm duct development (Vas Deferens Sequence Index = VDSI). However, this classification is not applicable for the masculinization in all species. For example in the periwinkle (*Littorina littorea*) it was found that females do not develop male sexual characteristics next to the female sexual organs, but the pallial oviduct becomes transformed, thereby directly impairing the reproductive output. This different process is termed '*intersex*' (Bauer *et al.*, 1995).

The effects of the development of male sexual organs in females can be deleterious. In *N. lapillus* sperm duct development may lead to a blockage of the genital opening of the female (VDSI $\geq$ 4). This prevents reproduction in two ways; fertilisation by males has become impossible as well as the ability to lay egg-capsules. Obviously, at this stage females have become mechanically sterilised, although egg production may still occur. In that case the capsule gland can rupture resulting in the premature death of the female (Gibbs *et al.*, 1987). Other reported effects in severly affected females are altered ovarian tissues with suppressed oogenesis or even spermatogenesis. The absence of a sperm ingesting gland and the presence of ripe sperm in the vesicula seminalis were also observed (Gibbs *et al.*, 1988).

Female gastropods which become (irreversibly) sterile due to this masculinisation process will lower the recruitment potential of the population. When all females have become sterile, the population will decline and might eventually become extinct, if no migration from adjacent populations occurs.

Sexual development and steroid hormones in marine gastropods

In the marine environment, snail species are either hermaphroditic or gonorchoric (separate sexes). What determines the gender of the species is poorly investigated. Sex chromosomes have been identified only for a few snail species (Vitturi & Catalano, 1984; 1990), but this genetic predisposition of sex determination is rather the exception than the rule (Vitturi, pers. comm., 1995). For some hermaphroditic species factors in their environment (e.g. population density, presence of females, food availability) influence the differentiation to the male or female sex. Others start as male to become female at a later stage (Hoagland, 1978).

The physiology determining the differentiation of sexual organs in marine gastropods is not well understood. Neuropeptides or neuroendocrine mitogenic factors from the cerebral ganglia control cell differentiation in (at least) gonads (leGall *et al.*, 1987; Féral *et al.*, 1987) and the dorsal bodies attached to the cerebral ganglia are considered endocrine organs involved in the control of vitellogenesis and the differentiation of the female reproductive tract (Geraerts & Joosse, 1975). Teunissen *et al.* (1992) identified a cytochrome P450 (CYP10) enzyme in the dorsal bodies of *Lymnaea stagnalis*, which showed the highest similarity with the mitochondrial cytochrome P450 enzymes, known to be involved in the synthesis of hormones such as steroids and vitamin D. Also, in *in vitro* incubations with the dorsal body complex of *Helix pomatia* the presence of the enzyme 3β-hydroxysteroid dehydrogenase was demonstrated (Krusch *et al.*, 1979). However, the endogenous formation of (vertebrate) sexual hormones like testosterone and 17β-oestradiol has not been observed in marine gastropods, although Spooner *et al.* (1991) and Bettin *et al.* (1996) found these hormones in detectable concentrations in *N. lapillus* and the netted dogwhelk, *Hinia reticulata*. Also, in the hermaphroditic gland of the highly evolved terrestrial pulmonate snail *Euhadra peliomphala* testosterone, oestradiol and progesterone were detected by radioimmunoassay (Takeda, 1983). Studies to investigate the potential for the formation and metabolism of these sex hormones in adult gastropods (gonads) have been carried out (e.g. Krusch *et al.*, 1979; Ronis & Mason, 1996), but not in juveniles. Other studies with molluscs showed the presence of several enzymes involved in the metabolic pathway of cholesterol leading to steroid hormones (e.g. Voogt, 1967; deLongcamp *et al.*, 1974; Lupo di Prisco & Dessi'Fulgheri, 1975; Goad, 1976), but these studies could not indicate oestrogen formation in any invertebrate. The presence of steroids may well be dependent on the feeding pattern of the species, since for carnivorous animals it is not necessary to biosynthesize sterols (Voogt, 1967).

The physiological function of the measured sex hormones in marine gastropods is logically assumed to be similar to the function in vertebrates (Matthiessen & Gibbs, 1998), but the information on the endocrinology of this taxonomic group is scanty. In the sea star *Asterias rubens* (echinoderm) progesterone and oestrone in the testis may regulate gametogenesis (Voogt & Dieleman, 1984). In hermaphrodites or gonorchoric gastropods the primary germ cells self differentiate into oogonia and oocytes and the differentiation and maintenance of male cells depend upon factors secreted by cephalic ganglia, whereby dorsal bodies or the optic tentacle could modulate these actions (Lubet & Streiff, 1982). In *Euhadra peliomphala* (hermaphrodite) it has been demonstrated that addition of testosterone to the hermaphroditic gland and organ cultures accelerated spermatogenesis and stimulated male parts such as the prostate gland and sperm duct, while oogenesis was promoted and female parts developed under the influence of oestradiol (Takeda, 1983). Another study showed the necessity of the Central Nervous System (CNS) for the development of a penis in the sting-winkle, *Ocenebra erinacea* (Féral & leGall, 1983). In the latter study the formation of this male sexual organ was indicated to be a neuro-endocrinological process, since it was formed in the absence of gonadal tissue (regulation). Addition of TBT to the culture medium also caused the formation of a penis in females, suggesting TBT influences the neuro-endocrine mechanism responsible for the formation of a penis.

In conclusion it can be stated, that both steroid hormones and endocrinological factors can influence sexual development in marine gastropods, but the way they exert their functions and regulate other processes is not yet clear.

Inductive mechanism of imposex

The physiological mechanism leading to imposex is generally considered to be a disruption of the sex hormonal status by TBT (Matthiessen & Gibbs, 1998). In Bettin *et al.* (1996) and Spooner *et al.* (1991), an increased testosterone level in imposex females (*N. lapillus* & *H. reticulata*) was found. Furthermore, addition of testosterone or an aromatase inhibitor to the water promoted imposex. The addition of both TBT and a competitive androgen to testosterone did not promote imposex, suggesting a possible blockage of receptor sites. Aromatase (CYP19) belongs to the cytochrome P-450 enzyme system in vertebrates known to be involved in the metabolism of both endogenous (e.g. steroids) and exogenous (e.g. TBT) compounds (Fent, 1996). However, aromatase has not been demonstrated *in vivo* in marine gastropods which show imposex, although *in vitro* experiments with *Littorina littorea* indicated the potential for 17-β oestradiol production in that species (Ronis & Mason, 1996). The question remains which mechanism induces the masculine development, since in the study of Bettin *et al.* (1996) testosterone levels increased only after the increase in sperm duct development (VDSI), suggesting that the increase in testosterone level is a secondary effect.

Research with juvenile marine gastropods is scarce. Gibbs *et al.* (1988) and Davies *et al.* (1997a) studied the development of imposex in juvenile *N. lapillus* under chronic TBT exposure. In these studies adult females were collected from the field, at locations where imposex was already present. The juveniles from the control groups (TBT < Level of Determination, LOD) in these studies did show imposex development, which was assumed to be caused by TBT levels below the detection limit or other unidentified factors. Both studies have shown, that juveniles are more sensitive towards imposex development than adults. Although less TBT was accumulated, the incidence of occurrence, the VDSI and RPSI were all higher in the juveniles than in adults. Stroben (1994) found similar results for immature *H. reticulata*. Steroid analysis in juvenile marine gastropods in relation to sexual development has not been carried out.

Objectives and approach of the research

The objectives to elucidate the mechanism and causal relationship for the induction of imposex in *B. undatum* and to explain possible adverse population effects were to investigate:

1. the possible role of TBT in imposex induction in *B. undatum*,
2. the physiological consequences of the observed male sexual characteristics in females,
3. anthropogenic influences which could affect population development in the North Sea.

The approach was two-fold; both laboratory and field studies were performed.

In the laboratory experiments the emphasis was put on investigating a dose-effect relationship for the induction of imposex by long-term exposure to TBT for whelks of different age groups and possible effects on the reproductive performance of these animals. Such experiments with laboratory-reared juvenile *B. undatum* had not been carried out before, neither were any longer term exposure studies with *B. undatum* performed.

In the field studies, the occurrence of imposex in coastal and off-shore waters has been studied in relation to organotin exposure.

To study possible adverse effects of beam trawl fishery on whelk populations, whelks were investigated for direct trawling effects on board of a commercially equipped trawler and consequently were studied in the laboratory for longer-term effects.

CHAPTER 2

REPRODUCTION IN THE LABORATORY AND (SEXUAL) DEVELOPMENT OF JUVENILES

Before this project was started, long-term exposure studies to contaminants with *B. undatum* had never been done. The possibilities to maintain, rear and expose common whelks in captivity needed therefore special attention.

A prerequisite for studying the induction of imposex is the selection of individuals from populations where imposex was absent. The unaffected adult females from the exposure studies described in this thesis were collected in an area (54'29''N, 4'00''E) at the Dogger Bank in the North Sea, where no imposex was found during cruises in 1991 and 1992 (ten Hallers-Tjabbes *et al.*, 1994). Adult whelks were collected in September 1993 and September 1995. After transfer to the laboratory, many whelks died within the first weeks of the acclimation period. This was probably due to the consequences of beam trawling, see also *Chapter 6*. The acclimated whelks successfully reproduced in the storage tanks and in the experimental aquaria. Unique observations on copulation behaviour and egg-disposition were recorded. After hatching, (sexual) development of juvenile whelks was also studied.

Copulation behaviour in the laboratory

At least one male and five females (undamaged shells) were placed together in the experimental aquaria. Directly after transfer, the male was often seen to explore actively the new environment, whilst the females remained more static. A few weeks after transfer of the whelks, in October, copulations were observed. Females attached to the wall of the aquaria as well as females on the floor were visited by the male. The male attached to the shell of the female firmly and seemed to pull round the female rather aggresively. The male extended its penis and inserted it into the mantle cavity of the female. During the copulation, the male proboscis touched the foot of female several times. Duration of the copulation was at least half an hour. The male copulated with several females.

Egg disposition, development and hatching

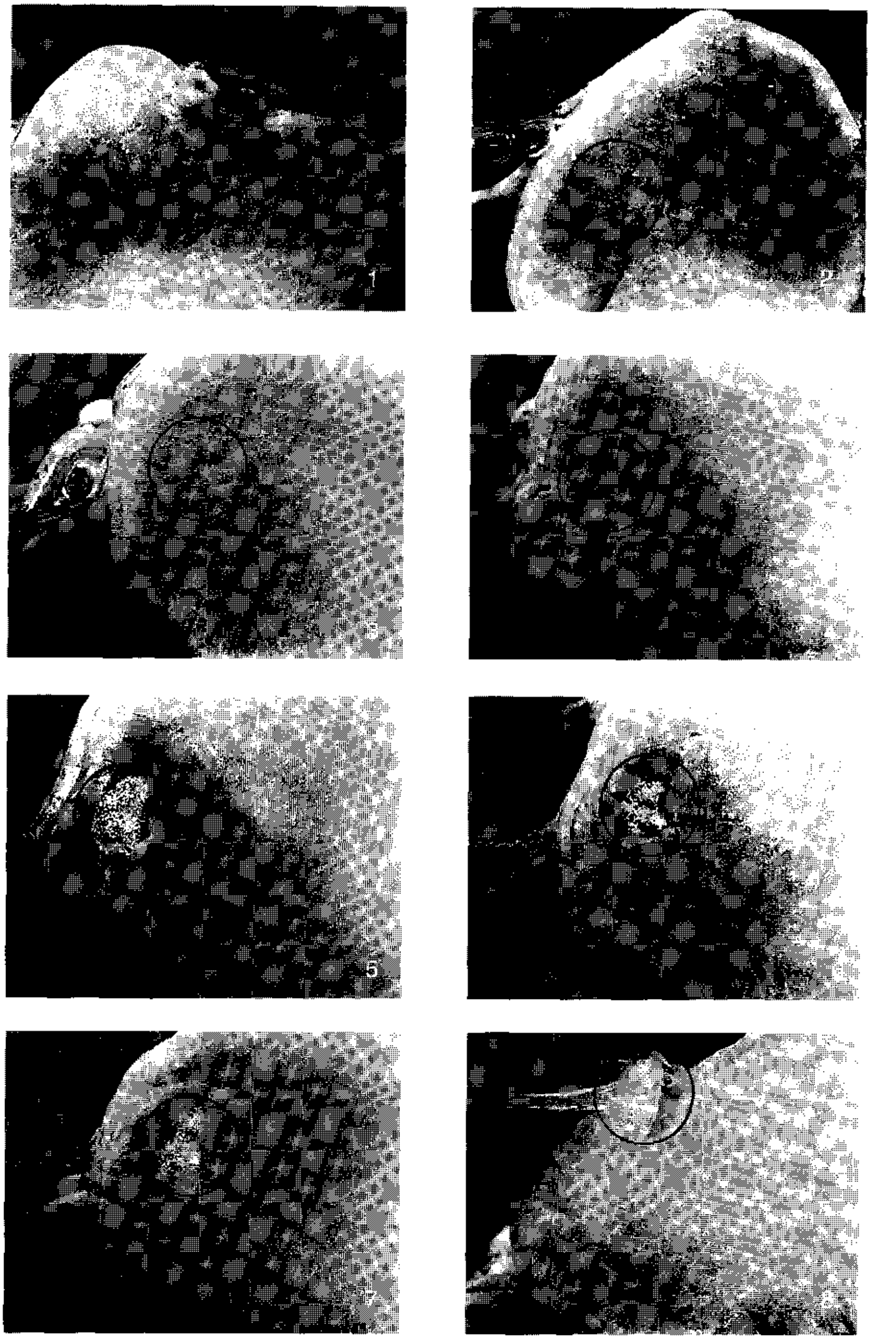
It can not be excluded that the above described females had copulated in the North Sea prior to capture. Females produced egg-masses in the laboratory from the end of October until mid-December (1993) or January (1996), whereas an optimum of produced egg-masses was found in November. In 1996-1997 females from (other parts of) the North Sea were found to produce egg-masses until February.

Both in the storage tanks and in the aquaria the females explored the best sites for egg-disposition. This disposition occurred at the wall close to the water surface and always submerged. There were no apparent characteristics at this elevated position, although water movement was obvious. There was no preference towards light exposure or water flow directions.

When a female selected a favourable site for egg-disposition, a certain period of time elapsed before the actual egg-disposition started. Although not detectable by eye, but visible on photographs (fig. 2.1) the female prepared the position thoroughly. In the foot of the female, assumed glandular tissue became visible and with this part of the foot the glass of the aquaria was (pre-) treated for more than an hour. This specific tissue was located at the anterior part of the foot sole, and measured a diameter of approximately 0.5 cm. When the treatment was completed the female folded the skin to a groove on the right side of the body. The groove connected the genital pore with the glandular spot at the foot. Then, a flexible gelatinous egg-capsule containing hundreds of eggs was transported towards this specific spot, where the egg-capsule was moulded and finished. During the finishing, the assumed glandular cells of the foot were somewhat darker, indicating a different state. After the first egg-capsule the disposition of subsequent egg-capsules took less time, since the location at the glass-wall was already prepared. The indicated female (fig. 2.1) did not attach the second capsule to the first. An egg-mass of at least 50 capsules was deposited within 72 hours.

When one female started its egg production, other females within the same aquaria almost immediately followed, possibly triggered by the release of chemical stimuli. Often these females aggregated and even fought for the 'best' position. When a female had completed her egg-mass, other females attached their egg-capsules to the already present egg-mass. Thus, egg-masses of *B. undatum* can very well originate from more than one female. After disposition the (feeding) activity of the females decreased, suggesting a recovery period, although easily available mussel flesh was still consumed by these females.

Figure 2.1. Egg disposition of a female *Buccinum undatum* in the laboratory. The foot of the female is attached to the glass wall of the aquarium. The photographs display the different steps in the disposition process. In the first photograph (1) the female has taken position at the water surface. In the second and third (2 & 3), assumed glandular tissue in the foot (circle) becomes clear. After about 1 hour the female folds the skin into a groove via which an egg-capsule is transported to the spot (photographs 4 & 5). Then the egg-capsule is shaped (6) and when this egg-capsule is attached to the wall (7), the female starts the process all over, at a position nearby (8).

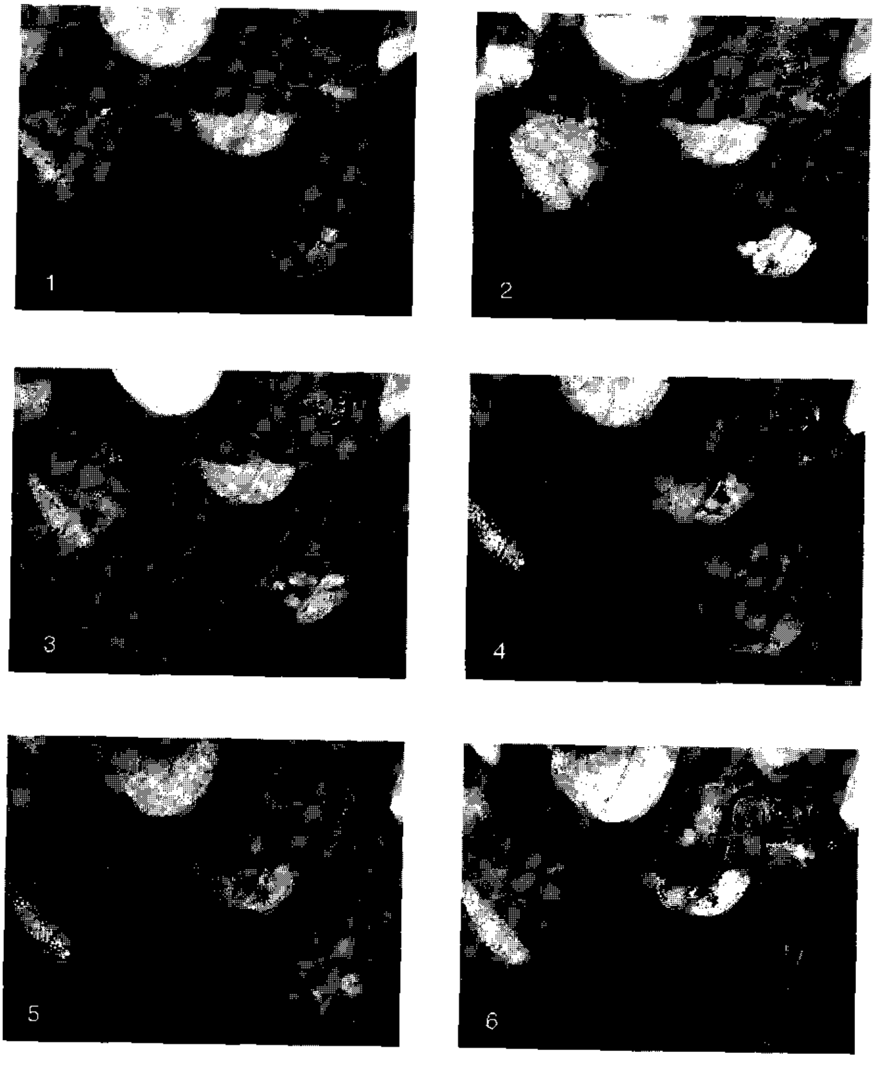


Most egg-masses consisted of at least 50 egg-capsules, which in turn contained hundreds of eggs. The majority of the eggs was used as nurse eggs to feed the embryos (see fig. 2.2). Only five juveniles from the egg-capsule would hatch after they completely consumed the other eggs. In the capsules observed, the numbers of developing young whelks varied from zero to a maximum of 10 individuals.

The time for development in the capsule and for hatching was approximately three months (Nov/Dec-Feb/March) at an ambient temperature of 12°C. When the nurse eggs had all been consumed, the whelks ate their way out and stayed confined to the egg-mass for a few weeks. At hatching the young whelks were approximately 2 mm in length (< 0.01 g in weight). However, as juvenile density increased at the egg-mass, the juveniles started to spread in the aquarium, but were rarely observed at the sediment. Only when mussel flesh was administered as food, the juveniles went actively searching for the food at the sediment. Afterwards most of them (re)climbed the sides of the aquaria. The larger juveniles also burrowed themselves in the thin layer of sand on the bottom. Some juveniles went exploring their environment above the water surface but were sometimes unable to find their way back into the water and dessicated. Also, whelks exploring the effluent tubes did not survive, and the holes of these tubes were later closed for visiting juveniles by placing fine meshed nets in front of the hole.

When an egg-mass was detached from the wall of the aquaria and kept moving freely in the water, juveniles did not develop at all. This may have serious consequences in nature, when whelk egg-masses are torn loose from hard substrates e.g. by strong currents or fishery activities.

Figure 2.2. The development of juvenile *Buccinum undatum in ovo* in the laboratory. The photographs show the development in time of juveniles of an egg-capsule attached to the glass wall of the aquarium. The first photograph shows the development 5 weeks after egg-mass disposition, subsequent photographs were taken weekly. Five juveniles develop in the indicated egg-capsule, of which the nurse eggs are consumed. When all the nurse eggs are eaten, whelks eat their way out of the capsule, but stay confined to the egg-mass.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Juvenile growth and sexual development

Some data on common whelk (> 3 cm) development in north European waters are available (Kideys, 1996), but studies on the growth and sexual development of juvenile *B. undatum* are absent. In the laboratory the juveniles were kept under very favourable conditions, i.e. no need to search for food, no predators present and a relatively constant climate. A comparison with juveniles from the North Sea could not be made, since in the studies it was impossible to collect very young whelks from the sea floor.

Data on growth and sexual development presented here originate from the reference groups of the TBT exposure studies (for details see *Chapter 3*). In Table 2.1 average length and weight with standard deviations (± SD) are given for whelks from the different studies during the first three years of development. From these data a linear relationship between length and age can be seen, while the weight appears to have an exponential relationship with age (see fig. 2.3).

Because whelks extend out of their shell when inverted and exposed to air, the examination of some typical male sexual characteristics can be performed non-invasively. Therefore the development of these male sexual organs could be recorded over time in an elegant non-destructive manner.

The first sexual characteristic to be observed is the start of penis development in juvenile males. This development started in males with a shell length of at least 8 mm and was characterized by a small round knob (stage 1) a few millimeters behind the right tentacle. Another male sexual organ which could be easily detected was the sperm duct *(vas deferens)*. The presence of the sperm duct is characterized by a thin white line on the epithelial surface running from the base of the penis into the mantle cavity. In the scheme for classification of male sexual development for *B. undatum* (see *Chapter 3*, fig. 3.1) the presence of a sperm duct is indicated with a "+" sign additional to the stage of penis development (in numbers). It was observed only in males > 9 mm in length. The increase in penis development coincided with an increasing length of the animals. However, stage 3 (complete penis with penis duct and subterminal papilla) without a sperm duct was never observed in reference males from the laboratory (< 3 years old). Most likely after stage 1+ or 2 (characterized as a small undefined structure, bigger than a knob which can be "lifted" with a pair of tweezers) all males have developed/will develop a sperm duct, hence the final observed stage was 3+.

Histological examination of juvenile whelks from the laboratory (2 years old, 1-4 cm) and from the North Sea (3-4 cm) showed no gonadal development in these animals. These juvenile male whelks from the North Sea (partially) developed a penis, while the seminal vesicle was also being developed. The latter was characterized by development of a convoluted duct in the inner curve of the digestive gland. In these males no sperm duct was observed, while in juvenile males from the laboratory a sperm duct was (sometimes) present. In the examined males and females from the laboratory no other sexual characteristic could be distinguished. In females penis development was always absent and the development of a capsule gland, albumin gland or seminal receptacle had not started at this age (size). It is therefore concluded that in juvenile males secondary sexual characteristics are developed prior to the development of primary sexual organs and that in common whelks < 4 cm the gonads have not yet developed.

A fully developed penis included a penis nerve and duct (extended sperm duct) which ended subterminal at the penis papilla. The penis and sperm duct were both muscular organs. The duct was covered with connective tissue on the outside, followed by two layers of longitudinal and circular muscle fibres respectively; the lumen of the sperm duct was covered by a layer of ciliated epithelial glandular cells.

Table 2.1. Growth of juvenile *B. undatum* during the first three years of development in the laboratory.

study	age (months)	n	average length (mm) ± stdev	average weight (g) ± stdev
TBT exposure exp. > 1993	8 (a)	91	9.3 ± 2.1	0.12± 0.08
	8 (b)	70	10.6 ± 2.2	0.17± 0.08
	14 (a)	17	15.0 ± 3.3	0.45 ± 0.25
	14 (b)	32	15.4 ± 2.9	0.46 ± 0.23
	18 (a)	17	21.4 ± 5.8	1.21 ± 0.94
	18 (b)	29	21.1 ± 4.7	1.17 ± 0.80
	23 (a)	15	29.4 ± 8.6	3.20 ± 2.53
	23 (b)	28	28.1 ± 5.4	2.52 ± 1.13
TBT exposure exp. > 1996	6 (a) *	58	7.2 ± 1.7	0.08 ± 0.04
	6 (b) *	69	7.2 ±1.6	0.07 ± 0.05
	6 (c) **	61	6.9 ± 1.6	0.07 ± 0.04
	6 (d) **	60	7.0 ± 1.6	0.06 ± 0.04
	10 (a)	84	10.2 ± 3.0	0.18 ± 0.14
	10 (b)	80	10.8 ± 2.9	0.21 ± 0.17
	10 (c)	80	9.3 ± 2.2	0.14 ± 0.09
	10 (d)	77	9.8 ± 3.0	0.16 ± 0.14
	21 (a)	114	15.2 ± 4.4	0.57 ± 0.47
	21 (b)	100	15.3 ± 5.1	0.59 ± 0.61
	21 (c)	91	15.1 ± 4.8	0.54 ± 0.47
	21 (d)	78	16.4 ± 6.1	0.75 ± 0.75
egg-exposure experiment	7	64	8.7 ± 2.1	0.10 ± 0.05
	11	61	12.3 ± 2.3	0.24 ± 0.12
	18	60	17.5 ± 4.4	0.74 ± 0.52
TBT two-year old females > 1996	28	30	29.8 ± 6.9	3.48 ± 2.44
	30	29	29.9 ± 7.0	3.79 ± 2.76
	32	27	31.8 ± 7.6	4.43 ± 3.16
	35	17	36.4 ± 8.1	6.04 ± 3.63

* a,b,c,d represent replicates per reference treatment

** c,d are artificial seawater replicates (see *Chapter 3*)

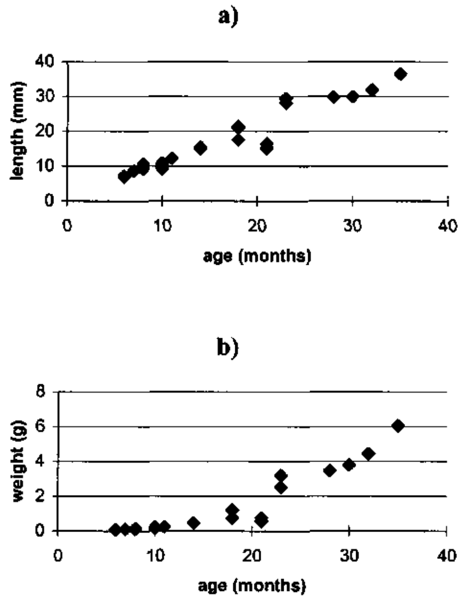

Figure 2.3 Relationships between the length (a) and weight (b) and the age of juvenile *B. undatum* during the first three years of development in the laboratory.

CHAPTER 3

IMPOSEX INDUCTION IN LABORATORY REARED JUVENILE *BUCCINUM UNDATUM* BY TRIBUTYLTIN (TBT).

Environmental Toxicology and Pharmacology (submitted)
Co-Authors : H. Kralt, A.D. Vethaak, C.C. ten Hallers-Tjabbes, J.H. Koeman, B. van Hattum & J.P. Boon

Abstract

Here we report a series of experiments on the development and occurrence of imposex in the common whelk, *Buccinum undatum*, under the influence of (chronic) exposure to butyltin compounds. The main objective of the experiments was to obtain more information about the effects of organotin compounds in the marine environment, which possibly relate to the reported decline of *B. undatum* in Dutch coastal waters.

In these studies tributyltin (TBT) dose-dependently induced the development of male sexual organs in juvenile whelks. A TBT concentration > 7 ngSn/l induced imposex in juvenile whelks, indicating that *B. undatum* is less sensitive than *N. lapillus*. Growth in TBT-exposed juvenile whelks was significantly reduced compared to the reference group at a nominal TBT dose ≥ 4 ngSn/l in one of the exposure studies. After 5 years in the laboratory, reproduction (egg-laying) was only observed in both reference aquaria. Thus, TBT might impair whelk reproduction through growth reduction. The consequences for common whelks at a population level in their natural environment need to be investigated.

The study showed differences in sensitivity towards imposex development in different life-stages. Juveniles were the most sensitive, adolescent females also responded, but adult females did not respond to TBT exposure, although they dose-dependently increased their organotin (OT) body-burden when exposed. TBT exposure of developing whelks only in the *in ovo* stage, did not result in an increased masculinisation compared to non-exposed whelks.

Histological studies showed no sterilisation due to mechanical blockage of the (adult) female genital opening by sperm-duct tissue. Gonadal development in two year old juveniles was not observed in common whelks. This means, that hormones synthesized in the gonads can not play a role in the differentiation of a penis and a *vas deferens*, which already occurred in the first few months after hatching. No other sexual characteristics than those already visible with the eye were found.

Microsomal fractions of common whelks were exposed to TBT to investigate a possible influence of TBT on the cytochrome P450 enzyme system. Inactivation of CYP450 to its inactive form CYP420 was observed in the same manner as occurring in fish.

Introduction

Common whelks *(Buccinum undatum)* from the open North Sea show imposex: the development of male sexual characteristics (i.e. a penis and a sperm duct) in female prosobranch gastropods. The active biocide in anti-fouling paints, tributyltin (TBT), was expected to be the cause, since a good correlation with shipping intensities was established (ten Hallers-Tjabbes *et al.*, 1994) and similar effects due to TBT exposure have been reported for other marine gastropods. Laboratory experiments with the dog-whelk, *Nucella lapillus* (Gibbs *et al.*, 1987) and the netted dog-whelk *Hinia reticulata* (Stroben *et al.*, 1992) which live in the immediate vicinity of the coast-line, have clearly shown that TBT promotes imposex in these species at concentrations ≥ 1 ng Sn/l. However, laboratory exposure studies on a possible cause-effect relationship for imposex are limited. Because TBT concentration levels were assumed to be low in the open North Sea, common whelks were expected to be at least as sensitive to TBT as *N. lapillus* and *H. reticulata*. The responses of snail species to TBT exposure can differ and depend on the prevailing TBT concentration (Gibbs *et al.*, 1997). Severe adverse effects on reproduction (sterility) of the imposex females of *N. lapillus* and *Ocenebra erinacea* are well known and described (Gibbs *et al.*, 1991). The common whelk has been reported to be in decline in some areas in and around the North Sea (ten Hallers-Tjabbes *et al.*, 1996a; Cadée *et al.*, 1995). A possible role of TBT in this decline needed investigation.

To date no laboratory exposure studies have been reported for *B. undatum*. Special efforts to optimize the conditions to breed whelks had to be made, since it was the objective to study several life stages of whelks, including the early life stages.
To study the cause-effect relationship for *B. undatum*, adult and juvenile common whelks were experimentally exposed to TBT. In a first experiment (*Exp.1*) common whelks were exposed to three different TBT doses. Adult *B. undatum* reproduced and their developing juveniles (*in ovo*) were also exposed to test the importance of life-stage with respect to imposex development. Animals did not have to be sacrificed during the experiments, since whelks crawl out of their shell when exposed to air. Therefore the developmental stages of male sexual characteristics could elegantly be recorded over time. A confirmation experiment with artificial seawater (*Exp.2*) was performed to see whether Wadden Sea water had confounded the results from *Experiment 1*. Another experiment was conducted to test whether exposure to a high TBT concentration *in ovo* (*Exp.3*) already has an influence on the development of juvenile whelks. To test whether sensitivity towards TBT exposure differs in different life stages (*Exp.4*), 2½ year old female whelks were also exposed.

Because external characteristics give insufficient information about the health status of the animal, adult and juvenile whelks were sampled and processed for (organ) structural and functional analysis. Morphological changes possibly affecting reproduction could in this way be discriminated.

Also, observations were made regarding possible effects of TBT on the activity of biotransformation enzymes, which are known to be involved in the transformation of both endogenous (e.g. fatty acids and steroids) and exogenous (e.g. PCB and PAH) compounds in vertebrates. Bettin *et al.* (1996) suggested that TBT inhibits aromatase (CYP19) activity in marine gastropods thereby disturbing steroid hormone levels, which would lead to imposex. Fent & Stegeman (1993) and Morcillo & Porte (1997) reported interactions of TBT with different elements of the cytochrome P450 system in fish, bivalves and *Thais haemastoma* (gastropod). To study a potential interaction of TBT with the whelk CYP450 enzyme system, microsomal fractions of *B. undatum* were exposed to different doses.

Methods

I Exposure studies

ORIGIN OF ANIMALS

Experiment 1

Adult *B. undatum* were collected from the North Sea near the Doggerbank in September 1993. At this station (54°29'N; 4°00'E) no imposex had been found during former cruises (ten Hallers-Tjabbes *et al.*, 1994).

Adult females produced egg masses during the acclimation period in all groups (all 8 aquaria) in November/December 1993. Each experimental aquarium contained five adult females and at least one male. In February 1994, two months after the start of TBT administration and the production of egg-masses by the adult whelks, the young whelks hatched.

Experiment 2

In the confirmation experiment with only juvenile whelks, a similar set-up was chosen as in *Exp.1*. Adults were collected near the Doggerbank in September 1995, they produced their egg-masses in the experimental aquaria (Dec 1995-Jan 1996) after which the adults were removed and the juveniles equally (120) and at random divided among the experimental aquaria. Juvenile whelks were 6 months old when the TBT exposure was initiated.

Experiment 3

The egg-masses used in this *in ovo* experiment were produced by the same adult whelks as in *Exp.2*.

Experiment 4

Other adult females (Doggerbank, September 1993) also produced egg-masses in storage tanks and their juveniles were reared in similar physical and nutritional conditions as described below for the experiments (except now the aquaria only received Wadden Sea water) for two and a half years before they were first used in a TBT exposure experiment.

EXPERIMENTAL CONDITIONS

In all experiments, a waterflow of 80 l/day per aquarium (50 dm^3), a 12h light-12h darkness regime and an air temperature of 12°C was applied. In each aquarium a thin layer (± 1 cm) of course sand was used as sediment. Fresh mussel meat (*Mytilus edulis)* was fed *ad libitum* once a week.

Experiment 1

TBT was administered to the experimental aquaria by dissolving tributyltinacetate (TBTAc, > 97.5% purity, Aldrich Chemical co., Wisconsin, USA) in demineralised water (stock solutions); these solutions were diluted 1 : 100 with Wadden Sea water (salinity 30‰, pH 8.3) in a mixing chamber to nominal concentrations of 1 µg TBTAc/l, 100 and 10 ng TBTAc/l respectively. A reference group received only a 1 : 100 dilution of demineralised water. These solutions were continuously administered in duplicate for each concentration level. The exposure to the different TBT concentrations started in the second week of December 1993 and ended in April 1998.

Experiment 2

Artificial seawater was prepared by dissolving sea salt (Aqua Medic Sea Salt, Bad Essen, Germany, 20 kg-600 l) in tap water to obtain sea water comparable to Wadden Sea water (salinity 30±1 ‰, pH=8.0-8.5). TBT nominal concentrations were prepared as described in *Exp.1*. Four different groups were exposed to nominal concentrations of 500 ngTBTAc/l, 100, 50 and 10 ngTBTAc/l respectively. One reference group received a 1: 100 dilution of artificial sea water with demineralised water (reference), the other reference received a 1: 100 dilution of Wadden Sea with demineralised water (Wadden Sea). All treatments were carried out in duplicate. TBT exposure was initiated August 1996 and lasted till November 1997, when whelks were sacrificed.

Experiment 3

Two egg masses were exposed to either a 1 : 100 dilution of demineralised water with Wadden Sea water or a nominal TBT concentration of 1000 ngTBTAc/l prepared as described above. The egg-masses were exposed during two months (December 1994-February 1995). When the first juvenile whelks emerged from these egg-masses (Feb 1995), two egg-masses were translocated. One egg-mass of the reference group was transferred to a high TBT dose aquarium and an egg-mass of the high TBT dosed group was transferred to a reference aquarium. Whelks were sacrificed when they were 38 months old (April 1998).

Experiment 4

In July 1996, two and a half year old laboratory-reared common whelks which showed no male sexual characteristics prior to exposure to TBT, were divided in two groups of about thirty individuals. One group received a 1 : 100 dilution of demineralised water with Wadden Sea water, the other a nominal dose of 100 ngTBTAc/l prepared as described above. The exposure lasted till January 1997 (7 months).

ANALYSIS OF BUTYLTIN COMPOUNDS

To determine the actual butyltin concentrations, water samples were acidified with 30 % HCl (1 ml per litre) (Merck, Germany) prior to storage at 4°C. Then, water samples were analysed by means of gas-chromatography combined with atomic absorption detection after on-line hydride generation and cryogenic trapping on a gas chromatographic column (Ritsema & Laane, 1991). Analyses were conducted at the RIKZ (Rijkswaterstaat) laboratory in Haren, The Netherlands.

Butyltin concentrations in animals from the first experiment were measured in pentylated extracts applying combined gas-chromatography and mass-spectrometry with ion trap detection (GC/ITD-MS). Details of the method, including quality control, performance characteristics and detection limits are extensively described in Mensink *et al.* (1997a), see *Chapter 5*. Analyses were carried out at the Institute for Environmental Studies (IVM, Vrije Universiteit), Amsterdam, The Netherlands.

DETERMINATION OF SEXUAL DEVELOPMENT

The animals could be sexed by exposing them to air; since they react by extending out of their shells, thereby showing some of their sexual organs. The different stages of (sexual) development of a penis were characterized as follows: (see also figure 3.1)

0 : no male sexual characteristics
1 : small round bud at the site where males grow a penis
2 : enlargement to various shaped structures, the tip of the structure is loose from the body
3 : development of a curved penis similar in shape but of smaller size than in adult males

The presence of a (partial) *vas deferens* in addition to a penis is indicated with a "+" sign.

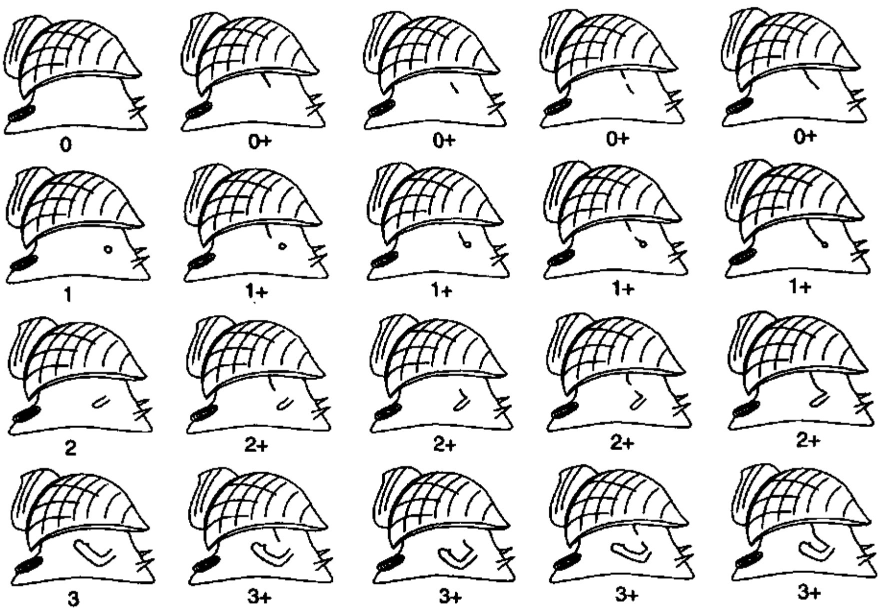

Figure 3.1. Possible stages of the development of male sexual characteristics in *Buccinum undatum*.

STATISTICAL TESTING

Statistical analysis of the data of *Exp.1* and *Exp.2* was performed by one-way ANOVA. For sexual development, a log(P/1-P) transformation of the percentages with male sexual characteristics was used. To test significance of differences in length, also log transformation of the data was used. Since the differences in weight were greater than the differences in length and because weight is closely related to the length, only length has statistically been tested.

For *Exp.3* and *Exp.4* 2x2 Chi-square testing of the log transformed data was performed.

II Histological study

ORIGIN OF THE ANIMALS

Adult females (>6 cm), adolescent (4-6 cm) and juvenile whelks (3-4 cm) from the Dogger Bank area in the North Sea were used as reference animals. No imposex had been found during former cruises in this area (ten Hallers-Tjabbes *et al.*, 1994). Females with imposex were collected at the Eastern Scheldt ('Hammen'). In this coastal area, more than 90% of the females showed imposex of which over 50% showed the more advanced stages of imposex (stage 3 or 3+) (Mensink *et al.*, 1996a; 1997a).

Juveniles (1-4 cm) from the reference and exposed groups of *Experiment 1* (**part I**) were used to determine the development of genital ducts and thus their sex. These juveniles were 2 years old when used in the histological study.

Adolescent animals from the Dogger Bank (North Sea) were used as reference; all reproductive organs were present but not fully developed/matured. Animals were all sampled in the reproductive period (November 1995-February 1996).

PREPARATION OF THE SAMPLES

The whelks were narcotised in a 7% $MgCl_2.6H_2O$ solution for at least 30 min. prior to dissection. Of adult and adolescent animals, only the reproductive organs were investigated. Juveniles were sampled as a whole. Tissues were fixed in Bouin's fluid. Dehydration was performed using increasing concentrations of ethanol. Dehydrated samples were embedded via isopropanol and xylene in paraffin. Serial sections (7 µm) were made using a Reichert (nr. 15403) microtome. Rehydration was achieved by a reverse order of the dehydration procedure after which sections were stained with haemaluin-eosine and enclosed with entellan (Merck). Sections were examined using a Carl Zeiss microscope (nr. 65266).

III Cytochrome P450 investigations

Common whelks were collected at the Dogger Bank and then kept under the laboratory conditions as previously described. For the preparation of microsomal samples, the shells were cracked with a vice or adjustable wrench and the whelks were killed by decapitation. Digestive glands (without ovary or testis) and kidneys were removed from the visceral mass. All procedures were carried out on ice. The fresh tissue samples were weighed and homogenised in a freshly prepared (pH= 7.4) potassium phosphate buffer (1:3) containing 0.1 M K_2HPO_4, 0.15 M KCl, 1 mM EDTA, 1 mM DTT, 20% v/v glycerol. An Ultra Turrax and Potter teflon homogenizer were used to homogenise the samples.

The homogenate was then centrifuged for 20 min at 12,500g in the ultra centrifuge Centrikon T-1080. The supernatant was centrifuged at 100,000g for 1 hour. The pellet was then resuspended in resuspension buffer (homogenisation buffer without KCl) and again centrifuged at 100,000g for 1 hour. The final pellets were resuspended in resuspension buffer and homogenized with a Potter homogenizer. The fractions were directly frozen in liquid nitrogen and stored at -80 °C.

BSA (bovine serum albumin, Sigma diagnostics) and PMSF (phenyl methyl sulphonyl fluoride, Sigma diagnostics) were also used in efforts to improve the CYP450/CYP420 ratio in favour of CYP450. Dab (*Limanda limanda*) liver microsomes were also used to check whether this method showed results comparable to those reported by Fent (1996). Protein content of the microsomal fraction was determined according to Bradford (1976) using BSA as standard protein.

To determine the cytochrome P450 spectra, 2.5 ml microsomal samples (protein content 1-2 mg/ml) were used. Two types of difference spectra were tested and recorded using a dual-beam spectrophotometer (Perkin-Elmer, λ-6):
I. CO-difference spectrum of 2 to 2 sodium dithionite reduced samples
II. Sodium dithionite difference spectra of two CO treated samples.

Incubation studies were performed to investigate whether TBT would bind to or react with CYP450. This could be observed by substrate induced difference spectra and substrate induced cytochrome P450 spectra, using varying concentrations of TBTAc dissolved in ethanol (96%). Incubations with TBT were carried out at 26 °C for a maximum of 30 min. according to Fent & Stegeman (1993). The maximal ethanol concentration in the cuvette was 3%.

To study an effect of a substrate on the CYP450 activity of the microsomal fraction, a CYP450 spectrum was recorded (400-500 nm). Then the substrate (TBTAc) was added and a new CYP450 spectrum was directly determined. A reaction with or inactivation of CYP450 could be determined by comparison of the peaks occurring at 450 and 420 nm. To enable a good comparison between several spectra the absorbances at 490 nm were mathematically standardised at 1.

Table 3.1. Whole body butyltin concentrations (in ng Sn/g wet wt.) of experimentally exposed common whelks, *B. undatum*. Analysis was done after 1, 2, 3, 4 and 11 months of exposure to different doses of TBTAc.

Butyltin	nominal dose (ng TBTAc.l^{-1})	month 1	2	3	4	11
	control	<2*, <2	<2, <2			<2
	10				<2,<2,<2	**4**,<2,<2,<2,<2
TBT	100	<2	<2, <2		<2	**3**, <2
	1000	<2, **6**	**8**	**32**		**140, 107, 68**
	control	<1, <1	<1, <1			**6**
	10				**8**, <1, <1,	**8,8**,<1,<1, <1
DBT	100	<1	<1, <1		<1	**25, 22**
	1000	<1, **14**	**26**	**95**		**503, 536, 509**
	control	<1, <1	<1, <1			**3**
	10				**2, 3**, <1	**6**,<1,<1,<1,<1
MBT	100	<1	**5, 3**		**5**	**13, 14**
	1000	<1, <1	**21**	**41**		**244, 197, 227**

*<1, <2 means below detection level of 1 or 2 ng Sn/g wet wt.

Results

It should be mentioned, that for the first time attempts to rear, maintain and expose different life stages of the common whelk in the laboratory over a period of several years succeeded.

I Exposure studies

Experiment1

None of the adult females exposed to nominally 10, 100 or 1µg/l TBT showed any sign of imposex after 11 months, although a dose-dependent increase in body burden of TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) over time was observed (Table 3.1). In the control group, only low concentrations of DBT (6 ng Sn/g wet weight) and MBT (3 ng Sn/g ww) could be detected, but TBT concentrations were below detection limit (< 2 ng Sn/g ww). In the low dose group a similar pattern as in the control group could be observed, whilst in the 100 ng TBTAc/l group concentrations of 23 and 14 ng Sn/g ww were found after 11 months for DBT and MBT respectively. The high dose group showed detectable TBT body burdens already after 1 month, which increased over time to an average of 100 ng Sn/g ww. after 11 months of exposure. Metabolites also increased to around 520 ng Sn/g ww. and 225 ng Sn/g ww. for DBT and MBT respectively.

In contrast to these adults, their offspring showed significant ($P < 0.05$) differences in sexual development (fig. 3.2), when exposed in the same manner as their parents. The juveniles from the control and the low dose (10 ng TBTAc/l) groups showed comparable penis development. After two years of exposure the male to female ratio (based on the animals with male sexual characteristics) converged to 1. This pointed to a life stage where these sexual organs are basically present and only a further growth of these organs will occur in the future (figures 3.3a-d).

When exposed to a nominal dose of 100 ng TBTAc/l for 8 months, 54% of the juveniles showed the development of a penis and/or *vas deferens*. This percentage increased to 100 % after 14 months of exposure. During the investigations, a steady increase in the stages of penis development was observed (fig. 3.3c). After 8 months, when actual TBT concentrations had not exceeded 20 ngTBTAc/l (fig. 3.5c), 34 % of the animals showed the first stage of penis development. Nearly all animals showed a small structure or a penis-homologue both with a *vas deferens* after 14 months, whilst two years after the start of the experiment 90 % of the juveniles showed a penis with a *vas deferens*.

At the highest experimental dose of 1 µg TBTAc/l, virtually all animals had a fully developed stage of penis development in all exposed animals already after 8 months. After 10 months of exposure even animals with a double penis were found in this group as well as other aberrations such as a split end of the penis and a thick penis"bed". In this group even the smallest whelk formed a penis, whilst in the other groups only the larger specimens showed penis development. During this study, no decrease in the percentage of animals (100%) with male sexual characteristics was observed nor a decrease in the developmental stage of male sexual organs. Nearly all animals showed a penis and a *vas deferens*, but one whelk developed a *vas deferens* only.

One-way ANOVA testing on the percentages of juveniles with male sexual characteristics showed significant ($P \leq 0.001$) differences between groups at all sampling times. *Post-hoc* comparisons showed no differences ($P > 0.5$) between the reference and low dose group. However, the high dose group at all times had significantly ($P < 0.001$) higher % of juveniles with male sexual characteristics than the control group.

After 14, 18 and 23 months significant differences ($P < 0.05$) in growth (fig. 3.4) occurred. The average length in the control group was significantly higher than in the TBT exposed groups ($P < 0.01$), but all TBT-exposed groups did not differ significantly from each other ($P > 0.3$).

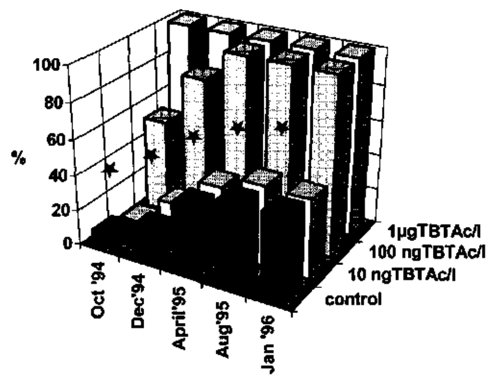

Figure 3.2. Sexual development, expressed as the percentage with male sexual characteristics, of juvenile *B. undatum* during the first two years.
** means significantly lower than the TBT exposed groups ($P < 0.01$).*

The dose levels mentioned above concern the theoretically calculated exposure levels. The actual levels in the experiment however, can differ considerable from these values for reasons of e.g. adsorption and/or degradation. Actual butyltin concentrations in water varied for the different groups (figs 3.5, 3.6 & 3.7). In the beginning of the experiment both the reference and the low dose group occasionally showed detectable (1-10 ng/l) levels of TBTAc. During the experiment, these concentrations reached TBT levels equivalent to 30 ng TBTAc/l at the most for the control group and 40 ng TBTAc/l for the low dose group (fig. 3.5a,b). The concentrations in the medium dose group ranged from below detection limit to 20 ng TBTAc/l in the first 40 weeks to 40 ng/l after 11 months. Only after one full year of exposure concentrations increased to the nominal dose of 100 ng/l (fig. 3.5c). In the highest dose actual concentrations ranged from 35 to about 2000 ng TBTAc/l (fig. 3.5d). The presence of TBT, DBT and MBT in the control group might be caused by the use of Wadden Sea water in the experimental design, since butyltin levels measured were comparable with other butyltin measurements in the Wadden Sea (A. de Jong, pers. comm., 1996).

So, in the low and medium dosed groups the actual TBT concentrations were often below the nominal concentrations in the beginning of the experiment, probably due to adsorption and metabolism, since analysis of stock solutions showed that these were correctly prepared.

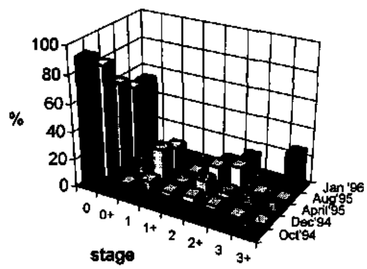

Fig. 3.3a. Control group.

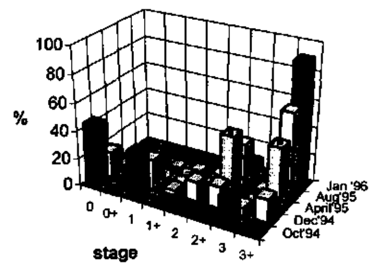

Fig. 3.3c. Dose : 100 ng TBTAc/l.

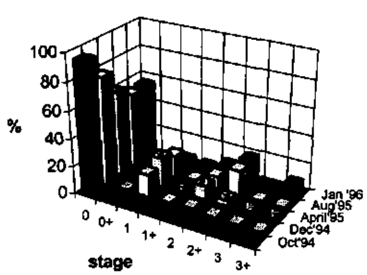

Fig. 3.3b. Dose : 10 ng TBTAc/l

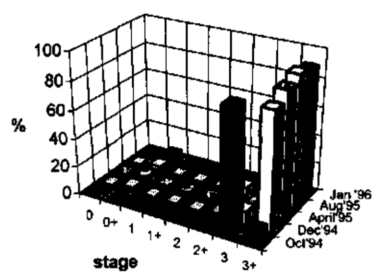

Fig. 3.3d. Dose : 1 µg TBTAc/l

Figures 3.3a-d. Stages of the development of male sexual characteristics in juvenile *B. undatum* for different TBT doses during the first two years.

After two years of exposure the survival percentages were 66 % (n = 212) for the low dose group, 74 % (n = 120) for the medium dose group and 60 % (n = 27) for the highest TBT dose. An aeration failure after 9 months caused the death of many juvenile whelks in the control group. The remaining whelks were then again equally divided over the two aquaria. This was the main reason that only 28 % (n = 43) of the initial number of whelks survived in this group.

After 5 years in the experimental design, only two reference females (one in each aquarium) produced egg-capsules in April-May 1998. These females were the largest animals (> 7 cm) in the aquaria. However, no juveniles hatched from the eggs. None of the TBT-exposed animals produced eggs.

This experiment shows, that TBT dose-dependently induced the development of male sexual characteristics in juvenile whelks at actual TBT concentrations > 7 ngSn/l, however adult females did not show any male sexual development when exposed.

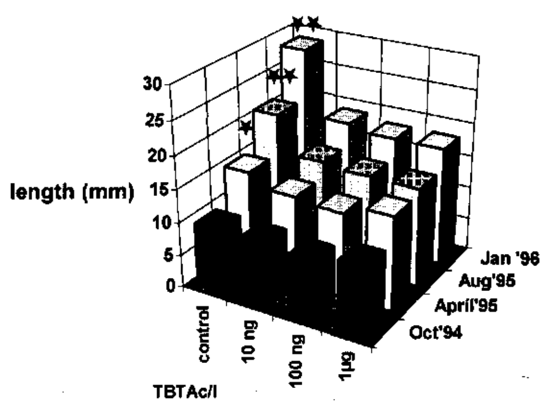

Figure 3.4. Average length for the experimental groups.
** means significantly higher than TBT exposed groups (P< 0.01).*
*** means significantly higher than TBT exposed groups (P = 0.001)*

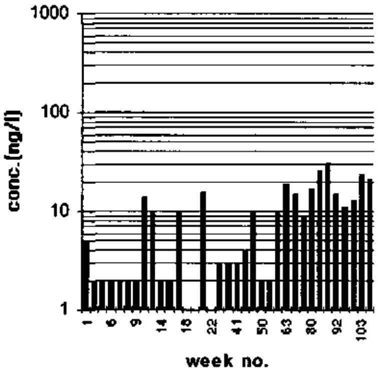

Fig. 3.5a. Control group

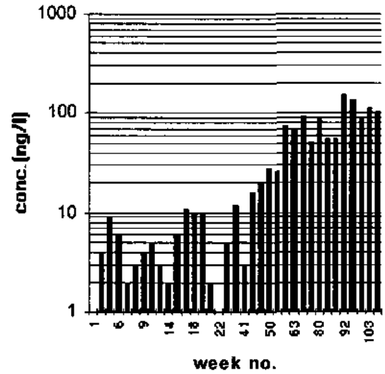

Fig. 3.5c. Dose : 100 ng TBTAc/l

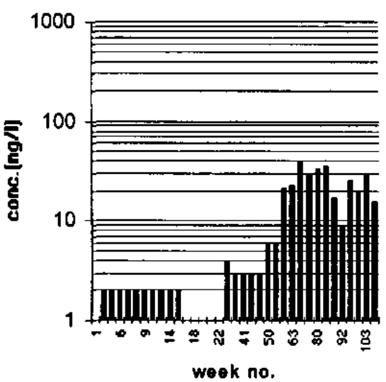

Fig. 3.5b. Dose : 10 ng TBTAc/l

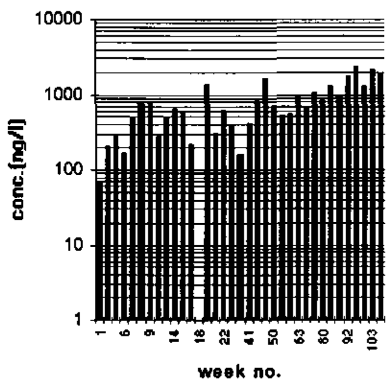

Fig. 3.5d. Dose : 1 μg TBTAc/l

Figures 3.5a-d. Actual TBTAc concentrations (in ng/l) for the TBT exposed groups during the experimental period.

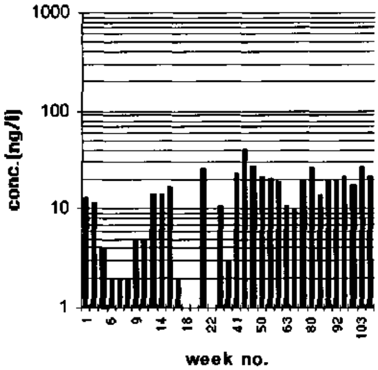

Fig. 3.6a. Control group

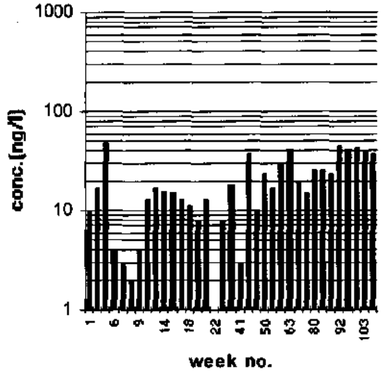

Fig. 3.6c. Dose : 100 ng TBTAc/l

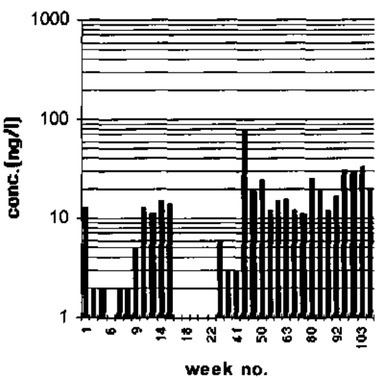

Fig. 3.6b. Dose : 10 ng TBTAc/l

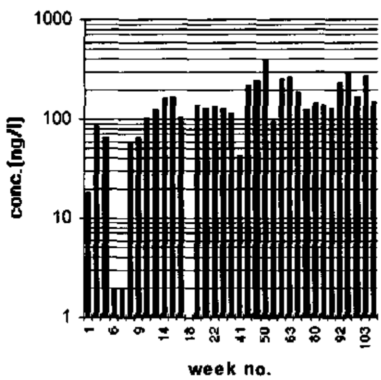

Fig. 3.6d. Dose : 1 μg TBTAc/l

Figures 3.6a-d. Actual DBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

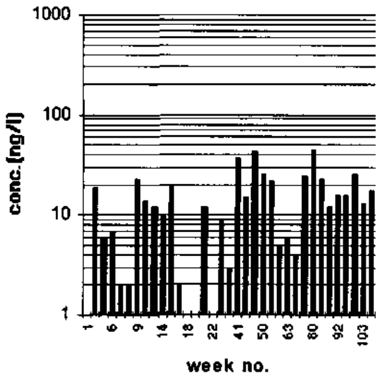

Fig. 3.7a. Control group

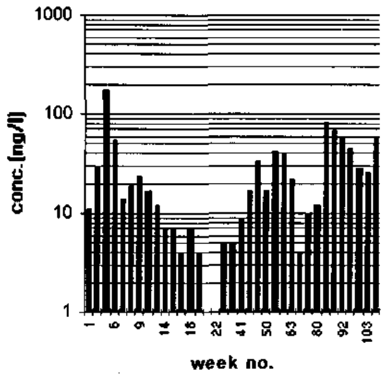

Fig. 3.7c. Dose : 100 ng TBTAc/l

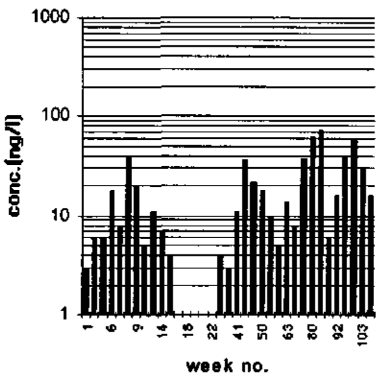

Fig. 3.7b. Dose : 10 ng TBTAc/l

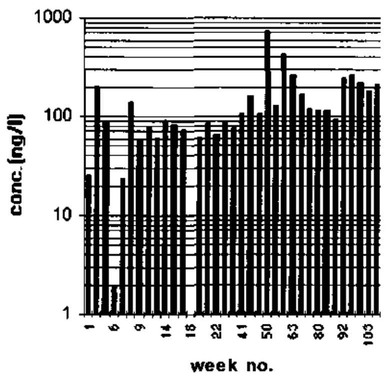

Fig. 3.7d. Dose : 1 µg TBTAc/l

Figures 3.7a-d. Actual MBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

Experiment 2

Male sexual development in the different groups is shown in figure 3.8. A steady dose-dependent increase in the percentage of animals with male sexual characteristics and in the stage of male sexual development of these juveniles could be distinguished (figs. 3.9 a-f). After 4 and 14 months of exposure, significant ($P \leq 0.001$) effects in sexual development between the groups occurred. The Wadden Sea and reference group showed a significant lower percentage of animals with male sexual characteristics than the TBT exposed groups ($P < 0.001$), but they were not different ($P > 0.3$) from each other, as were the 100 and 500 ng TBTAc/l groups ($P > 0.2$). The 10 ng TBTAc/l group showed significantly ($P \leq 0.001$) lower % with male sexual characteristics than the 50 ngTBTAc/l group.

After 14 months of exposure, nearly all (≥ 99%) juveniles in the 50, 100 & 500 ngTBTAc/l groups showed male sexual development. Only in the highest dosed group, abnormalities in penis development were observed, e.g. double penises, excrescences and a penis with two penis openings were found. The reference group, Wadden Sea group and 10 ngTBTAc/l group showed virtually the same percentage of juveniles with male sexual characteristics (38, 40 and 39% respectively).

Only in the highest dosed groups (≥ 50 ngTBTAc/l), juveniles with only a developing *vas deferens* (stage 0+) were observed. In the low dosed group, Wadden Sea group and reference group neither stage 3 or 3+ were found, and a more advanced stage of penis development coincided with an increased shell length in these categories. This relation was, however, not found for the high-dosed groups, which showed an equal average length distribution among the stages of male sexual development.

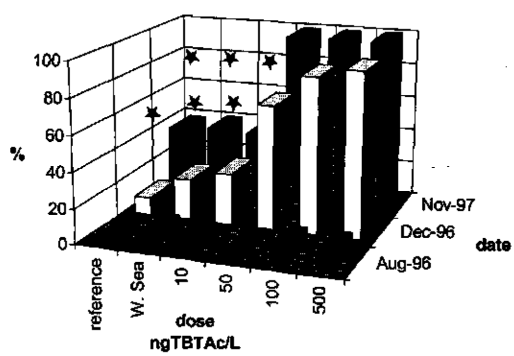

Figure 3.8. Percentage of juvenile *B. undatum* with male sexual characteristics for different treated groups.

** means significantly lower (P ≤0.001) than TBT exposed groups or 50 ng TBTAc/l (see text for explanation).*

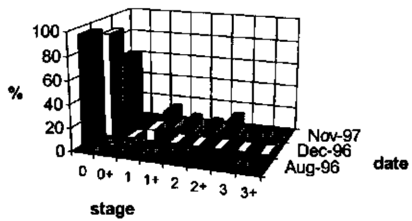

Fig.3.9a = reference

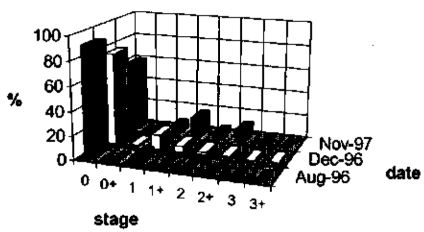

Fig.3.9b = Wadden Sea

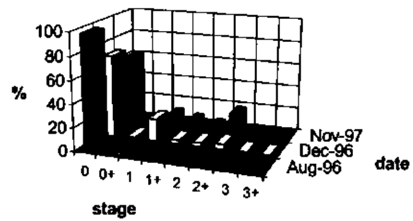

Fig.3.9c = 10 ngTBTAc/l

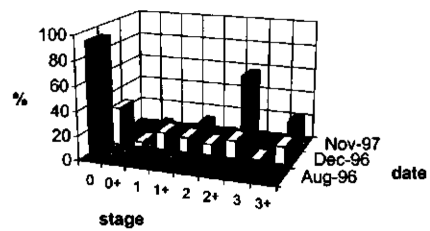

Fig.3.9d = 50 ngTBTAc/l

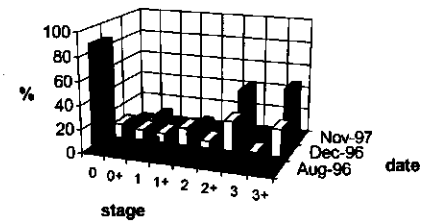

Fig.3.9e = 100 ngTBTAc/l

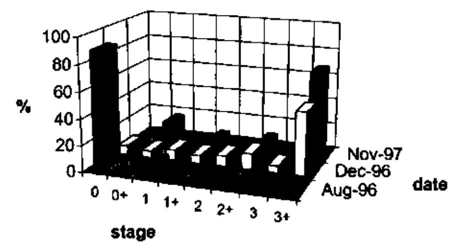

Fig.3.9f = 500 ngTBTAc/l

Figures 3.9a-f. Stages of the development of male sexual characteristics in juvenile *B. undatum* for different treated groups.

Average lengths for the different groups did not significantly ($P > 0.1$) differ. After 14 months, for all doses at least 118 juveniles per dose group were examined. Due to a technical problem after 4 months, only 49% of the 50 ngTBTAc/l dosed group survived, while in the other groups survival was > 68%.

During the experiment, average tributyltin concentrations for the reference, Wadden Sea, 10, 50, 100 and 500 ngTBTAc/l groups were 6.2, 9.0, 12, 46, 30 and 150 ngSn/l respectively. The increased average TBT concentration for the 50 ngTBTAc/l group was a result of the technical problem already mentioned.

So, within 4 months of exposure at increasing actual TBT concentrations up to 46 ngSn/l, juveniles in this experiment dose-dependently developed male sexual characteristics.

Experiment 3

The juveniles ($n = 31$) from the egg-mass which had been transferred from 1000 ngTBTAc/l to the reference treatment developed normally compared to the juveniles from the reference group ($n = 64$), except there was a slightly higher percentage of animals with male sexual characteristics in the *in ovo* exposed group (figs 3.10 & 3.11) during all examinations. Only after 11 months this difference was significant ($P < 0.05$). The percentages never exceeded 50% and mortality in the two groups was equal (6.5%).

The translocation of the egg-masses when juveniles emerged from the egg-capsules caused a total mortality (100%) in the group transferred to the 1000 ngTBTAc/l aquarium. Also, the juveniles of the egg-mass which developed in 1000 ngTBTAc/l, all died within the first week of exposure after emergence from the egg-capsules. So, for juveniles which just hatched, a nominal concentration of 1000 ngTBTAc/l proved lethal, irrespective of a possible exposure during the development within the eggs.

The juveniles exposed only *in ovo* were on average always the largest (and heaviest) compared to the non-exposed. Only after 18 months the difference (fig.3.12) was statistically significant ($P < 0.05$).

At all moments of sampling an increase in the percentage of animals with male sexual characteristics and in stage of male sexual development was observed in both groups. Final sampling when the animals were 38 months old showed a majority of animals without male characteristics in both the reference (67%) and the *in ovo* (55%) exposed group. Animals exposed *in ovo* also showed a slightly enhanced stage of penis development compared to the reference group, this occurred already in the first months of development and continued during further development.

Thus, there is no evidence for a substantial masculinization effect due to an actual exposure to a high TBT (720-1800 ng TBTAc/l) dose *in ovo*, nor for an effect on the development of these juveniles when reared in Wadden Sea water after hatching.

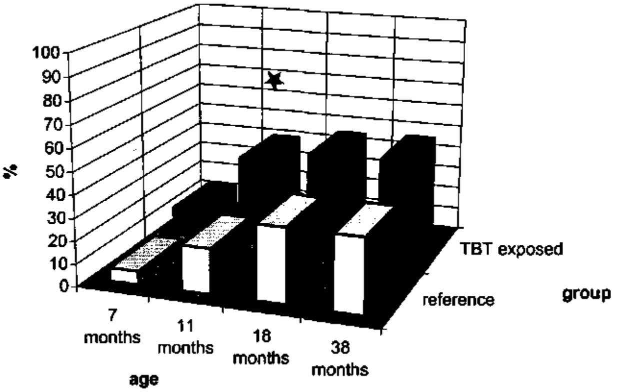

Figure 3.10. Percentage of juvenile *B. undatum* with male sexual characteristics in the *in ovo* treated groups.
** means significantly higher than reference group (P < 0.05)*

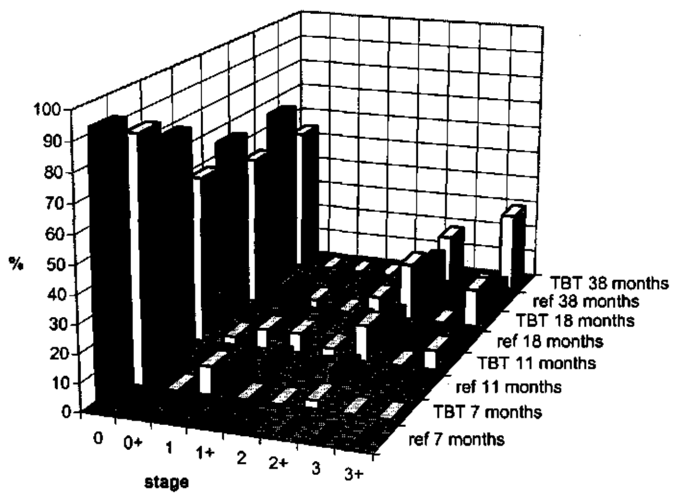

Figure 3.11. Stages of male sexual development in juvenile *B. undatum* in the *in ovo* treated groups.

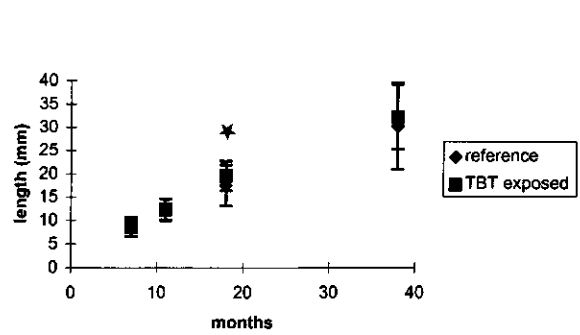

Figure 3.12. Average length (± SD) of juvenile *B. undatum* in the *in ovo* treated groups.
** means significantly higher than reference group (P < 0.05)*

Experiment 4

In this experiment no induction of imposex could be observed in the first 3 months. Neither the exposed, nor the control group showed any development of male sexual characteristics. In both groups length as well as weight of the animals increased in time.

However, after 7 months of exposure (January 1997) in the TBT exposed group, 53% of the animals showed masculine development. Fourteen of the affected females showed only *vas deferens* development (0+), the other two showed the start of penis development with a *vas deferens*. In the reference group no signs of male sexual development were observed (significantly different, P < 0.001).

Average organotin concentrations in the TBT exposed group were 32, 23, 18 ngSn/l for TBT , DBT and MBT respectively. In the reference group actual TBT, DBT and MBT levels were on average 2, 7 and 7 ng Sn/l respectively.

There was no significant difference in average shell length between the different groups (P > 0.5).

In conclusion, 2½ year old females are (still) susceptible to develop male sexual characteristics after exposure to an average TBT concentration of 32 ngSn/l for 7 months.

II Histological studies

Although two juvenile whelks (length ≤ 4 cm) from the North Sea showed initial penis formation, no gonadal differentiation could be observed. Development of the seminal vesicle was already in progress, as indicated by the presence and development of a convoluted duct in the inner curve of the digestive gland. There was a clear *vas deferens* development, which ran from the penis towards the seminal vesicle. In juveniles without male sexual characteristics, it was virtually impossible to distinguish typical female sexual tissues/organs at this age (size).

Juvenile whelks from the laboratory exposure experiment also showed no signs of gonadal development. However, the animals with a penis and *vas deferens* from different exposure groups did not differ with respect to these male sexual organs. Reproductive organs other than penis and *vas deferens* were not observed. In the highest dose group abnormal penises were characterised by either an additional penis formation, which was similar in structure and tissue composition as the single penis, or by an extra tissue formation on or close to the base of the penis.

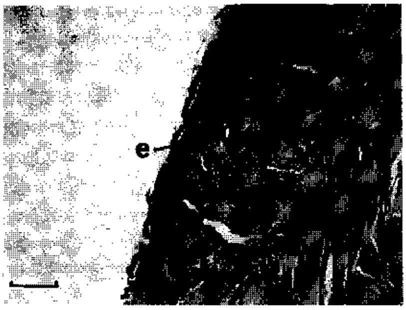

Fig. 3.13a. Imposex stage 0, epithelial cells form the interface between connective tissue and the environment.

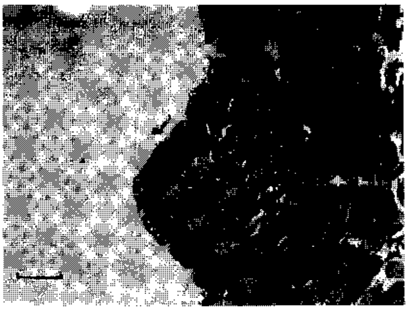

Fig. 3.13b. Imposex stage 1, first development of a penis by differentiation of the connective tissue, forming a knob.

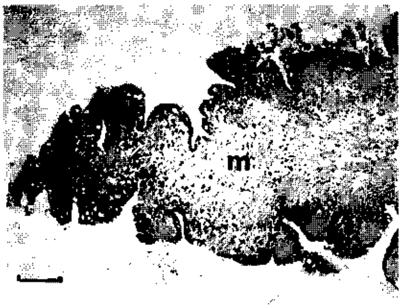

Fig. 3.13c. Imposex stage 2, enlargement of the knob into a structure with muscle tissue

Fig. 3.13d. Imposex stage 3+, the penis is now characterized by the presence of a penis opening (not shown) and a sperm duct and has increased in size.

Figures 3.13a-d. Imposex stages of *Buccinum undatum*. — = 40 µm. e = epithelial cells, m = muscular tissue, sd = sperm duct

Sometimes excrescences on penis tissue were found. Differences in penis size for juvenile males or juvenile females with imposex were not observed.

No sexual characteristics were discovered additional to those already macroscopically visible and used for classification (figs. 3.13a-d).

The studied adolescent (4-6 cm) whelks had partially developed gonads, which could be recognised from immature oocytes and the highly proliferative testis tissue. The developing gonads were small compared to the much larger digestive gland. In males a seminal vesicle was present, although it was much smaller in size than in adult males and its lumen was empty. Although the adolescent females were immature (developing oocytes), attemps already had been made to fertilise these females as could be deduced from the presence of spermatozoa in the seminal receptacle, where normally the eggs are being fertilised.

In these adolescent female whelks, the absence of male sexual characteristics was histologically confirmed. No penis growth and/or sperm duct development could be observed.

The ovaries of adult females (≥ 6 cm) from the North Sea (reference) and Eastern Scheldt (imposex) did not differ in reproductive stage. Animals from both locations showed mature oocytes filled with yolk (fig. 3.14a,b). Testis tissue development could not be observed even in females with the most advanced imposex stage (3+) from the Eastern Scheldt. Studying the seminal receptacle of these females, spermatozoa were observed. These were actually envelopping (fertilising) the eggs of the females and did not have an endogenous origin (fig. 3.15).

Clearly, these females were not sterile although they suffered from the most advanced stages of imposex observed in *Buccinum undatum*. Whether imposex affected the fertility could not be studied in these sections. Following the sperm duct from penis to capsule gland in females with imposex stage 3+, the transition of the duct into the capsule gland could not be observed. It appeared to end at the ventral mid section of the egg capsule gland without invading the glandular tissue.

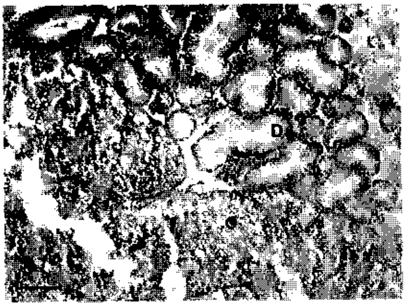

Fig. 3.14a. Unaffected female from the North Sea

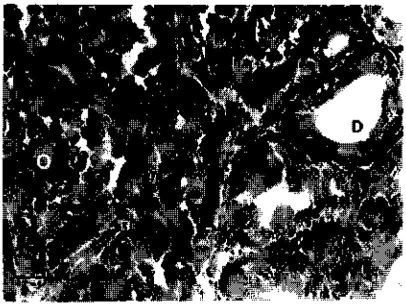

Fig. 3.14b. Imposex stage 3+ female from the Eastern Scheldt

Figures 3.14. Ovary with oocytes (O) and digestive gland (D) of adult female *B. undatum*. a — = 40 µm, b — = 20 µm.

Kidney microsomes of the common whelk showed a similar pattern of change. With increasing TBT concentrations the CYP420 peak became more pronounced and at 1.5 mM TBT only CYP420 was present. With increasing TBT concentrations the base-line first became horizontal and finally decreased with increasing wavelength. Incubation time had virtually no influence on the decrease of CYP450 and increase of CYP420. After every addition of TBT, the spectrum changed directly in favour of CYP420 (fig. 3.19).

These results give clear indications for a dose-dependent inactivation of CYP450 due to TBT-exposure.

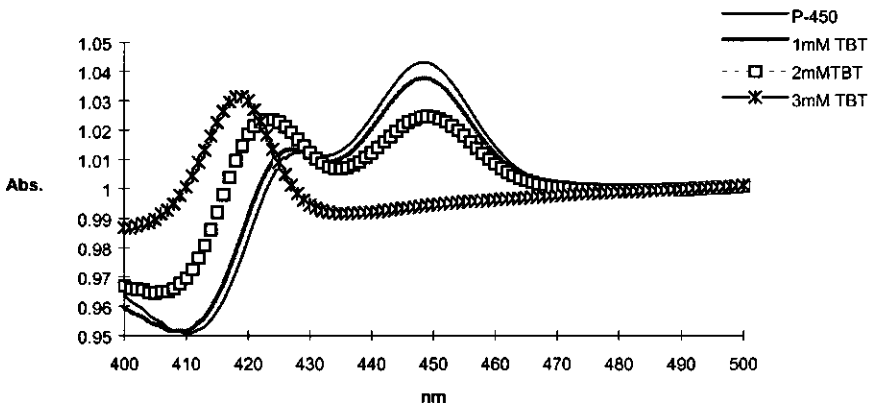

Figure 3.18. Cytochrome P450-TBT incubation spectrum of dab, *L. limanda*, liver microsomes.

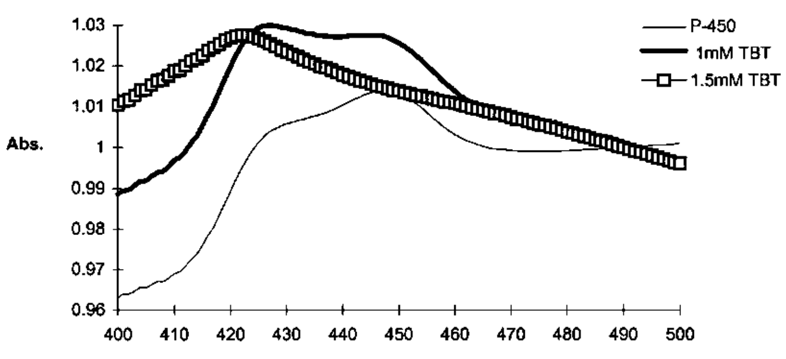

Figure 3.19. Cytochrome P450-TBT incubation spectrum of *B. undatum* kidney microsomes.

Discussion and conclusions

I Exposure studies

Experiment 1

In this study we could only find an effect of TBT on the development of male reproductive organs in juvenile common whelks, but not in adult females during an exposure of 11 months. Most likely, adult females are less sensitive to TBT exposure than juveniles, because they already have a fully developed genital system. The young animals develop their sexual organs in their first life stages and any agent which interferes in this development may cause a different development.

Although in *N. lapillus* the masculine development already starts at concentrations of 2-3 ng TBT/l (Gibbs & Bryan, 1994), we observed a significant increase in the percentage of animals with male characteristics in this study at (actual) concentrations of ≤ 17 ng TBT/l after 8 months of exposure. This indicates a sensitivity of the same order because during the first four months concentrations were on average even below 10 ng TBT/l (see fig. 3.7c.).

The use of continuous flow conditions made it very difficult to obtain and maintain the nominal TBT concentrations. Especially it took long to achieve the lower doses.

Exposure to TBT also seems to affect the growth of juveniles. A significantly lesser shell length was observed at a dose of 10 ng TBTAc/l (8.3 ngTBT/l). However, there was no observed dose-dependent effect. We cannot exclude the possibility of a population density effect being responsible for the effects observed. However, if there was such an effect, it would be expected that aquaria containing less animals should have a greater average length (and weight) than the aquaria containing more individuals. This is not the case, because within the exposure groups, the aquaria containing the largest number of individuals show the greatest average lengths for all periods. Thus, the observed effect is contrary to what would have been expected in the case of a density dependent effect.

In the adult common whelks, a dose dependent increase in butyltin body burden was observed. Based on the physical-chemical properties of the butyltins concerned, theoretically one would expect TBT to show the highest body burdens. In the high dose group water concentrations of TBT>DBT≥MBT and the $\log K_{ow}$ value for TBT (3.75) is two units of magnitude higher than the $\log K_{ow}$ value for DBT (1.5)(Fent, 1996), which consequently should result in a much higher body burden for TBT than for DBT. Our results show a body burden of DBT>MBT>TBT. Biotransformation of TBT is the most likely explanation for the concentrations observed. In the medium dose group, the increase of DBT in body burdens after 11 months can be explained by the increase of TBT body burdens, because DBT levels in water have remained constant and did not result in detectable DBT body burdens earlier. After 1 month in the high dose group, no detectable MBT levels in whelk tissue were found, although aqueous MBT concentrations were on average as high as or equal to those 2 months later, when a whole body burden of 41 ngSn/g ww. was measured. Moreover, DBT and MBT body burdens increased with increasing TBT body burdens. Therefore, the uptake of DBT and MBT from water seems to contribute little to DBT and MBT body burdens compared to the endogenous metabolism of TBT.

If equilibrium conditions are assumed after 11 months of exposure, in the medium dose group the observed butyltin body burdens below or just above the detection limit (<2-3 ng Sn/g ww.) for TBT, 23 ng Sn/g for DBT and 14 ng Sn/g for MBT are comparable to body burdens of common whelks from the Eastern Scheldt (Mensink *et al.*, 1997a) showing average values of 0.7-2.4, 2.3-12 and 1.8-6.0 ng Sn/g for TBT, DBT and MBT respectively. In particular the butyltin body burdens in the Eastern Scheldt whelks show similar (1.14-2.40 ng Sn/g ww.) TBT levels in February and June 1995, whilst comparable DBT levels (12 ng Sn/g ww.) and MBT levels (6 ng Sn/g ww.) were found in June 1995 (Mensink *et al.*, 1997a). This could indicate TBT concentrations between 10 and 80 ng TBT/l in the Eastern Scheldt. The calculated concentrations are in the same range of values reported for the marina at Colijnsplaat (Eastern Scheldt), where water analysis from 1990-1992 showed average levels of 32-61 ng TBT/l. In this marina dredging activity occurs (3 times/year), which very likely reduces the TBT concentration in the water phase (Ritsema, 1994). These levels of TBT will certainly cause a masculinisation effect in developing juvenile whelks.

After 5 years, eggs were produced in the experimental groups. Although more females were present, only two reference females longer than 7 cm (one in each aquarium) produced eggs. Males in these aquaria had not reached the fertile stage, neither had any of the TBT exposed animals. The observed effect of TBT on development (growth) resulted in whelks which take longer to reach the fertile stage. This indicates that TBT exposure can affect reproduction in *Buccinum undatum* indirectly. Whether populations can be affected in this manner needs to be investigated more thoroughly.

Experiment 2

Results obtained clearly confirmed the findings from *Exp.1*, whereby no differences could be observed in the development of male sexual characteristics between the reference group in the artificial sea water and the group which received Wadden Sea water. Both groups showed the same percentage of animals with a penis and/or *vas deferens* and showed an equal development of these organs. Concentrations of organotins as measured in the non-exposed groups did not cause a masculinization effect. As in *Exp.1* nominal concentrations > 10 ngTBTAc/l caused the strong increase in the percentage of animals with male sexual characteristics. It should be noted, that animals from this study were exposed from an age of 6 months onwards.

In contrast to *Exp.1* no (significant) effect on growth was observed in this experiment at the lower TBT doses.

Experiment 3

In this long-term experiment, no evidence for a masculinization effect due to exposure to TBT *in ovo* was found, nor was an effect on the development of these animals observed. However, exposure to a TBT dose of 1000 ngTBTAc/l after hatching proved lethal irrespective of a possible exposure during the development of the eggs.

Although a slightly increased percentage of animals with male sexual characteristics (45%) was observed after 18 months in the *in ovo* exposed group compared to the reference group (33%), this percentage was not different from the percentage in the reference group from *Exp.1* (47%) after the same experimental period. The observed difference in percentage of juveniles with male sexual characteristics between the two groups in this third experiment, possibly reflects a variation in sexual development between juveniles from various egg masses. In *Exp.1* several egg-masses contributed to the number of juveniles per group, so differences per egg-mass could not be observed.

This type of research has never been conducted before and shows the importance of exposure to TBT at the moment of hatching, when the animals were no longer shielded from their environment. This can be deduced from the fact that juveniles within the eggs developed normally, when their environment was heavily contaminated (actual water TBT concentrations 720-1800 ng TBTAc/l). Once the juveniles had hatched and were exposed via the waterphase, these concentrations proved lethal. This suggests a relatively "clean" egg-environment.

It is therefore interesting to study TBT levels within the egg masses during the very first development of juvenile whelks. These concentrations can result either from diffusion from the environment into the egg capsules, which seems unlikely (this study), or are related to the environment passed on by the adult female whelks, when they produced the egg capsules with fertilised and nutritional eggs. The egg masses in this experiment were produced by females from the Dogger Bank, where no imposex occurred. Disposition of TBT in these egg-masses seems therefore unlikely.

Experiment 4

The results suggest, that adolescent females are less sensitive to TBT compared to juveniles which just emerge from eggs; it takes them longer to develop male sexual characteristics at higher TBT concentrations. Furthermore, the male sexual development is different, since the adolescent females mostly developed only *a vas deferens* and just a minimal penis. Juveniles first start to develop the penis, afterwards the *vas deferens* is formed. Possibly stages of male sexual development would increase in time in adolescent females, however no evidence for this is available and this hypothesis needs further investigation.

In conclusion these laboratory exposure studies showed that:
Although it is expected that 50% of young whelks will eventually develop as normal males, exposure to doses of TBT > 7 ng Sn/l induced and accelerated the development of male sexual characteristics in a dose-dependent manner, when *B. undatum* were exposed just after hatching. Exposure to nominal TBT concentrations ≥ 4 ng Sn/l may lead to reduced growth in juvenile common whelks and consequently affect reproduction. Adults seem to be less sensitive to TBT than juveniles. TBT levels encountered in the Eastern Scheldt can cause a masculinisation effect in developing juvenile whelks living in this area. Exposure to high water TBT concentrations *in ovo* does not cause a substantial increase in the percentage of animals with male sexual characteristics when juvenile common whelks are transferred to Wadden Sea water directly after hatching. The egg environment can therefore be considered to be protective against TBT. Exposure to a nominal concentration of 100 ngTBTAc/l causes imposex in adolescent (2.5-3 years old) females, but the expression of imposex is different compared to just after hatching. The fact that juveniles develop imposex at TBT concentrations > 7 ngSn/l and imposex is found in adults from the North Sea, points to a substantial TBT contamination in open sea.

II Histological studies

Although in the Eastern Scheldt a high percentage of female common whelks (>90%) sufferred from imposex (Mensink *et al.*, 1996a), the highest stages of imposex did not mechanically sterilize these females as could be observed in the seminal receptacle of a female with stage 3+ with the sperm duct entering the capsule gland. Here, apparent fertilisation of the eggs by spermatozoa was found and the ovary and capsule gland were not different from reference females from the North Sea. Ide *et al.* (1996), who studied imposex in female common whelks from the North Sea, could not find pathological alterations in the digestive gland-gonad complex of these females, which is in accordance with our findings from the Eastern Scheldt area. Stroben *et al.* (1992) gave an overview of imposex in *Hinia reticulata*. They found the masculinisation effect of exposure to environmental TBT concentrations to be expressed not only by an enlargement of the female penis length, but also by a reduction in size of the albumen, the digestion and the capsule gland. Sterilisation was not found. The ovary and vaginal opening were unaffected and restrictions on fertility were not obvious.

Although TBT exposure may indirectly affect reproduction in common whelks by reduced growth (*Exp.1*), direct effects on reproduction due to structural changes are not obvious.

So far, few histological data on developing prosobranchs have been published. Laboratory-reared two year old *Nucella lapillus* (adult stage) showed a normal oogenesis when reared at TBT concentrations 3-5 ng Sn/l, but were virtually sterile due to the mechanical occlusion of the genital pore. When reared at concentrations of 20-100 ng Sn/l, the gonads showed a very different appearance: Oogenesis was suppressed or had ceased and even testis tissue could be distinguished (Gibbs *et al.*, 1988). Histological data showed that it was virtually impossible to distinguish the sexes of our TBT exposed juvenile (< 4 cm) common whelks, because after two years no primary sexual characteristics had been developed, despite the complete formation of a penis and *vas deferens*. This supports the hypothesis by Féral & leGall (1983) and Lubet & Streiff (1982) that the differentiation and maintenance of male cells in marine gastropods depend upon factors secreted by the nervous system and that it is not controlled by hormones produced by gonads as has been suggested for the terrestrial snail *Euhadra peliomphala* (Takeda, 1983). Adolescent animals from the North Sea (4-6 cm) showed development of immature gonads. However, spermatozoa were already found in the seminal receptacle of such a female. This indicates that copulation had already taken place before the female could reproduce successfully.

From the detailed analysis of common whelks the following conclusions are drawn :

Adult female whelks from the Eastern Scheldt with imposex are not mechanically sterilised by imposex phenomena. The vaginal opening is unaffected as are the ovary and pallial glands. Juveniles could not be sexed using histological techniques when they were 2 years old, since besides a penis and *vas deferens*, no other reproductive organ was developed. Development of a penis and *vas deferens* can therefore not be controlled by steroid hormones produced by gonads and points to a mechanism with neuro-endocrinological importance. A histological study in whelks of 4-6 cm in shell length can give evidence for the gonadal development and the development of other reproductive organs. From this stage it is possible to say to which sex the animal belongs/develops.

III Cytochrome P450 studies

In this study we showed the presence of a cytochrome P450 enzyme system in the digestive gland as well as in the kidney of the common whelk. The digestive gland primarily contained the inactive CYP420 form, whilst the kidney showed a more equal CYP450/CYP420 ratio. An explanation for this difference could be that the digestive gland contains lysosomes filled with hydrolases. Use of the Potter teflon homogenizer could destroy these lysosomes and the enzymes released could break down a part of the P450 to P420. The exact amounts of CYP450 or CYP420 in whelks were difficult to quantify due to the varying base-lines and the overlapping CYP450 and CYP420 peaks in the spectra obtained. The amount of CYP450 in the digestive gland and the kidney was (as an estimate) between 0.01-0.1 nmol/mg protein. Dab liver microsomes were used as a positive control to test the experimental procedures. These microsomes showed a type I binding spectrum after incubation with TBT. This has been observed in freshwater fish species as well (Fent & Bucheli, 1994). Whelk microsomes however, did not show such a binding spectrum.

Comparable results for the two species were obtained looking at CYP450 spectra after incubation with TBT. A clear dose dependent transformation of the CYP450 enzyme into the inactive form CYP420 was observed after addition of TBT. This inactivation of CYP450 by organotins has been described for fish and several molluscs (Fent, 1996; Morcillo & Porte, 1997). TBT showed an inhibition of both NADH and NADPH cytochrome c reductase activity in *Thais haemastoma* (gastropod), whereas triphenyltin (TPT) showed little effect (Morcillo & Porte, 1997). In the latter study, problems with molluscs similar to this study (e.g. shifts of baseline at higher organotin (OT) levels, low CYP450 levels) occurred. This effect on the CYP450 enzyme system is thought to play a role in the induction and promotion of imposex in gastropod species, leading to an imbalance in steroid hormone titres, which consequently would result in the formation of male sexual characteristics in female specimens of *N. lapillus* and *H. reticulata* (Oehlmann *et al.*, 1993; Stroben, 1994; Spooner *et al.*, 1991; Bettin *et al.*, 1996). In the biosynthesis pathway of steroid hormones in mammals and fish, many steps are regulated by isoenzymes of the CYP450 family. Thus, an effect on these enzymes could theoretically cause hormonal disturbances in common whelks. In this study we present additional evidence to support the above hypothesis, although it is not certain the effects observed in the *in vitro* incubations will also occur *in vivo*. Also, TBT concentrations used in this study are relatively high. It is questionable whether these concentrations will actually occur in the organisms. Furthermore it should be kept in mind that the CYP450 described here originates from the kidney from the whelk, whereas this may not be the organ where hormonal metabolism occurs.

However, so far biosynthesis of steroid hormones and the role of CYP450 in this process has never been properly investigated in marine gastropods. Elucidation of the formation and functional meaning of steroid hormones in relation to CYP450 enzyme activities is therefore essential before the phenomenon of imposex can be considered an example of endocrine disruption (in common whelks).

From these findings it can be concluded that :

The microsomal fraction of the digestive gland as well as the kidney contain the CYP450 enzyme system, which is held responsible for the degradation of xenobiotic compounds like TBT. CYP450 is transformed by TBT; a dose dependent decrease in the amount of CYP450 was observed whilst the inactive CYP420 form increased with increasing TBT concentrations (> 1 mM). Substrate (TBT) induced difference spectra did not show a type I binding spectrum for the common whelk.

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CHAPTER 4

ASSESSMENT OF IMPOSEX IN THE COMMON WHELK, *BUCCINUM UNDATUM* (L.) FROM THE EASTERN SCHELDT, THE NETHERLANDS

Marine Environmental Research (1996) : 41 (4) : 315-325
Co authors : C.C. ten Hallers-Tjabbes, J. Kralt, I.L. Freriks and J.P. Boon

Abstract

Several cruises were carried out on the Eastern Scheldt between September 1992 and March 1995 to study the frequency and appearance of imposex in the common whelk, *Buccinum undatum* L. Incidences of imposex were always > 90 %. For the first time several stages of imposex in *Buccinum undatum* are reported. The evolution of imposex in the whelk seems to follow a similar route as in *Hinia reticulata* belonging to the same superfamily (Buccinacea).

Tissue organotin concentrations of animals collected in February 1995 showed the highest organotin concentrations in the digestive gland and lowest in the foot of the animals. Concentrations of phenyltin compounds (up to 625 ngSn/g dry weight) were much higher than those of butyltin compounds (up to 40 ngSn/g dry weight).

Introduction

The common whelk, *Buccinum undatum*, is a benthic predator that lives subtidally at the seabed. It preys predominantly on live benthic bivalve molluscs and dead animals. In the coastal waters of the Netherlands, common whelks can nowadays only be found in the Eastern Scheldt, whilst 25 years ago whelks were also abundant in the Wadden Sea and coastal areas of the North Sea (ten Hallers-Tjabbes *et al.*, 1993). So far, no conclusive explanation for the decline of whelk populations in these areas has been given.

In the Southern North Sea, imposex was found in *B. undatum* (ten Hallers-Tjabbes *et al.*, 1994). Imposex is defined as the development of male sexual characteristics in female gastropods (Smith, 1971), also called pseudo-hermaphroditism (Fioroni *et al.*, 1991). This phenomenon was first described for the dog-whelk, *Nucella lapillus* L. (Blaber, 1970). Imposex in *N. lapillus*, *Nassarius obsoletus* (Smith, 1981) and *Ocenebra erinacea* (Féral & leGall, 1983) can be caused by exposure to tributyltin (TBT). TBT is the active agent in antifouling paints used on ship hulls. Concentrations as low as 1 ng/l have been reported to cause adverse effects in *N. lapillus* (Gibbs *et al.*, 1988). Severe stages of imposex at higher TBT concentrations can lead to an overgrowth of the genital papilla by the *vas deferens*, resulting in sterilization and premature death (Gibbs & Bryan, 1986). Ultimately, this will lead to a local disappearance of the species (Bryan *et al.*, 1986). Phenyltin compounds are also used in anti-fouling paints, but its major application is in agriculture. The fungicide triphenyltin (TPT) is mostly used in potato culture, which is common in the surroundings of the Eastern Scheldt.

The population of *Nucella lapillus* in the Eastern Scheldt is severely affected by imposex, and the species is even expected to become extinct in this area (Mertens & van Zwol, 1988). This seems to be confirmed by data on live dog-whelks from surveys by Harding *et al.* (1992) and Sips & Waardenburg (1992). The latter authors question the relation with organotin compounds, because some disappearances of dog-whelk populations took place 10 years before organotin was introduced in the aquatic environment. They state furthermore that a lack of findings does not necessarily mean a complete disappearance or even a local extinction.

The state of the dog-whelk population in the Eastern Scheldt and the occurrence of imposex in common whelks from the North Sea lead us to investigate the state of the population of *B. undatum* in the Eastern Scheldt. This paper describes the results of several cruises carried out from 1992 to 1995. During the last cruise in February 1995, samples were taken for the determination of tissue concentrations of butyltin and phenyltin compounds.

Methods

Cruises were made with RV Biezelinge of the directorate "Zeeland" of Rijkswaterstaat and with RV Navicula (NIOZ). Specimens of ***Buccinum undatum*** were caught at the location 'Hammen' (figure 4.1) during one day cruises in 1992-1994 with a 4 m wide beam trawl. The mesh size of the trawl net was 7.5 cm. In 1995 several days were used in order to study more individuals and to prepare samples for tissue analyses of butyltin and phenyltin compounds.

Since they crawl out of their shell when exposed to air, thereby exposing the penis and/or *vas deferens* development, *B. undatum* could generally be sexed by examining the sexual parts without breaking the shell. Males were characterized by the presence of a full-grown penis and a clearly recognizable *vas deferens*. Individuals were determined as normal females if neither a penis homologue nor a *vas deferens* was present. Imposex in female whelks was characterized by the presence of a penis homologue, which is always considerably smaller than a normal male penis and often differs in shape from it. In a number of cases a *vas deferens* was also observed. The shells of whelks with a small penis homologue and a *vas deferens* were cracked and the reproductive organs were studied. The presence of an egg capsule gland, albumen gland, ovary and bursa in females were checked as well as the testis and seminal vesicle in males. Several stages of imposex could be distinguished (figures 4.2a-e).

The length of the shell was measured and the shape of the penis homologue was recorded, as were the number of empty whelk shells and hermit crab inhabited shells. The latter was done to assess whether whelks had been abundant in areas where nowadays few or none could be found.

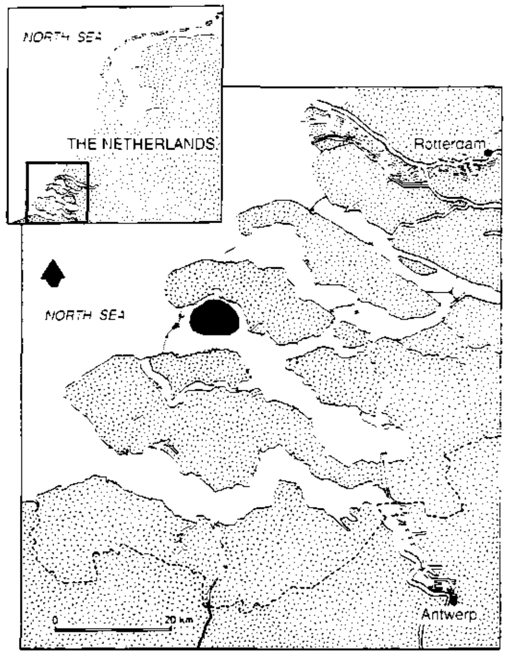

Figure 4.1 : The black area represents the sampling location in the Eastern Scheldt (Hammen).

Analysis of organotin compounds

Female *Buccinum undatum* showing a penis and a *vas deferens* were used to determine tissue organotin concentrations. For whole body, digestive gland and foot analyses two females were pooled. Five young individuals were also pooled for whole body analysis.

Organotin concentrations in *B. undatum* tissue have been determined with GC-MS after pentylation of the organotin compounds. Extraction, derivatisation and clean-up procedure have been described extensively elsewhere (Stäb *et al.*, 1994). The GC-MS and GC-MS-MS analyses were carried out with an upgraded Varian Saturn II ion trap detector, equipped with a Wave-Board (Varian, Walnut Creek, CA, USA). The GC-column was a 30 m * 0.2 mm I.D., film thickness 0.2 µm DB-5 column (J&W Scientific, Folson, CA, USA). A retention gap of 2 m x 0.53 mm I.D. deactivated silica (Chrompack, Middelburg, the Netherlands) was used in all cases. In order to achieve lower detection limits, in some cases GC-MS-MS was used. GC-MS-MS was carried out using non-resonant ion ejection, providing a greater linear dynamic range (Schachterle *et al.*,1994).

Results

Occurrence of imposex

Imposex was observed in most of the female *Buccinum undatum*, only one visually unaffected female was found in September 1992 and 1993 and May 1994; four unaffected females were recovered in February 1995 and three in March 1995 (Table 4.1). The size of the penis homologue varied from a very small bud (c. 1 mm) up to about 2 cm; still considerably smaller and thinner than the penis of males (c. 5 cm). Some females also showed a *vas deferens*, which sometimes seemed to enter the egg-capsule gland. The penis homologue of such animals was always among the largest observed in these pseudohermaphrodites (figures 4.2a-e). In February and March 1995 the percentage of females with a large penis (about 2 cm) and some with *vas deferens* was 65% and 62% respectively. In the classification system of imposex developed for *N. lapillus* (Gibbs *et al.*, 1987) and later adjusted by Fioroni *et al.* (1991), the presence of a penis and a fully developed *vas deferens* represent stage 4 or higher, when females can become sterile. However, no indications for sterility of common whelks have been found in the present study. During the cruise of May 1994, one empty egg mass was recovered, whilst in February and March 1995 more than twenty egg masses with developing young *B. undatum* were found, indicating that reproduction is still taking place in the Eastern Scheldt.

Many empty shells and shells inhabited by hermit crabs were found, often more than live whelks and most of these shells were undamaged.

Table 4.1. Number, mean length, sex and imposex incidence of *Buccinum undatum* at location Hammen. SD= standard deviation.

month	mean length ± SD (cm)	male (n)	female (n)	ratio m/f	% females with imposex
Sept 1992	7.56 ± 0.45	34	29	1.2	97
Sept 1993	7.45 ± 0.85	45	11	4.1	91
April 1994	6.78 ± 0.68	12	5	2.4	100
May 1994	7.03 ± 0.58	53	21	2.5	95
Feb 1995	6.76 ± 0.89	114	106	1.1	96
March 1995	7.18 ± 0.59	46	55	0.8	95

Organotin concentrations

Tissue organotin concentrations are given in Table 4.2. In all samples TBT could be detected. The digestive gland and the foot of some females were seperately analyzed. The highest levels were found in the digestive gland. The metabolites dibutyltin (DBT) and monobutyltin (MBT) were enhanced compared to TBT in all samples. The ratio DBT+MBT : TBT was at least 2 and highest (6) in the digestive gland of the animals.

Phenyltin concentrations were much higher than butyltin concentrations with an exceptionally high TPT level (625 ngSn/g dry weight) in the digestive gland of adult females. The metabolites diphenyltin (DPT) and monophenyltin (MPT) were also detected, but often in lower concentrations than the parent compound.

Table 4.2. Tissue organotin concentrations (ngSn/g dry weight) in pooled samples of n individuals of *Buccinum undatum* collected at location Hammen in February 1995.

Sample (pooled)	n	Tissue	TBT	DBT	MBT	TPT	DPT	MPT
young whelks (38.4-49.1mm)	5	WB	10.0	19.6	10.0	76.9	104	10.8
young whelks (37.6-49.5mm)	5	WB	5.5	16.2	10.7	58.6	< 52**	2.8
adult females*	2	DG	11.8	32.5	40.0	625	185	60
adult females*	2	F	2.6	4.8	5.2	35.2	< 34**	2.6
adult females*	2	WB	11.5	15.8	14.6	92.3	< 42**	8.1

* Females with imposex (showing a penis and a *vas deferens*),
** the values of the detection limit differ between samples because different amounts of tissue were used, WB = whole body, DG = digestive gland, F = foot,
TBT = tributyltin, DBT = dibutyltin, MBT = monobutyltin, TPT = triphenyltin, DPT = diphenyltin and MPT = monophenyltin

Discussion

The imposex incidence reported here is high (> 90%), although ten Hallers-Tjabbes *et al.* (1994) found an imposex incidence of 100 % in the North Sea in 1991 at a location directly north of the 'Eurogully', which is the deepened entrance to the harbour of Rotterdam. It should be noted that at this station in 1992 one live male specimen of *B. undatum* was caught despite extensive trawling effort. No other station during the North Sea cruises of 1991 and 1992 showed an incidence greater than 30 %. The only other case of imposex in *B. undatum* is reported for the White Sea in the area of the Solevetski isles, where 15 out of 19 females showed penis homologues (Kantor, 1984).

Although in 1993 (n=56) and 1994 (n=91) the male to female ratio is higher than 1, results from 1992 (n=63) and 1995 (n=321) did not show a difference from an expected 1: 1 ratio, as found in reference (no observed imposex) populations from the central North Sea and earlier findings in the North Sea and estuaries (ten Hallers-Tjabbes, 1979; ten Hallers-Tjabbes *et al.*, 1995). Severely affected populations of *N. lapillus* show far more males than females (Gibbs & Bryan, 1986), while the majority of the remaining females show the highest stages of imposex. These stages could lead to premature death and consequently result in the presence of more males than females. In the Eastern Scheldt we have observed all stages of imposex but so far not a single female with an occluded genital pore. It seems therefore unlikely that the 'dominance' of males over females in 1993 and 1994 is caused by premature death of females suffering from imposex.

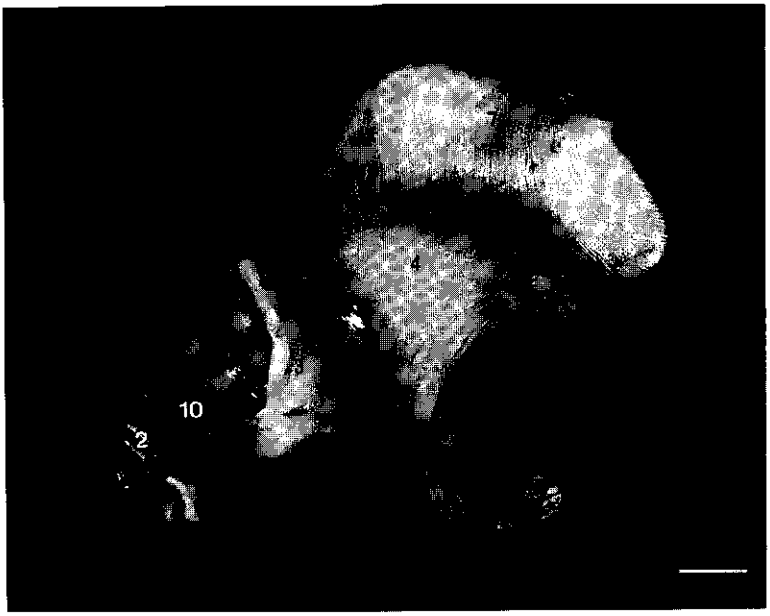

Figure 4.2a : Normal male whelk, *B. undatum*

Figures 4.2 a-e : *Buccinum undatum* removed from their shell. Stages of imposex according to Fioroni *et al.* (1991). 1 = ovary, 2 = testis, 3 = columnar muscle, 4 = foot, 5 = albumin gland + seminal receptacle, 6 = egg capsule gland, 7 = penis, 8 = penis homologue, 9 = vas deferens, 10 = seminal vesicle. Figures 4.2a-d : bar = 0.5 cm, figure 4.2e : bar = 0.02 cm

Two stages of imposex in *B. undatum* are shown (figures 4.2 c+d+e); a stage where only a penis homologue is visible, and a stage where both a *vas deferens* and a penis homologue are visible. The most advanced stage of imposex observed in *B. undatum* in the Eastern Scheldt showed the presence of a penis and a *vas deferens*, which seemed to enter the egg-capsule gland. Fioroni *et al.* (1991) have given an overview on the different possibilities of the development of imposex (pseudohermaphroditism) of prosobranchs, where the occurrence of a penis homologue and a *vas deferens* (stage $\geq$ 4) follows the occurrence of only a penis homologue. According to their evolution schemes, the *vas deferens* develops from the penis homologue to the genital pore of the female whelk, or the *vas deferens* develops synchronously from the penis homologue towards the genital pore and from the genital pore towards the penis homologue. The common whelk follows both evolution schemes, but so far no occlusion of the genital pore was found. This is partly supported by Stroben *et al.* (1992), who reported an imposex evolution for *Hinia reticulata* (Buccinacea), following the first evolution scheme. In their study the final stage of imposex in *H. reticulata* was stage 4+, when the *vas deferens* enters the capsule gland, but does not occlude the genital pore of the female. Also no sterilization was found in *H. reticulata*.

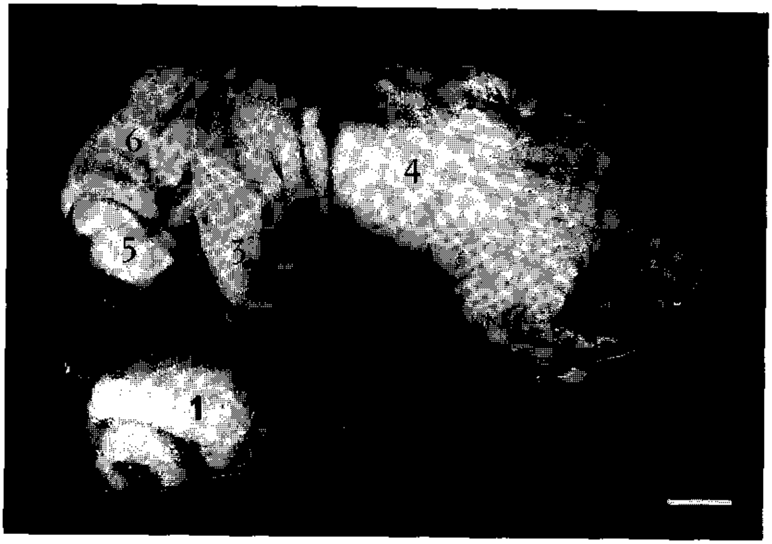

Figure 4.2b : Normal female whelk, *B. undatum*

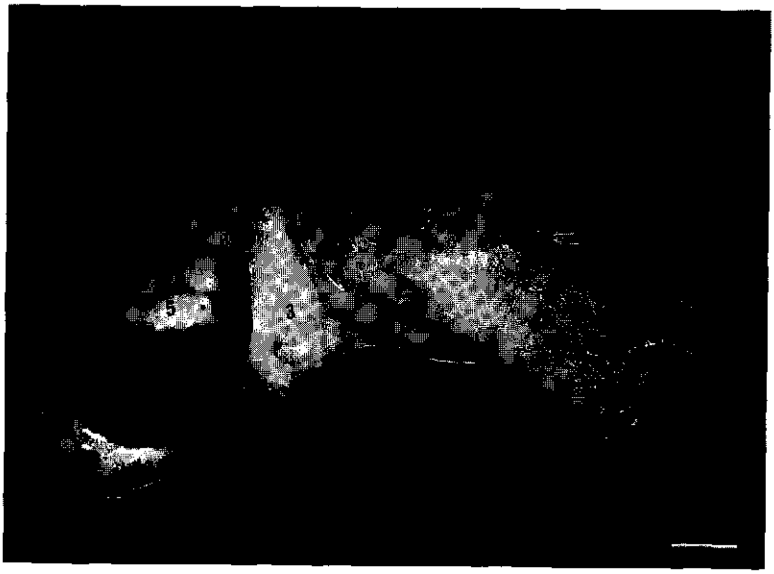

Figure 4.2c : Female *B. undatum* with imposex, only a penis homologue with penisduct is present (stage 2a)

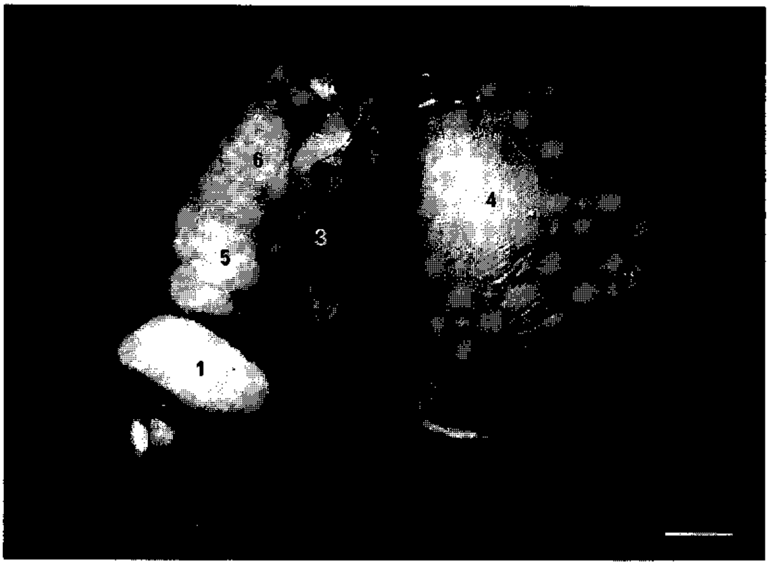

Figure 4.2d : Female *B. undatum* with imposex, a penis homologue with penis duct as well as a vas deferens are present (stage 4)

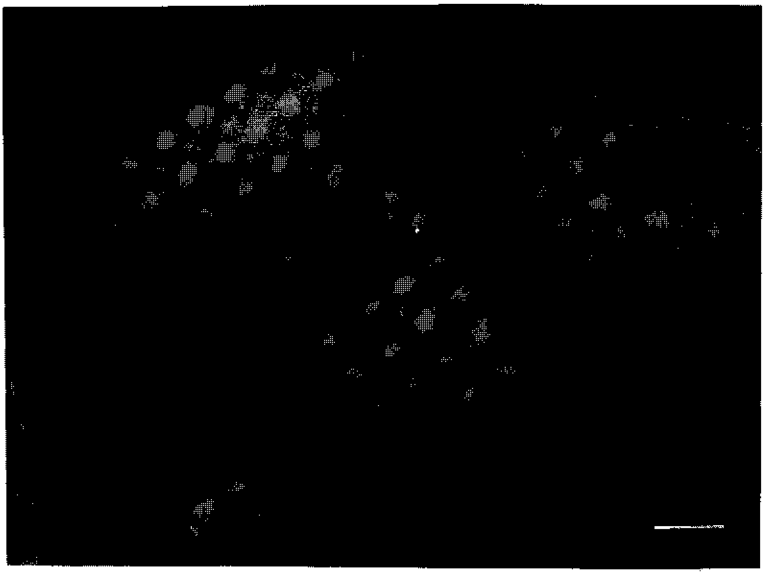

Figure 4.2e : Detail of figure 4.2d.

In spring 1994 and 1995 several egg masses were caught, and thus most likely young whelks hatched in the Eastern Scheldt at that time. Thus the important question remains whether imposex causes a similar effect on the population of the common whelk as it does on the dog-whelk population.

The Eastern Scheldt is a high leisure boating activity area with many marinas. Despite the ban in 1990 on the use of TBT containing antifouling paints for ships and vessels up to 25 m in length, TBT concentrations in water near marinas have remained more or less constant from 1990 to 1993 (mean 30-60 ng/l) due to the leaching of the butyltins already adsorbed to the sediment to the waterphase to maintain equilibrium conditions. All TBT concentrations measured were >> 1 ng/l; the reported threshold concentration for the induction of imposex in *N. lapillus* (Bryan *et al.*, 1988). DBT (mean 20-40 ng/l) and MBT (mean 4-9 ng/l) were also measured, but always at lower concentrations than TBT. Every year in spring a peak in TBT concentrations occurred, coinciding with the launching of freshly painted boats (Ritsema, 1994; Smedes, 1994). At present no data on aqueous concentrations of phenyltin compounds in the Eastern Scheldt are available.

Tissue organotin concentrations in *B. undatum* showed a different pattern from water. Although in the water TBT was the dominant butyltin compound, in tissue DBT > MBT > TBT. The highest relative metabolite levels were measured in the digestive gland, an important organ for the biotransformation of TBT (Bryan *et al.*, 1993). Phenyltin compounds however, showed higher TPT levels compared to the degradation products DPT and MPT. These TPT levels could point to a recent input into the area or indicate that biotransformation is slower for phenyltin than for butyltin compounds. Horiguchi *et al.* (1994) also found TPT to be dominant over its metabolites in *Thais clavigera* and *T. bronni*, but less pronounced than in this study.
It remains to be investigated whether *B. undatum* is as sensitive to TBT pollution as *N. lapillus,* although the presence of imposex in the open North Sea suggests at least a sensitivity of the same order.

Laboratory studies with newly hatched *B. undatum* have shown that TBT exposure promotes the development of male sexual organs in a dose dependent manner. Concentrations between 10 and 100 ng tributyltin acetate (TBTAc)/l caused a steady increase in the percentage of animals showing male sexual organs. After 10 months already 80 % of the animals showed male sexual characteristics. A dose of 1000 ngTBTAc/l caused 100 % penis and/or *vas deferens* development some even with a malformed penis already after 8 months of exposure (Mensink *et al.*, 1996b).

A comparison of the effectiveness of several organotin compounds in inducing imposex was made by Bryan *et al.* (1988). Here TPT alone did not induce imposex in *N. lapillus*. An exposure to a combination of organotins was not tested. Thus, it remains to be investigated, whether TPT has an additive effect on imposex promotion. However, Horiguchi *et al.* (1994) state that injection of TPT had an inducing and promoting effect on the development of imposex in *Thais clavigera* and *T. bronni.*

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CHAPTER 5

BIOACCUMULATION OF ORGANOTIN COMPOUNDS AND IMPOSEX OCCURRENCE IN A MARINE FOOD CHAIN (EASTERN SCHELDT, THE NETHERLANDS).

Environmental Technology (1997) : 18 (**12**) : 1235-1245
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Abstract

During several seasons in 1995 common whelks (*Buccinum undatum*), mussels (*Mytilus edulis*) and sediment were analysed for organotin compounds. For butyltin compounds, the order of concentrations in whole body homogenate of common whelks was DBT>MBT>TBT. TBT was usually just above the detection limit except for the samples of the neural ganglia (nerve centre) taken in September. Here, only TBT could be detected, while the TBT/TPT ratio was >1. This could be important since the induction of imposex concerns the involvement of neuropeptides and/or steroid hormones. TPT concentrations in whole body homogenates of common whelks were 4-100 times higher than those of TBT. TPT clearly showed much higher levels than its metabolites DPT and MPT. No structural differences in organotin contamination were found between the sexes, different stages of imposex, or adult and juvenile common whelks.

In mussels, TBT>DBT>MBT, but phenyltin ratios were comparable to those in the common whelk. Phenyltin whole body concentrations, however, were 5-10 fold below those of *B. undatum*. Biomagnification of butyltin compounds is not expected, whereas comparison of phenyltin ratios between the two species suggests biomagnification.

In total sediment samples organotins were usually below their detection limit.

The percentage of adult and juvenile female *B. undatum* showing imposex was > 95% throughout the year.

Introduction

Imposex is a phenomenon found along many coastal areas, not only in Europe, but all over the world. Imposex is caused by tributyltin (TBT) in littoral species such as *Nucella lapillus* and *Hinia reticulata* (Gibbs *et al.*, 1987; Stroben *et al*, 1992). Sublittoral species like *Buccinum undatum* and *Neptunea antiqua* from the open North Sea also show imposex (ten Hallers-Tjabbes *et al.*, 1996a), which for *B. undatum* is positively related to shipping traffic intensities (ten Hallers-Tjabbes *et al.*, 1994). Many gastropod species suffer from this masculinisation effect in females, which can cause a decline in population density and eventual may lead to local extinction of certain species (Gibbs *et al.*, 1991). Indirectly imposex can have effects on other organisms as well. For instance, adult hermit crabs *(Pagurus bernhardus)* depend for their protection on shells of bigger snail species, like the common whelk (*B. undatum*).

The Eastern Scheldt is an area likely to be affected by organotins. TBT input can be expected from the high leisure boating activity (Ritsema, 1994) and triphenyltin (TPT) input from its use in agriculture (potato culture) is likely. *N. lapillus* was thought to become extinct in this area in 1988 (Mertens & van Zwol, 1988). Oysters in the same area showed shell deformities, which are also caused by butyltin compounds (van Zwol & Mertens, 1988). More than 90% of female *B. undatum* in the Eastern Scheldt are known to suffer from imposex, but only a few organotin concentrations have been reported so far (Mensink *et al.*, 1996a).

These strong indications for toxic effects of organotin compounds in the Eastern Scheldt led us to investigate the levels of organotin compounds in common whelks, their food (mussels) and their habitat (sediment). Due to the fact that organotins are seasonally applied, we sampled in February, June and September 1995. Tributyltin is mostly introduced in the Eastern Scheldt in spring and summer when freshly painted vessels enter the water, while triphenyltin mostly reaches the Eastern Scheldt area from agricultural (potato culture) use in the period spring-autumn.

Methods

Cruises were made with the RV Navicula (NIOZ) from 6-9 February, 7-8 June and 6-8 September 1995. Specimens of *Buccinum undatum* and *Mytilus edulis* were caught at the location 'Hammen' in the Eastern Scheldt (Mensink *et al.*, 1996a) with a 4 m beam trawl at a maximal depth of 25 m. The mesh size of the trawl net was 6 cm. Several days were used to study imposex in a sufficient number of individuals and to prepare samples for tissue analysis of butyltin and phenyltin compounds. The length of the shell was measured for all *B. undatum*. Sediment samples were taken with a van Veen grab in the same fishing lane as where the common whelks and mussels were collected.

Determination of organotin compounds

a. Preparation of samples

Most samples were prepared for whole body analyses in individual and pooled samples. In a number of samples the specimens were dissected and the digestive gland, the foot and the ganglia were analysed separately. Animals were separated into adults and juveniles and also according to stages of imposex. Animals < 55 mm in length were considered to be juveniles. In February, juveniles used for analysis were in the range of 37.6-54.5 mm, in June they ranged 28.8-54.0 mm and in September they ranged 25.3-53.2 mm.

Five sediment samples were homogenised and divided in two subsamples. Mussels were pooled, 5 specimens in February, 12 in June and September.

b. Analysis of organotin compounds

Organotin concentrations in tissues of *B. undatum* and *M. edulis* were determined with GC-MS after pentylation of the organotin compounds. Extraction, derivatisation and clean-up procedures have been extensively described elsewhere (Stäb *et al.*, 1994). The method is based on acid (pH 1.5-2) extraction with diethylether (15 ml) of 2-3 g of homogenated sample in the presence of a complexing agent (tropolon 0.3%). Derivatisation of the positive organotin ions (pentylation) was performed with Grignard-reagent (pentyl magnesium bromide) and clean up was conducted with basic alumina. The GC-MS and GC-MS-MS analyses were carried out with a Varian 3400 gaschromatograph equipped with a CIS3-Gerstel on-column injector; retention gap 2 m x 0.53 mm I.D., deactivated fused silica; SGE-BPX5 column 25 m x 0.22 mm I.D., filmthickness 0.25 μm and an upgraded Varian Saturn IV ion trap mass spectrometer, equipped with a Wave-Board (Varian, Walnut Creek, CA, USA). In order to achieve lower detection limits, GC-MS-MS was used in some cases as this resulted in much more favourable signal-to-noise ratios especially for phenyltin compounds.

Table 5.1.a Detection limits and reproducibility- animal tissue

	Quantitation mass (m/z) GC-MS-MS	Detection limit* Sn ng g^{-1} (dry wt.)	Sn ng g^{-1} (wt wt.)	Variation** coefficient MO-94/OT	Concentration wet wt. Sn ng g^{-1} MO-94/OT
TBT	385	*2*	*0.4*	*21%*	*48 ± 10*
DBT	319	*1*	*0.2*	*35%*	*2.5 ± 0.9*
MBT	319	*1*	*0.2*	*28%*	*3.1 ± 0.8*
TPT	351	*0.5*	*0.1*	*23%*	*33 ± 8*
DPT	345	*1*	*0.2*	*19%*	*4.0 ± 0.8*
MPT	319	*0.5*	*0.1*	*13%*	*1.2 ± 0.1*

* determined with 99% confidence interval (3 x sd) of the signal-to-noise ratio in chromatogram MO-94/OT, for 2.5 g (wet wt.) sample and 20 % dry weight.

** variation coefficient analysis reference sample (*Dreissena* homogenate; n = 6; Nieuwkoopse plassen, MO-94/OT).

Table 5.1.b Detection limits and reproducibility- sediment

	Detection limit sediment* Sn ng g^{-1} (dry wt.)	Sn ng g^{-1} (wt wt.)	Variation coefficient** sediment S-94/OT
TBT	*0.5*	*0.3*	*15%*
DBT	*0.4*	*0.2*	*23%*
MBT	*0.3*	*0.2*	*21%*
TPT	*2*	*1*	*18%*
DPT	*0.2*	*0.1*	*13%*
MPT	*0.3*	*0.2*	*25%*

* determined with 99% confidence interval (3 x sd) of the signal-to-noise ratio in chromatogram for 10 g (wet wt.) sample and 50 % dry weight.

** variation coefficient analysis reference sample (harbour dredge spoil S-94/OT; n = 7)

Quantitation was based on SIM (Single Ion Monitoring), quantitation ions are indicated in Table 5.1a. GC-MS-MS was carried out using non-resonant ion ejection, providing a greater linear dynamic range (Schachterle *et al.*, 1994). Calibration was done with standard curves, these were sufficiently linear over a four orders of magnitude range. Concentrations of the organotins are expressed as ng Sn g^{-1} fresh or dry tissue.

Total sediment samples of 10 g fresh weight were first acidified to pH=2 with HCl. Then the same steps as described above for biological tissues were performed. Sediment characteristics were on average 0.6 % organic carbon content and average cumulative percentages of the size fractions < 2 µm, < 20 µm, < 200 µm and < 2000 µm were 5.5%, 18%, 71% and 100% respectively.

c. Quality control of organotin analysis

Quality control included analysis of procedural blanks, control samples (IRM, internal reference samples), spiking experiments and the application of internal standards (dibutyl-dihexyltin and PCB103). All data were corrected for overall recovery of internal standards. The recovery range was 60-90% for the different compounds. Detection limits were determined with the variability of signal-to-noise ratios in the chromatogram (see Table 5.1a,b). Variability within and between sample series was determined by using internal laboratory reference samples of zebra mussel (*Dreissena polymorpha)* homogenate and harbour dredge spoil (see Table 5.1a,b). The repeatability of TBT and TPT were 21% and 23% respectively, for DBT 35% (highest) and for MPT 13% (lowest). Up till now, no suitable certified reference material for organotin analysis in animal tissue is available.

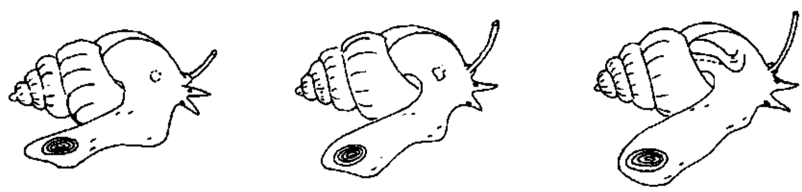
Determination of imposex

Since the animals crawl out of their shell when exposed to air, thereby showing the penis and/or *vas deferens* (sperm duct) development, *B. undatum* could generally be sexed without being sacrificed. Males were characterised by the presence of a full-grown penis and a clearly recognisable *vas deferens*. Individuals were determined as normal females if neither a penis homologue nor a *vas deferens* was present. Imposex in female whelks was characterised by the presence of a penis homologue, which is always considerably smaller than a normal male penis and often differs in shape from it. Another imposex characteristic, the development of a *vas deferens* (in addition to penis development) was also checked. According to the size and shape of the penis homologue, 3 stages of imposex were discriminated (fig. 5.1).
Stage 1 resembles the presence of a small knob at the site where males have their penis.
Stage 2 shows a differently shaped small structure, larger than a knob.
Stage 3 can be recognised by a penis homologue of about 2-3 cm in length (in adults).
In a number of cases a *vas deferens* was also observed. This was indicated with a "+" sign after the number indicating the stage of penis development.

The shells of whelks with a small penis homologue and a *vas deferens* were cracked and the other reproductive organs were studied. The presence of an egg capsule gland, albumen gland, ovary and *bursa* in females were checked as well as the testis and seminal vesicle in males. The length of the shell was measured and recorded. Indications for the presence of parasites (trematodes) have not been found.

Statistical testing

Two way ANOVAs were performed with the factors whelk groups (5 levels) and months (3 levels), including the interaction effect. The log-transformed sum of the butyltin compounds and phenyltin compounds were used as the dependent variables. In these analyses, values below the detection limit were replaced by half the reported detection limit.



Stage 1 Stage 2 Stage 3+

Figure 5.1. Example of the determination of imposex stages in female *Buccinum undatum*.

Results

Organotin concentrations

Organotin concentrations in common whelks are given in Tables 5.2 and 5.4. In general, whole body TBT concentrations in *B. undatum* are just detectable. DBT levels are highest in June and generally above or equal to those of MBT. The increase in metabolite levels in whole body analysis and results from the digestive gland in summer suggest an enhanced environmental TBT concentration and an increased metabolism.

TPT was generally present at the highest level of all organotin compounds. Low concentrations can be found in the foot of the animals (up to 11 Sn ng g^{-1} wet wt. in June), the highest are found in the digestive gland of the animals (up to 250 Sn ng g^{-1} wet wt. in February). DPT and MPT levels in whole body analysis were relatively low compared to those from the digestive gland. TPT, however, was always the dominant phenyltin, indicating that metabolism of this compound is slower than that of TBT, or that its metabolites are excreted extremely rapidly. In September, average whole body organotin concentrations ($\sum$OT = 29.0 ± 6.2) have dropped compared to those in February ($\sum$OT = 45.9 ± 13.0) and June ($\sum$OT = 55.3 ± 21.4). The decrease in the sum of whole body organotin concentrations in September appeared only to be significant ($P < 0.05$) for the sum of the butyltin compounds of the different whelk groups.

In September the ganglia and foot of the animals, however, show remarkable higher TBT concentrations (1.5 to 20 fold) than those of its metabolites (DBT and MBT) and are even higher than TPT concentrations (1.3 to 11 fold).

Although only in June males were analysed, levels of organotins did not differ from females then. Males and females appear to have a comparable organotin

Table 5.2. Whole body organotin concentrations (Sn ng g^{-1} wet wt.)* in *B. undatum* from the Eastern Scheldt in February, June and September 1995.

sex (imposex stage)	n	month	TBT	DBT	MBT	TPT	DPT	MPT
	2	Feb.	*3*	*4.1*	*3.8*	*24*	*<11*	*2.1*
	1	June	*1.7*	*6.5*	*4.0*	*25*	*1.2*	*1.6*
	1	June	*<1.5*	*23*	*10*	*28*	*3.2*	*28*
adult females (3+)	3	June	*1.4*	*11*	*3.8*	*19*	*3.2*	*1.7*
	3	Sept.	*<0.4*	*3.3*	*<0.9*	*17*	*6.4*	*3.2*
	1	Sept.	*4.9*	*1.7*	*2.6*	*20*	*2.8*	*3.0*
	1	Sept.	*<0.6*	*2.2*	*3.9*	*7.6*	*<0.4*	*3.1*
	1	June	*1.4*	*9.8*	*6.9*	*34*	*6.2*	*3.4*
adult female (0)	1	Sept.	*<1.1*	*<2.6*	*<2.2*	*25*	*4.1*	*4.2*
	1	Sept.	*<0.9*	*<2.1*	*2.9*	*21*	*1.9*	*1.9*
adult female (1)	1	June	*2.0*	*16*	*7.7*	*28*	*3.6*	*1.9*
adult males	3	June	*<0.8*	*12*	*6.0*	*39*	*4.5*	*2.7*
	5	Feb.	*2.6*	*5.1*	*2.6*	*20*	*27*	*2.8*
	5	Feb.	*1.6*	*4.7*	*3.1*	*17*	*<15*	*0.8*
juveniles	8	June	*<0.6*	*5.0*	*3.5*	*16*	*1.5*	*2.1*
	10	Sept.	*<0.6*	*3.0*	*<1.2*	*16*	*4.3*	*3.6*
	11	Sept.	*<0.8*	*3.2*	*<1.5*	*18*	*3.2*	*2.6*

* The values of the detection limits differ between samples because different amounts of tissue were used

Table 5.3. Relative amounts of TBT and TPT for *Buccinum undatum* and *Mytilus edulis* from the Eastern Scheldt throughout 1995. (Data of different months combined).

animal	TBT	TPT
M. edulis	3.4	1.0
B. undatum	0.5	8.1

metabolism. Also, no differences in concentrations were observed in relation to the stage of imposex. Juveniles show comparable organotin body burdens to adults. Thus, although developmental stages in these animals are different, the disposition of these xenobiotic compounds is very much alike. Statistical analysis of the data revealed no significant differences in organotin concentrations between the different whelk groups ($P > 0.05$).

In mussels, TBT is always found in higher concentrations than TPT, which is the opposite from the situation in common whelks (figures 5.2a,b). In February TBT and TPT show higher levels than in June and September. In June an increased level of metabolites points to increased metabolism of TBT. DPT/TPT and MPT/TPT ratios showed little differences between months and were always < 0.7. Total organotin concentrations in mussels showed (as in whelks) lowest concentrations in September.

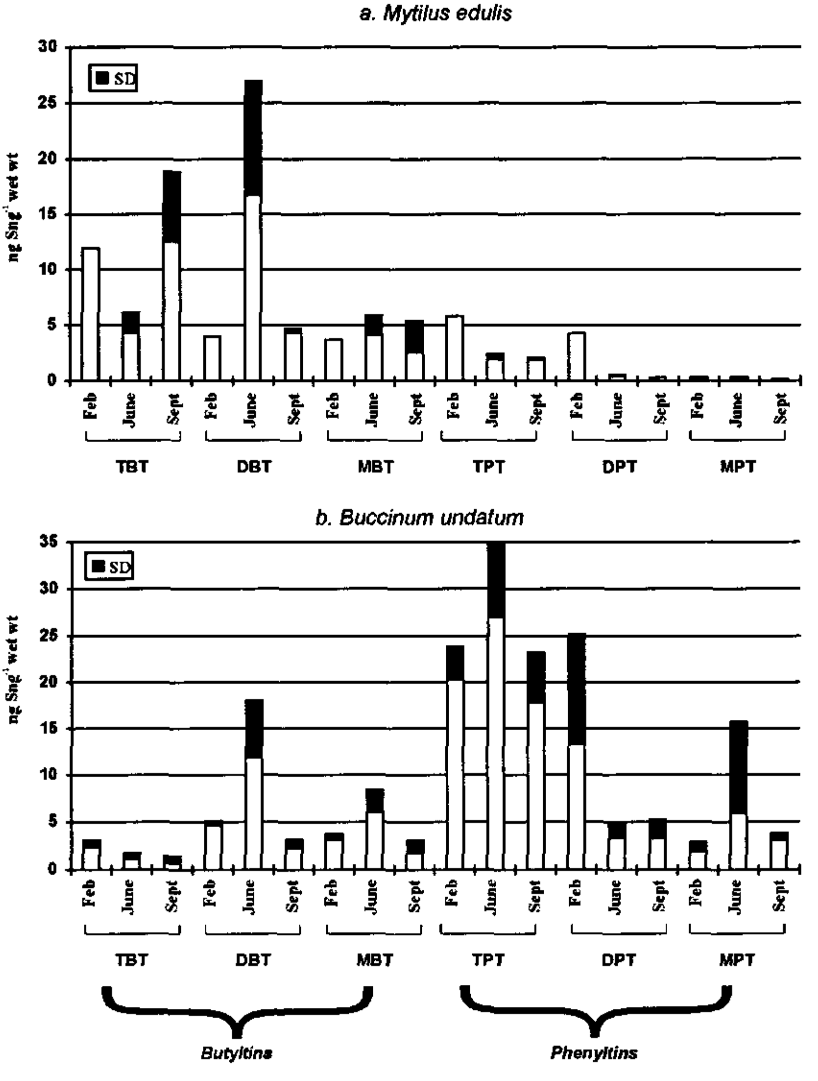


Figure 5.2 a,b. Average (± SD) whole body organotin concentrations (Sn ng g^{-1} wet weight) in *M. edulis* (a) or for all specimens of *B. undatum* (b) per month. (For values < DL, half of the reported DL was used.)

Organotin concentrations in the sediments could hardly be detected in the samples taken. The TBT concentration range was <0.1-1.4 ng Sn g^{-1} dry wt, DBT <0.1-2.4, MBT <0.3-6.6 and TPT ranged <0.2-0.6, DPT and MPT could not be detected (<1.3 and <1.1 ng Sn g^{-1} dry wt respectively).

To compare triorganotin burdens in mussels and common whelks, average TPT levels in whole body homogenate of mussels was used as reference (100%). Calculated relative amounts for common whelks and mussels are shown in Table 5.3. It is shown from these data that TPT biomagnifies, whereas TBT does not. Also, TBT/DBT and TBT/MBT ratios in *B. undatum* are generally <1. In contrast, TPT/DPT and TPT/MPT ratios are >1, indicating an enhanced butyltin metabolism compared to phenyltin metabolism in the common whelk (fig. 5.2b). For determining ratios, values below detection limits were replaced by a value of half the reported detection limit.

Table 5.4. Tissue organotin concentrations (Sn ng g^{-1} wet wt.)* in adult female *B. undatum* (imposex stage 3+) from the Eastern Scheldt in February, June and September 1995.

Tissue	n	month	TBT	DBT	MBT	TPT	DPT	MPT
	2	Feb.	*4.7*	*13*	*16*	*250*	*74*	*24*
	2	June	*<8*	*120*	*42*	*162*	*22*	*20*
	2	June	*<7*	*99*	*29*	*124*	*28*	*<2.6*
dig. gland	2	June	*19*	*98*	*37*	*221*	*33*	*19*
	2	Sept.	*<4.1*	*20*	*46*	*71*	*28*	*44*
	2	Sept.	*<3.6*	*15*	*40*	*84*	*19*	*41*
	2	Sept.	*<2.7*	*14*	*34*	*71*	*36*	*30*
	2	Feb.	*0.6*	*1.1*	*1.2*	*8.1*	*<7.9*	*0.6*
	2	June	*<0.9*	*2.7*	*1.9*	*11*	*0.8*	*<0.3*
	2	June	*<1.0*	*5.2*	*<0.5*	*11*	*0.7*	*1.3*
foot	2	June	*<0.6*	*4.7*	*<0.3*	*5.8*	*0.4*	*0.5*
	2	Sept.	*7.6*	*<2.1*	*<1.8*	*6.0*	*1.8*	*0.8*
	2	Sept.	*1.6*	*<2.2*	*<1.9*	*5.6*	*<0.7*	*0.4*
	2	Sept.	*<0.9*	*<2.0*	*<1.7*	*6.6*	*<0.7*	*0.9*
	6	June	*<5.4*	*<12.6*	*<10.7*	*17.3*	*<4.2*	*<1.8*
ganglia	6	June	*<3.5*	*<8.1*	*<6.8*	*11.4*	*<2.7*	*<1.1*
(nervous system)	6	June	*<2.2*	*<5.2*	*<4.4*	*5.9*	*<1.8*	*<0.7*
	6	Sept.	***21***	*<12*	*<10*	*9.1*	*<4.1*	*<1.7*
	6	Sept.	***79***	*<9.4*	*<8.0*	*7.0*	*<3.2*	*<1.3*

* The values of the detection limits differ between samples because different amounts of tissue were used

Occurrence of imposex in *Buccinum undatum*

The results are given in Tables 5.5 and 5.6. The imposex frequency is very high and virtually constant (> 95%). More than 50% of the females showed advanced stages of imposex (stages 3 and 3+), where relatively long penis homologues (up to 2-3 cm in length) and/or a *vas deferens* were present. Also females with abnormal penis structures (5%) were observed, some even with a double penis (1.5%). No occluded genital pores were found. Thus, despite the occurrence of the advanced stages of imposex, no indications for sterility of common whelks were found. Of the 43 investigated juvenile females (< 55 mm in length) 88% showed imposex, of which 92% was stage 1.

In February more than twenty egg-masses with developing *B. undatum* were caught, indicating that reproduction is still taking place in the Eastern Scheldt. In June only several empty egg-masses were observed.

Table 5.5. Number of *B. undatum* investigated, average length, male: female ratio and imposex frequency in February, June and September 1995 in the Eastern Scheldt.

month	number of individuals	average length (cm) ± SD	males (n)	females (n)	m/f ratio	imposex %
February	*220*	*6.76 ± 0.89*	*114*	*106*	*1.1*	*96*
June	*208*	*7.02 ± 1.00*	*94*	*114*	*0.82*	*96*
September	*345*	*6.66 ± 1.26*	*178*	*167*	*1.1*	*97*

Table 5.6. Stages of imposex in female *B. undatum* in February, June and September 1995 in the Eastern Scheldt (expressed as % of total number of females investigated).

month	females (n)	stage (%)	0	1	2	2+	3	3+
February	*106*		*4*	*12*	*17*	*2*	*39*	*26*
June	*114*		*4*	*6*	*22*	*3*	*41*	*25*
September	*167*		*3*	*24*	*17*	*<1*	*39*	*16*

Discussion

In this study we investigated organotin contamination and imposex incidence during a one year period in the Eastern Scheldt, a marine estuarine area with high leisure boating activity and agriculture in the surrounding land. The predatory gastropod *Buccinum undatum*, *Mytilus edulis* as primary food source and sediment were analysed for organotin compounds in several seasons during 1995.

For butyltin compounds, the order of concentrations in whole body homogenate of common whelks was DBT > MBT > TBT. No structural differences in organotin levels were observed between females with the most advanced stages of imposex and those without imposex. Females and males seemed to be exposed to organotins to the same extent in June and appear not to differ in organotin metabolism. Juvenile common whelks showed similar organotin body burden compared to adults. Juvenile females also showed imposex. However, tissue concentrations inducing imposex in *B. undatum* have not been established so far. Because sensitivity will differ between species and juveniles are more sensitive than adults (Stroben, 1994; Mensink *et al.*, 1996b), it seems impossible to draw conclusions concerning the exact body burden of common whelks which will induce imposex. Also, possible (synergistic) interfering agents in the mechanism of imposex induction will affect the level at which imposex is induced. As long as the mechanism for imposex development is not well understood, one should be careful drawing such conclusions.

In Japan (Horiguchi *et al.*, 1994), maximum observed TBT levels in *Thais clavigera* and *T. bronni* were 750 ng g^{-1} wet weight and estimated TBT levels inducing imposex in these species were 4-8 ng Sn g^{-1} wet weight. In south-west England (Gibbs *et al.*, 1987) for *N. lapillus* TBT body burdens of up to 633 ng g^{-1} dry weight were reported and the deducted body burden inducing imposex in the dog-whelk from this study was around 6-8 ng Sn g^{-1} wet weight (dry weight = 4-5 times wet weight). In France, *Hinia reticulata* showed maximum TBT levels up to 1400 ng Sn g^{-1} dry weight, while at sites away from sources of TBT contamination up to 100 ng g^{-1} dry weight was found (Stroben *et al.*, 1992). In the last study no body burdens inducing imposex in the species investigated were suggested. If the estimated levels inducing imposex from the two studies can be extrapolated to other gastropod families, levels encountered in the common whelks from the Eastern Scheldt can be regarded as imposex inducing.

TBT itself was often just above the limit of detection in the neural ganglia (nerve centre) except in September, when only the parent compounds (TBT, TPT) could be detected and the TBT/TPT ratio was even higher than 1. These high concentrations of TBT only in the ganglia of *B. undatum* are remarkable. In another study (Bryan *et al.*, 1993), high TBT levels in nervous tissue in *N. lapillus* were found and led to the speculation that TBT interferes with neurohormones, which may lead to the induction of imposex. It was concluded that TBT affected cerebropleural ganglia in *Ocenebra erinacea* causing a release of neural factors (hormones) inducing penis growth (Féral & leGall, 1983). The high affinity of the ganglia for TBT (no other organotin compound is enhanced) seemed to occur only in the interval prior to reproduction and could point to a seasonal sensitivity at a critical moment. This could be important in view of mechanistic aspects of the induction of imposex that might concern the involvement of neurohormones or steroids and needs more research.

Although Bettin and co-workers (1996) attributed imposex to be mediated by an increased androgen level, they failed to explain the most dramatic increase in VDS and penis growth in the first weeks, when hormone levels did not differ significantly between reference and exposed groups. Also, they could not refer to a control group which was unaffected; the observed effects are thus limited to promotion of imposex, not induction.

Phenyltins were present in all animal samples. TPT clearly showed the highest levels, followed by levels of its metabolites DPT and MPT, both in similar amounts.

TPT concentrations in whole body homogenates of whelks were 4-100 times higher than those of TBT. No clear seasonal trend could be observed for phenyltin levels.

TPT concentrations reported here are comparable with values reported for animals from the Western Scheldt (64 ng Sn g^{-1} wet weight, molluscs) and North Sea (26 ng Sn g^{-1} wet weight, fish) (Crijns *et al.*, 1992), but data on other phenyltin compounds in the marine environment are scarce. TPT and its metabolites are present in high levels in all Dutch fresh water systems (Stäb *et al.*, 1995), indicating that TPT is widely used in the Netherlands. Therefore, its presence in coastal waters is not surprising. In Japan, TPT levels are reported to be as high as 88 ng l^{-1} in coastal waters, 1100 ng g^{-1} in sediments and up to 2600 ng g^{-1} in fish (Yamada *et al.*, 1994). *Thais clavigera* and *T. bronni* from Japanese coastal areas showed TPT concentrations of up to 1770 ng g^{-1} and in these species TPT>MPT>DPT (Horiguchi *et al.*, 1994). In Spanish coastal waters, TPT concentrations of up to 94 ng l^{-1} were found next to TBT concentrations of up to 350 ng l^{-1} (Alzieu *et al.*, 1991).

Mussels, *Mytilus edulis*, representing an important food source for common whelks, showed a different accumulation pattern of butyltin compounds than *B. undatum*. Generally TBT > DBT > MBT, which is comparable to what has been found in mussels in the Eastern Scheldt and Grevelingen in 1988 (Ritsema *et al.*, 1991) and to water butyltin ratios in the Eastern Scheldt (Ritsema, 1994) and in other studies into TBT contamination in oysters and mussels (Garcia-Romero *et al.*, 1993; Uhler *et al.*, 1993; Skarphédinsdóttir *et al.*, 1996). The present TBT concentrations ranging from <6 - 17 ng Sn g^{-1} wet weight were comparable to or higher than those at location "de Val" in the Eastern Scheldt in 1988 (Ritsema *et al.*, 1991) and in the range as reported for mussels in Icelandic waters (Skarphédinsdóttir *et al.*, 1996) and the Gulf of Mexico (oysters) (Garcia-Romero *et al.*, 1993). On the basis of TBT concentrations measured in mussels from the Eastern Scheldt and literature data on bioconcentration factors (BCF values) for *Mytilus edulis* (Evers *et al.*, 1995), a prediction on water concentrations was made for minimal and maximal water TBT levels. This resulted in a (min-max.) concentration range of 0.4-70 ng Sn l^{-1} (1-170 ng TBT l^{-1}), which is supported by data reported by (Ritsema, 1994), where 12-25 ng Sn l^{-1} was measured at the marina Colijnsplaat during 1990-1993. A study by (Laughlin & French, 1988) also supports these water concentration predictions. In their study exposure of *Mytilus edulis* to TBT concentrations of 10-18 ng Sn l^{-1}, resulted in a TBT body burden of 8-16 ng Sn g^{-1} (wet weight) after 10 days (steady state). The Eastern Scheldt is an important shellfish culture area and the calculated TBT water concentrations are comparable to other shellfish plots in the Mediterranean region (<1-7 ng Sn l^{-1}) and in the Atlantic (3-23 ng Sn l^{-1}) (Tolosa *et al.*, 1992).

TPT was detected in all samples, but levels were 4 to 10 fold lower as in common whelks. DPT and MPT were often below the detection limit.

In total sediment samples from this study organotins were usually below their detection limit. Most studies on the TBT burden of sediments have focused on sediments of marinas or near harbours and docking activities, where concentrations were found to be very high (up to 38 μg g^{-1}) (Stewart & de Mora, 1992; Page *et al.*, 1996). In sediments of Arcachon harbour (France), where the organic carbon content was 5 %, sediment with a grain size < 60 μm contained 35 times more TBT (596 ng g^{-1}) as the sediment fraction > 60 μm (Quevauviller *et al.*, 1994). On the average in the present study the sediment fraction <63 μm comprised 26 % of the total sediment and the total organic carbon content was 0.60 %, which probably explains the low organotin levels.

Biomagnification of organotin compounds not only affects marine snails, but may also threaten other marine predators like fish and cetaceans. High levels of butyltins in, and effects of triorganotin compounds on the immune system of mammals, fish and several marine invertebrates have already been reported. (Kannan *et al.*, 1997; Fent, 1996).

The percentage of all female whelks from the Eastern Scheldt showing imposex was high (>95%) and adult as well as juvenile females showed male sexual characteristics. This percentage did not change throughout the year. From a laboratory study into the imposex inducing dose of TBT in *B. undatum* it was shown that TBT concentrations below 7 ng Sn l^{-1}, as found in the Eastern Scheldt, indeed induce imposex in juvenile common whelks in a dose-dependent manner. Adult females, however, did not show any sign of imposex during 9 months of exposure to nominal TBT doses up to 1000 ngTBTAc l^{-1} (340 ng Sn l^{-1}). This high dose also induced abnormalities in the male sexual characteristics formed by the juveniles (Mensink *et al.*, 1996b). Similar abnormalities, like a double penis and double penis openings, were found in adult females from the Eastern Scheldt in the present study, indicating that TBT concentrations might have been higher during previous years. Stages of *vas deferens* development only, as observed in a laboratory study (Mensink *et al.*, 1996b), have not been found in this study and in a previous study in the Eastern Scheldt (Mensink *et al.*, 1996a). Concluding from these studies, imposex development in *B. undatum* follows all possible routes of development indicated in (Stroben *et al.*, 1992), which is different from the assumption that the *vas deferens* would develop from the base of the penis to the vaginal opening in *B. undatum* (Stroben *et al.*, 1992).

No sterile females with occluded genital pores were observed in the present study, although more than 50% showed stage 3 or 3+ of imposex. Egg capsules with developing young animals clearly showed that reproduction is still taking place in the Eastern Scheldt population. It is unknown, however, which effects are caused by TBT concerning the reproductive success in common whelks. If hormonal imbalances are caused (Bettin *et al.*, 1996; Spooner *et al.*, 1991), effects on the reproductive potency of these females are likely to occur, like the inhibition or blockage of egg production. Both cited papers fail to relate the hormonal disorders to the reproduction level. It is therefore interesting to study all reproductive effects of TBT, including behavioural effects like the responses to or production of pheromones and effects on the fertility of the gastropods. More (basic) information on the production and function of steroid- and neurohormones in invertebrates is needed.

Recently (Horiguchi *et al.*, 1997) have shown that injection of TPT can also promote and even induce imposex in *Thais clavigera*. From this study it was concluded that TBT and TPT have similar effect concentrations for the promotion and induction of imposex in this muricid gastropod, when injected in the foot of the animals. Another study (Bryan *et al.*, 1988) into the effect of TPT and five other organotins on the induction of imposex in *N. lapillus* did not show an imposex inducing capacity of TPT, when TPT was administered via the waterphase and via injection. So, literature on the potency of TPT to induce imposex is not conclusive. If TPT would induce or promote imposex similar to TBT in *B. undatum*, its levels in the Eastern Scheldt might contribute substantially to imposex in this area. However, TPT levels in whole body analysis in this study do not show a significant ($P > 0.05$) difference between females with advanced stages of imposex and visually unaffected females.

The percentages of imposex reported here are in accordance with previous observations in the Eastern Scheldt (Mensink *et al.*, 1996a). Thus, the ban on the use of anti-fouling paints based on TBT which became effective already in 1990, failed to result in a decrease in the imposex incidence and in severity. This indicates that TBT concentrations (and possibly TPT) in the Eastern Scheldt are still well above the threshold level inducing imposex and thus a threat to the benthic fauna.

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CHAPTER 6

SHELL DAMAGE AND MORTALITY IN THE COMMON WHELK, *BUCCINUM UNDATUM*, CAUSED BY BEAM TRAWL FISHERY

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Abstract

Common whelks, *Buccinum undatum*, collected from the southern North Sea were investigated to study the amount of shell damage and mortality caused by beam trawl fishery. The ability of whelks to repair their damaged shells was studied in the laboratory. Whelks (n = 876) were caught with fine meshed 3m beam-trawl or with commercial 4m and 12m beam trawls, while in some areas whelks were also caught with baited traps (used as reference). Shell damage varied considerably for the different groups. For whelks collected by beam trawling minor shell damage was observed in 17-75% and severe damage (when protection against predators and scavengers is lost) was observed in 10-83% of the animals. Whelks caught with baited traps sustained only minor shell damage (0-27% of the individuals) and damage was significantly ($P < 0.005$) less than in beam trawled specimens. The majority of the whelks in all groups exhibited signs of former shell damage, which had since been repaired.

Whelk survival was studied in the laboratory over a six week period. Only 40% of the whelks caught with the 12m beam trawl survived, irrespective of the damage suffered. Whelks that survived and recovered had repaired their shell after six weeks. More than 95% of the whelks caught with baited traps survived the six week experimental period; this is significantly ($P < 0.05$) higher than the survival of animals caught with the 12m beam trawl.

At five locations females were screened for the presence and stage of imposex. Mild imposex development (mostly stage 1 and 2) was observed at all locations with incidences of 32-80%.

It is concluded that beam trawl fishery may be a much greater source of mortality for common whelks than previously thought.

Introduction

Recently a number of publications, based on fishery landings and data on distribution, have indicated a decline in populations of the common whelk, *Buccinum undatum*, in areas where it was once abundant (de Vooijs *et al.*, 1993; Cadée *et al.*, 1995; Michaelis, 1996; ten Hallers-Tjabbes *et al.*, 1996; Nicholson & Evans, 1997). Both fisheries and the phenomenon of imposex may form a serious threat to the development of whelk populations. In England and Ireland, whelk fisheries have increased since 1990 due to increasing demands of the commercial market in the Far East, and whelk stocks are now heavily exploited (Nicholson & Evans, 1997; Fahy *et* *al.*, 1995). However, there is no commercial whelk fishery in the eastern part of the southern North Sea and the number of whelks caught as by-catch in this area has declined in the last decades to almost none between 1980 and 1990 (de Vooijs *et al.*, 1993).

Imposex, the development of male sexual characteristics in female gastropods (next to their own sexual organs) is caused by tributyltin (TBT), the active biocide in anti-fouling paints (Gibbs *et al.*, 1988; Bryan *et al.*, 1988; Mensink *et al.*, 1996a). Imposex in *Buccinum undatum* has recently been reported in and around the North Sea, while in the early 1970's it did not occur (ten Hallers-Tjabbes *et al.*, 1994; Mensink *et al.*, 1996b; Ide *et al.*, 1997; Nicholson & Evans, 1997). Although imposex does not cause direct mortality, it can affect reproduction in gastropods. Sterility due to the occlusion of the female sexual opening by a developing *vas deferens* and reduced egg production have been reported for *Nucella lapillus* (Gibbs *et al.*, 1988). This form of mechanical sterility does not occur in *B. undatum* (Mensink *et al.*, 1996b; Ide *et al.*, 1997; Nicholson & Evans, 1997), but retarded growth due to TBT exposure can affect reproduction as juveniles take longer to reach maturity (Mensink *et al.*, 1997b).

The intensity of beam trawling has increased considerably since 1970's in the southern North Sea, where more than 1000 trawlers operate in flatfish fishery. About 600 small vessels (< 300 kW) fish with two 4m-wide beam trawls (each about 1500 kg) in the coastal areas, trawling with a speed of about 4 miles/hr. About 300 larger vessels (> 1000 kW) operate in the offshore areas with two 12m-wide beam trawls (each about 7000 kg) and a trawling speed of about 6 miles/hr. Both 4m and 12m beam trawls are fitted with about 10 tickler chains in front of the ground rope, digging into the upper 4-6 cm of the sediment which may inflict considerable damage to the benthic fauna (Bergman *et al.*, 1998). Beam trawl fisheries not only affect the commercial target fish species, like sole (*Solea solea*) and plaice (*Pleuronectes platessa*), but also (non target) invertebrates and non-commercial fish species. The discarded by-catch may amount to up to 80% of the total catch (Craeymeersch *et al.*, 1998). An even larger number of animals is not caught in the nets but remains damaged on the seabed in the trawl tracks. Scavenging species, including seabirds, benefit from the discards and damaged animals (Camphuysen *et al.*, 1995; Groenewold, 1996; Ramsay *et al.*, 1998). Evidence of the longer term effects of fisheries in the North Sea is provided by the historical decline of some species. Several larger fish species (rays, sharks) and invertebrates that used to be fairly common in the coastal areas until about 1970, have become scarce in the period between 1970 and 1990 (Walker & Heessen 1996; Philippart, 1998).

Trawling causes considerable mortality in many of the animals caught and discarded from beam trawls. However, in short-term (< 1 week) survival experiments on-board of trawlers, Kaiser & Spencer (1995), Fonds (1991 & 1994) and Fonds *et al.* (1992) found *B. undatum* to be very resistent to the effects of trawling, with survival percentages of 98%-100%.

In addition to whelks captured in trawls and landed on the deck of a trawler, many are either disturbed, or pass through the meshes of the net but remain on the seabed. Whelks that remain in the tracks of the trawl, can be rapidly attacked by predators such as star fishes (*Asterias rubens*) and hermit crabs (*Pagurus bernhardus*)

(Ramsay *et al.*, 1998). Mortality is also caused in whelk embryos, when egg capsules are torn loose from the substrate (Bergman *et al.*, 1998; *Chapter 2*).

Trawling leads to a high incidence of shell damage in a number of benthic molluscs, e.g. *Arctica islandica, Ensis* spp., *Solen* (Bergman & Hup, 1992; Witbaard, 1997). Depending on the type of shell damage, animals may be able to repair this damage if they are not consumed by predators (Cadée, 1995; Cadée *et al.*, 1997; Witbaard, 1997).

The present study was undertaken to investigate i) shell damage in whelks attributable to beam-trawl fisheries, ii) the survival capacity of beam trawled whelks in the absence of predators, iii) the potential for shell repair under laboratory conditions.

When possible, the frequency and stage of imposex occurrence were also investigated in the same animals.

Materials and methods

Between 1 and 12 May 1995, *Buccinum undatum* were collected from a number of locations in the southern North Sea, using a fine-meshed 3m beam-trawl and commercial 4m and 12m beam trawls towed by the R.V. Tridens of the Institute of Fisheries Research (RIVO-DLO, IJmuiden, The Netherlands). The whelks caught in this manner were compared to whelks caught with traps, baited with mollusc meat, which is the preferred food of *B. undatum* (S. Groenewold, pers. comm., 1998).

The fine-meshed 3m beam trawl had 6 tickler chains, a codend mesh size of 1 cm and was towed for 5 minutes. The 4m and 12m beam trawls (total weight 1500 and 7000 kg respectively) had 10 tickler chains. The 12m beam trawl also was provided with a chain matrix of 8 net-tickler chains. Codend mesh sizes of the nets were 8 cm for 4m and 12m beam trawl, the trawl durations were approximately 15 min.

Trap types used were transparent-pipes and commercial Danish crab traps (Fonds *et al.*, 1998, fig. 2.6.3 g+h).

Whelks were caught by alternation of the gears. During the cruise the 3m, 4m, 12m beam trawls and baited traps were used 4, 3, 6 and 4 times respectively, each time at different locations.

Field observations

Whelks were examined on board directly after capture. Whelks were measured to the nearest mm from the apex to the end of the siphonal canal.

Damage

The following types of shell damage were recorded:

- outer lip (light and severe damage)
- hole in the shell
- siphonal canal
- operculum missing

If the outer lip of the last whorl and/or the siphonal canal was damaged, severity was estimated by studying whether the animal could withdraw itself completely within the shell (light damage) or not. The latter situation was considered as severe shell damage, because protection of the animal against predators and scavengers was lost (fig. 6.1a).

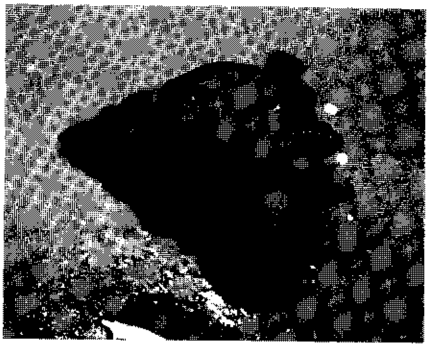

Fig. 6.1a. Severe outer lip damage in *B. undatum* from the North Sea.

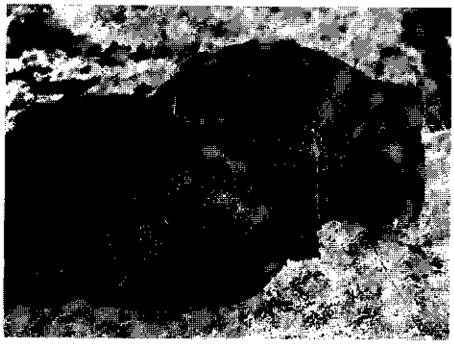

Fig. 6.1b. Hole in the final whorl of the shell of *B. undatum* from the North Sea.

Fig. 6.1c. Damaged outer layer of the shell (grazed shell) of *B. undatum* from the North Sea.

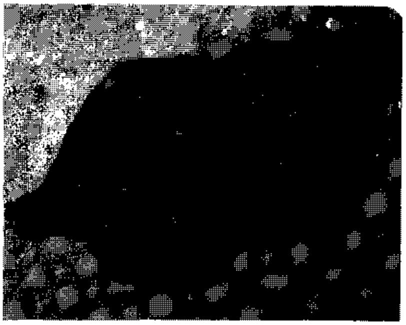

Fig. 6.1d. Scars from former shell damage of *B. undatum* from the North Sea.

Figures 6.1a-d : Examples of shell damage as observed in *B. undatum* caught with a beam trawl or baited traps in the North Sea.

When a hole in one of the whorls occurred this was noted as severe damage (fig. 6.1b), as soft parts or essential organs could be damaged and exposed to the environment. If only the outer shell layer was lost, but the thin inner, mother of pearl layer was still present (= grazed shell in Table 6.1, figure 6.1c), this was also considered as light shell damage. The absence of an operculum was considered as severe damage. The top of the shell (first whorl) was often broken off. This was considered as light damage and of very little importance when no opening to the inner space of the shell existed/was formed, but noted as severe damage if this exposed the animal to scavengers or predators. The presence of scars (repair marks) on the shell was noted as an indication for former (old) damage (fig. 6.1d).

Imposex

Imposex, the occurrence of male sexual characteristics in female *B. undatum,* was recorded at those locations where whelks were sexed (fig. 6.2). Due to the time-consuming sex determination, this was only possible for a limited number (5) of locations. The presence of a penis-like structure in females, at the spot where males grow their penis, and a partial or complete *vas deferens*/sperm duct in female *B. undatum* indicated the presence of imposex. Stages of imposex were determined according to Mensink *et al.* (1996a).

Laboratory survival experiment

Whelks were kept at 13°C in 6 aquaria of 275 l with a thin layer (1-2 cm) of sediment on the bottom and running Wadden Sea water (salinity 30‰), which contained virtually no particulate matter due to the passage through a settling basin. Three sides of the aquaria were painted black to reduce light exposure but to retain natural light conditions. Whelks were fed with mussels (*Mytilus edulis*) once a week.

They were divided into three groups according to the level of shell damage and capture method and each group was divided over two aquaria (duplicate);

- one group with no or light damage, caught with a 12m beam trawl (27 + 30 individuals)
- one group with severe damage, caught with a 12m beam trawl (19 + 20 individuals)
- one group caught with baited traps (reference group) (26 + 27 individuals)

Survival over a period of six weeks was examined for each group. When a whelk died, characterized by a bulged proboscis and/or the absence of a foot reflex, its length and the state of shell repair was recorded.

Statistical analysis

One way ANOVA was performed to test, whether differences in the percentages of damage and survival between the groups would be statistically significant. Log(P/1-P) transformation of the data was used to obtain homogenicity of variance. For the field study the sums of the percentages of light, severe and old damage were tested. In the laboratory survival experiment differences in survival percentages were tested at 3/6/95 (22 days) and 18/6/95 (37 days).

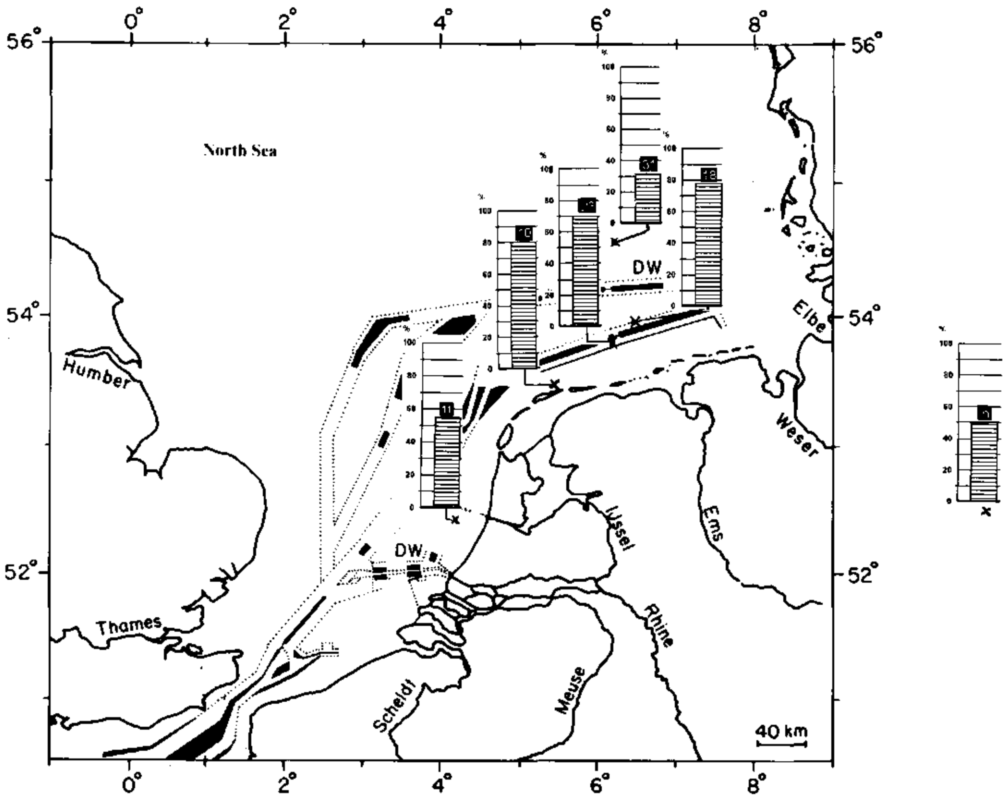

Figure 6.2. Sampling location (×), number (n) of female *B. undatum* investigated and imposex percentages in relation to shipping traffic routes. Dotted lines = demarcation of deep water routes. Solid blocks within dotted lines = traffic separation scheme.

Results

Field observations

Shell damage

A total number of 876 *B. undatum* have been investigated. Tables 6.1 & 6.2 present results as to shell damage for four groups of animals caught with different types of fishing gear. On average light damage was observed in 50% (31-75%) of the 3m beam trawled animals, whilst the average severe damage was 34% (22-46%). For the 4m trawled animals these percentages were 29% (17-35%) and 38% (15-83%) and for the 12m trawled animals 48% (38-58%) and 30% (10-56%) respectively. The animals caught in baited traps on average only showed 10% light damage (0-27%), but no severe damage. Statistical analysis of the data showed significantly less light and severe damage in the animals caught with the baited traps compared to the beam trawled whelks ($P < 0.005$ and $P < 0.001$ respectively). The beam trawled whelks did not differ significantly from each other with respect to the light damage ($P > 0.7$) and severe damage ($P > 0.5$).

Because juveniles have a thinner shell than adults, and are thus possibly more vulnerable, results are also calculated for adults (> 55 mm) only. In this way comparison is more accurate, because the percentages of juveniles varied between groups. Now, average percentages of light damage were 39% (14-83%), 29% (25-32%) and 48% (38-60%) for the 3m, 4m and 12m beam trawled whelks respectively. Severe damage was observed in 8% (0-14%), 34% (9-75%) and 23% (10-43%) for the 3m, 4m, and 12m trawled animals. Since only adult whelks were caught in the baited traps results are the same as those for all whelks.

The results again showed significantly less light ($P < 0.005$) and severe ($P < 0.001$) damage for the whelks caught with baited traps compared to the beam trawled whelks. Although no significant differences were observed between the beam trawled groups ($P > 0.4$) with respect to light shell damge, a significant difference was observed in severe damage between the 3m and 4m beam trawled animals. The animals caught with the 4m beam trawl showed significantly ($P < 0.05$) more severe damage than the whelks caught with the 3m trawl. There was no significant difference ($P > 0.7$) between the 4m and 12m beam trawled whelks with respect to the observed severe damage.

In all groups, the majority of whelks showed scars from former shell damage that had apparently been succesfully repaired. There was no statistical significant difference between the four groups ($P > 0.15$). More than 90% of the scars were repaired outer lips and siphonal canals. About 34% of the whelks with scars had more than one scar.

Imposex

The results (Table 6.3 & fig. 6.2) show high incidences of imposex at positions close to the Dutch coast (> 50%), whilst further off shore 32% imposex was found. Although the imposex incidences were all > 30%, the stages of imposex development were light to moderate (mostly stages 1 and 2). A partially developed *vas deferens* next to a penis homologue (stage 3+) was observed in only one female.

Table 6.1. Old and light shell damage in *B. undatum* from the North Sea (1995) for different types of fishing gear.

gear type	no.	total # whelks (= a)	# whelks > 55 mm (= b)	Σ % old damage	Σ % old damage	% LIGHT DAMAGE						Σ % light damage	Σ % light damage
						outer lip	outer lip	siphonal canal	siphonal canal	grazed shell	grazed shell		
				a[1]	b[2]	a[1]	b[2]	a[1]	b[2]	a[1]	b[2]	a[1]	b[2]
3m trawl	1	94	70	72	86	30	26	6	3	<1	<1	36	29
	2	13	7	62	86	8	<1	15	<1	8	14	31	14
	3	12	6	50	100	42	50	17	<1	17	33	75	83
	4	27	14	52	86	33	29	22	<1	<1	<1	56	29
4m trawl	1	49	46	67	70	22	20	6	7	6	4	35	30
	2	118	108	67	70	19	19	9	6	7	7	35	32
	3	6	4	33	50	<1	<1	17	25	<1	<1	17	25
12m trawl	1	89	88	94	95	29	30	20	20	<1	<1	49	50
	2	103	93	80	87	33	35	5	3	<1	<1	38	39
	3	40	35	68	77	30	34	20	17	8	9	58	60
	4	127	109	65	74	28	28	17	12	2	3	48	42
	5	79	79	68	68	32	32	6	6	<1	<1	38	38
	6	9	7	67	71	22	29	33	29	<1	<1	56	57
baited traps	1	15	15	93	93	27	27	<1	<1	<1	<1	27	27
	2	2	2	50	50	<1	<1	<1	<1	<1	<1	<1	<1
	3	21	21	81	81	5	5	5	5	<1	<1	10	10
	4	72	72	67	67	3	3	<1	<1	<1	<1	3	3

[1] calculated for the total number of whelks
[2] calculated for the number of whelks > 55 mm

Table 6.2. Severe shell damage in *B. undatum* from the North Sea (1995) for different types of fishing gear.

gear type	no.	total # whelks (= a)	# whelks > 55 mm (= b)	% SEVERE DAMAGE outer lip a[1]	outer lip b[2]	operculum missing a[1]	operculum missing b[2]	hole in shell a[1]	hole in shell b[2]	Σ % severe damage a[1]	Σ % severe damage b[2]
3m trawl	1	94	70	20	9	<1	<1	2	1	22	10
	2	13	7	46	14	<1	<1	<1	<1	46	14
	3	12	6	33	<1	<1	<1	<1	<1	33	<1
	4	27	14	26	<1	<1	<1	7	7	33	7
4m trawl	1	49	46	10	11	<1	<1	6	7	16	17
	2	118	108	10	5	1	1	4	5	15	9
	3	6	4	83	75	<1	<1	<1	<1	83	75
12m trawl	1	89	88	10	9	1	1	16	16	27	26
	2	103	93	21	14	<1	<1	3	3	24	17
	3	40	35	25	14	<1	<1	10	11	35	26
	4	127	109	15	6	<1	<1	10	11	25	17
	5	79	79	6	6	<1	<1	4	4	10	10
	6	9	7	33	14	22	29	<1	<1	56	43
baited traps	1	15	15	<1	<1	<1	<1	<1	<1	<1	<1
	2	2	2	<1	<1	<1	<1	<1	<1	<1	<1
	3	21	21	<1	<1	<1	<1	<1	<1	<1	<1
	4	72	72	<1	<1	<1	<1	<1	<1	<1	<1

[1] calculated for the total number of whelks
[2] calculated for the number of whelks > 55 mm

Table 6.3. Number and percentage of *B. undatum* from the North Sea (1995) analysed for sex and imposex stage.

location		# whelks	# females	% females with imposex	imposex stage (%) [a]			
					1	2	3	3+
52°27'N	4°15'E	29	11	55	67	33		
54°34'N	6°15'E	70	31	32	30	70		
53°56'N	6°33'E	54	18	78	36	50	14	
53°48'N	6°09'E	33	23	70	44	50		6
53°28'N	5°26'E	16	10	80	13	88		

[a] according to Mensink *et al.* (1996a)

Laboratory experiment

Survival

All whelks but one caught with baited traps (control) survived the six-week experimental period (fig. 6.3). In contrast, the 12m beam trawled specimens with light or severe damage had a survival rate of only 40%. Mortality rates were highest for beam trawled specimens during the first two weeks. This indicates that whelks might have recovered and became acclimated to laboratory conditions. After one month a few whelks with no/light shell damage died without a clear cause. At the end of the six-week period all remaining whelks had repaired their damage as shown in figures 6.4a-c. In general, the survival of whelks in both aquaria of the same type of experimental group showed close agreements.

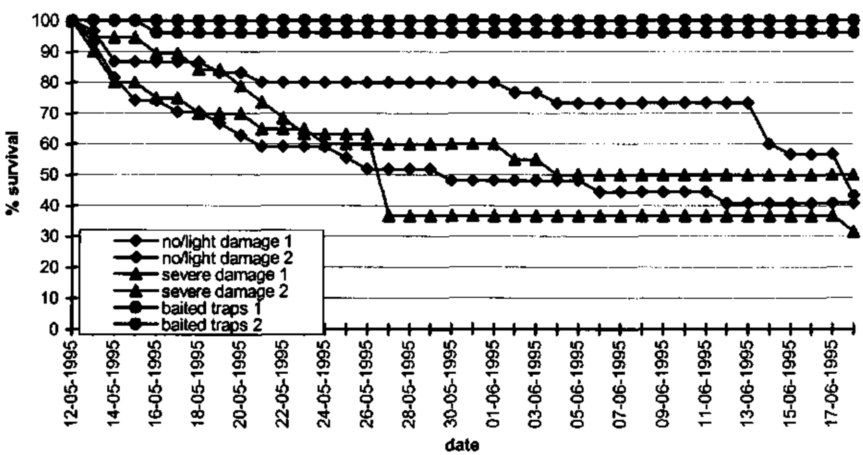

Figure 6.3. Survival (%) of *B. undatum* from the North Sea (1995) in the laboratory after being caught with commercial 12m beam trawl (light/severe damage) or baited traps.

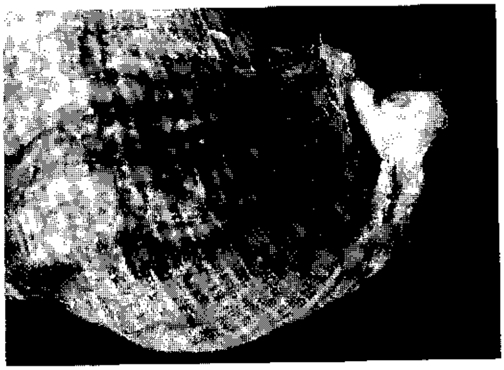

Figure 6.4a. Repaired siphonal canal of *B. undatum*.

Figure 6.4b. Repaired light outer lip damage of *B. undatum*.

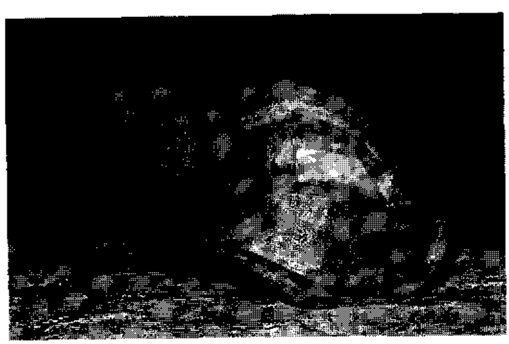

Figure 6.4c. Repaired severe outer lip damage of *B. undatum*.

Figures 6.4a-c : Repaired shell damage of *B. undatum* from the North Sea, caught with a 12 m beam trawl or baited traps.

Statistical analysis on 3/6/1995 (22 days in captivity) revealed an almost significant difference ($P = 0.051$) between the three groups, whilst at the end of the study period (18/6/1995) differences were significant ($P < 0.05$). Post-hoc comparison showed that survival in the group caught with baited traps was significantly higher ($P < 0.05$) than in the groups caught with the 12m beam trawl. There was no significant difference in the survival percentages of the severely damaged or the lightly damaged beam trawled groups ($P > 0.9$).

Discussion

Field observations

Damage

Most of the existing shell damage observed in *B. undatum* caught during this study was probably caused by the beam trawl fishery. Nielsen (1975) and Cadée (1995) describe the possibility that the light outer lip damage could also be inflicted, when whelks unsuccessfully attack larger bivalves. Such light damage can also originate from attacks by predators such as crabs and lobsters; this results in a characteristic shell damage pattern due to crushing and chipping of the shell (Thomas & Himmelman 1988; Cadée, 1995). However, such characteristic patterns were not found in the whelks in our studies. A high incidence of self-inflicted damage from attacking large bivalves also seems unlikely, as large bivalves were not abundant at the locations examined. A more plausible explanation for light outer lip damage may be that it can be caused during handling on board, as indicated by the light damage in whelks caught with baited traps (although this damage was significantly less frequent than in beam trawled whelks). The beam trawl fishery is likely to be the main cause for the other damages described. The damage is probably caused by direct contact with the tickler chains and net-tickler chains and the weight and composition of the catch is also likely to affect damage rates. Another possible factor affecting damage rates might be the nature of the sea floor; the dragging of the net on a hard (rocky) bottom probably causes a different damage pattern than a soft sandy sea floor. This could have affected the results of this study. However, since in this study gears were used one after the other, the significance of bottom composition on the results was reduced. A significant difference was observed in severe damage between the 3m and 4m trawled adult whelks, which possibly can be explained by the gear type and/or fishing time. The 3m beam trawl was only towed for 5 minutes compared to 15 minutes for the 4m and 12m trawl and the latter also contained more chains. The majority of all whelks showed scars from former shell damage in all groups, indicating that damage is caused to the same extent.

Imposex

The percentage of imposex was between 30 and 80% at all of the five locations. Locations closest to the coast line, in areas with intense shipping and fishing (Rijnsdorp *et al.*, 1997) show the highest percentages of imposex (up to 80%), although the stages of imposex were mild. Effects on reproduction of *B. undatum* due to morphological changes (mechanical sterility) are not to be expected (Mensink *et al.*, 1996b). Our results are in good agreement with results of other studies in the North Sea (ten Hallers-Tjabbes *et al.*, 1994; Ide *et al.*, 1997; Nicholson & Evans, 1997). However, in this study we present additional data on whelks collected close to and in the shipping lane and close to the coast line, where whelks were not sampled before.

TBT causes imposex in *B. undatum* (Mensink *et al.*, 1997b) and at stations in the North Sea with intense shipping and imposex, organotin levels are elevated compared to other stations (ten Hallers-Tjabbes *et al.*, 1996b; 1997). Because growth can already be impaired at TBT concentrations below the levels which induce imposex in juvenile *B. undatum* (Mensink *et al.*, 1997b), we can expect an indirect effect of TBT exposure on reproduction as juveniles take longer to reach maturity. Shell damage may exacerbate this effect, since the repair of such damage is energy consuming (Vermeij, 1993).

Laboratory experiment

Survival

From the laboratory survival experiment it is clear that many whelks died as a consequence of beam trawling. After six weeks, there was no difference in the survival of whelks with no/light shell damage or with severe shell damage (both 40%). In whelks caught with baited traps, used as reference, only one animal died. After capture, these animals were treated similarly to beam trawled animals and therefore the difference in survival percentage observed can only have been due to the effect of the 12m beam trawl. The results are in contrast to those of Fonds (1991 & 1994), Fonds *et al.* (1992) and Kaiser & Spencer (1995), who reported little (2%) or no mortality of whelks caught with beam trawls and kept for several days on board in survival tanks with running seawater. However, in their studies survival of whelks was observed for less than one week, whereas the results suggest a critical period of at least two weeks for whelks after being caught with a 12m beam trawl (and discarded). For whelks caught with baited traps, the high survival percentage shows that laboratory conditions and the transport from the ship to the laboratory had little effect on mortality, although the transport certainly had caused some stress in these animals.

Moribund whelks invert themselves in the laboratory (own observations). It often takes several days before the animal actually dies; when it is slightly touched, a live whelk in poor condition still retracts its foot. It is evident that such animals are extremely vulnerable to predators at this stage under natural circumstances.

At Red Wharf Bay in July 1995, Ramsay *et al.* (1998) and Ramsay & Kaiser (1998) observed that a large proportion of inverted whelks were attacked by sea stars and hermit crabs in an area fished by a scallop dredge. This suggests that the survival percentages in our experimental aquaria might have even been lower in the presence of predators. In future studies it would be interesting to examine the potential for shell repair and consequent survival with and without predators.

The number of whelks with scars in our samples indicate that shell damage can be quickly repaired if the animal recovers. In our laboratory experiment, surviving animals repaired the damage within six weeks.

Whelks in areas with high predation pressure have stronger, thicker shells (Thomas & Himmelman, 1988) and it might therefore be expected that whelks with thicker shells would be more resilient to the effects of beam trawling. However, from our results in the laboratory there appears to be no substantial difference in mortality between animals with no/light shell damage and severely damaged individuals in the absence of predators. Therefore, the assumption that whelks with thicker shells suffer less shell damage than thin shelled specimens and therefore have better chances for survival when beam trawled, is not confirmed.

Hermit crabs (*Pagurus bernhardus*) which depend for their protection on shells from gastropod species, might also suffer indirectly from beam trawl fishery when shells are severely damaged. In such cases shells only become less useful as protection and hermit crabs have to find another suitable shell. A strong reduction, due to trawling, in the numbers of larger whelks may finally set a limit to the growth of hermit crabs because larger specimens can no longer find sufficient large shells.

Whelk fisheries can seriously threaten whelk populations. Whelks are nowadays mainly caught with baited traps, which can affect all sizes of whelks. In Canada and Ireland concern about overfishing (also due to the catch of immature whelks) is reflected in discussions about a minimum landing size of whelks (Gendron, 1992; Fahy *et al.*, 1995). In areas where whelks are caught by beam trawling and landed as by-catch, egg-masses (produced in winter) are often torn loose from the substrate as indicated by the number of egg-masses in the catches (own observations). This may severely affect the reproductive output, since (our) laboratory observations showed that eggs, which were kept in motion by currents, did not develop into juveniles (unpublished results, see also *Chapter 2*).

In conclusion, we have shown that mortality in ***Buccinum undatum*** after capture by a 12m beam trawl can be as high as 60% over a six-week recovery period in the laboratory. The presence of predators and scavengers may further increase this figure. This suggests that the beam trawl fishery could be having a major adverse impact on whelk populations, rather than that the species would profit as a scavenger, as was suggested by Evans *et al.* (1996). The increased beam trawl intensity might well be a main cause of the observed decline in the last 20-25 years of whelks in several areas in the North Sea, as indicated by ten Hallers-Tjabbes *et al.* (1996), Nicholson & Evans (1997), Cadée *et al.* (1995), Rumohr *et al.* (1998) and Philippart (1998).

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CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

The research described in this thesis concerned the perhaps best known and studied common gastropod from the open North Sea of which only limited information was available. With the present research more insight has been obtained concerning this long-lived, off-shore snail species, which existence is threatened by human activities. To date, no other laboratory exposure studies with *Buccinum undatum* of different developmental stages than those reported in this thesis have been performed.

Summary of results

Laboratory maintenance and rearing (Chapter 2)

The attempts to optimize conditions to study adult whelks in the laboratory have resulted in a successful method for the reproduction of *B. undatum* in captivity and for the rearing of the off-spring. Juvenile whelks with a well-known background became subsequently available for the exposure studies to realize the objectives of the studies.

Laboratory exposure studies (Chapter 3)

Imposex was induced in juveniles at concentrations > 7 ngSn/l, which showed *B. undatum* to be less sensitive than *N. lapillus*. On the other hand this indicates that TBT contamination of the North Sea can be substantial.

This research has clearly shown age-related differences in sensitivity for the development of imposex. Developing juvenile whelks were affected by TBT, whilst fully matured adult females were not, when exposed for 11 months. Adolescent females showed a deviant response to TBT from juveniles which had just hatched, this points to a sensitivity where the stage of development influences the trigger for the formation of male sexual organs.

Exposure of egg-masses with developing whelks (*in ovo*) to TBT did not result in an increased masculinization.

Two year old juveniles had not yet developed gonadal tissue. The formation of a penis and a *vas deferens* in TBT-exposed juveniles can therefore not be related to or influenced by any steroid hormone produced in the gonads. This points to a different, possibly neuroendocrinological, mechanism.

TBT inhibited general cytochrome P450 activity in whelks. If certain cytochrome P450 iso-enzymes are involved in steroid hormone metabolism or synthesis in whelks, TBT might influence these processes.

Although TBT exposure resulted in imposex, the consequences for populations of *B. undatum* showed no sterilization effects on reproduction as opposed to the development in *N. lapillus*. However, indications were found that exposure to TBT can reduce growth which might affect reproduction indirectly when juveniles take longer to mature.

No other masculine sexual organs than a penis and a *vas deferens* were formed in females after two years of TBT-exposure. Histologically these structures consisted of the same cell types normal for males. Juvenile whelks first developed their secondary sexual organs, followed by the differentiation of primary sexual organs.

The research has contributed to the decision of the Marine Environmental Protection Committee (MEPC) of the International Maritime Organization (IMO) to ban the application of TBT by 1st January 2003 and to ban its presence by 1st January 2008 (Meeting MEPC 42, 1998).

Method for the identification of masculine sexual development in B. undatum (Chapters 2, 3, 4 & 5)

The laboratory and field studies demanded the development of a non-invasive method to investigate masculine sexual development or imposex. This method has been put forward in the OSPAR-SIME (Oslo and Paris commissions-Substances in the Marine Environment) workshop of the Joint Assessment Monitoring Programme (JAMP) to be used as guideline for TBT-specific biological effects monitoring in off-shore species (Davies *et al.*, 1997b).

Both laboratory and field studies resulted in a comprehensive overview of the possible stages of male sexual development/imposex in *B. undatum*.

Histological analysis of whelks from the laboratory and field studies showed the structure of a penis and a *vas deferens* in animals showing masculine development and imposex females to be similar to the cell types found in males, however, in adult imposex females the penis remained smaller than the penis of an adult male. It was found that no other sexual organ than already visible with the eye was present, which validated the method from the first paragraph.

Imposex and organotin levels in the Eastern Scheldt (Chapters 4 & 5)

The ban on the use of TBT based anti-fouling paints on ships < 25 m of 1990 has not resulted in a decrease in imposex incidence in females from the Eastern Scheldt in the period 1992-1995. More than 90% of the females showed imposex of which the majority showed the presence of the most advanced stages known for *B. undatum*.

Organotin whole body analysis of whelks and mussels from the Eastern Scheldt during 1995 showed TBT levels ≤ 17 ng Sn/g wet weight in animals.

High levels of specifically TBT were found in neural ganglia of whelks in September 1995 exceeding all other organotins and this may be relevant with respect to the mechanism of induction of imposex.

In total sediments TBT could hardly be detected (≤ 1.4 ng Sn/g dw). It is advisable to measure TBT in the small grain size fractions of sediments.

No statistical differences were observed between the organotin levels of different groups of whelks, i.e. between males and females, adults and juveniles, and between females with advanced stages of imposex and unaffected females/females with initial stages of imposex. However, a significant decrease in butyltin levels in whelks was observed in September 1995.

In general, TPT levels were higher than TBT levels in whelks from the Eastern Scheldt, but in mussels the opposite was found.

The levels of DBT and MBT were higher than TBT in whelks indicating biotransformation. In mussels TBT levels often exceeded those of its metabolites.

TPT levels in whelks were increased compared to mussels, indicating biomagnification.

Consequences of beam trawl fishery for B. undatum (Chapter 6)

Beam trawl fishery caused different types of shell damage. Significantly less shell damage was observed in whelks caught with baited traps compared to beam trawled specimens. The majority of all whelks showed repair marks from former damage.

A significantly higher percentage of whelks caught with baited traps survived a six weeks recovery period in the laboratory compared to whelks caught with a 12m-beam trawl (>95% vs 40%). Beam trawl fishery is considered to affect populations of *B. undatum* adversely in the North Sea.

Concluding remarks

For the first time laboratory exposure studies have been performed with the commercially fished, long lived, off-shore common whelk, *Buccinum undatum*. The successful efforts to maintain whelks, the ability to let them reproduce and to rear juveniles in the laboratory, allowed long-term experiments with whelks of different age with a well-known life history. This in contrast to the many field studies for imposex with mostly intertidal (= coastal) snail species. The laboratory exposure experiments proved unequivocally that TBT induces imposex in a dose-dependent manner in *B. undatum*. This was also shown for the species *Nucella lapillus*, *Ocenebra erinacea* and *Hinia reticulata* (Oehlmann, 1994; Gibbs, 1996; Stroben, 1994). Although this masculinization phenomenon, concerning the superimposition of a penis and sperm duct on female gastropods, is known for over a decade, information on subtidal species was limited and thus research projects concerning open sea species provide new insights on TBT pollution from large ocean going ships and associated effects.

Although the laboratory studies proved *B. undatum* not to be the most sensitive species to develop imposex after exposure to TBT and since imposex is wide-spread in *B. undatum* from the North Sea (ten Hallers-Tjabbes *et al.*, 1994; Ide *et al.*, 1997; Nicholson & Evans, 1997; *Chapter 6*), TBT contamination of the North Sea can therefore be substantial.

Juvenile and adolescent females showed masculine sexual development after exposure to TBT, but adults did not. This showed an age-dependence towards the sensitivity for the development of male sexual characteristics. Although in general juveniles are more sensitive than adults (e.g. Gibbs *et al.*, 1988; Stroben, 1994; Gibbs, 1996; Davies *et al.*, 1997), adult females totally lacking this morphogenetic change have only once been reported for *Littorina littorea* (intersex) (Bauer *et al.*, 1995). The generally accepted hypothesis that the induction of imposex is due to increased testosterone levels by aromatase inhibition (Bettin *et al.*, 1996; Matthiessen & Gibbs,

1998) is questioned, since steroid sex hormones are thought to be produced by the gonads, which were still lacking in the juveniles investigated.

Aromatase is a specific form of cytochrome P450 which catalyzes the reaction of male (testosterone) to female (oestradiol) steroid hormones. Since this biochemical reaction is common in all vertebrates, it does not explain the initiation of imposex solely in marine gastropods. The hypothesis that increased androgen presence causes imposex rather than an inhibition of aromatase needs more investigation. 17-β Oestradiol levels or testosterone/17-β oestradiol ratios from TBT-exposure studies with *H. reticulata* did not significantly differ between control and exposed animals (Bettin *et al.*, 1996). TBT inhibits the activity of many iso-forms of cytochrome P450 in several aquatic species *in vitro*, including *B. undatum*. Therefore, this inhibition could disturb a multitude of physiological processes related to the function of cytochrome P450 iso-enzymes.

At present attempts are made to identify steroid sex hormones in *B. undatum*. Regrettably, little is known about the production and function of sex steroids in (marine) gastropods. Further research on the (neuro-)endocrinology of this taxonomic group will lead to a better understanding of the mechanism of the masculinization process. This is even more important, taking into account that in general, in gonochoristic gastropods (including *Buccinidae*) there is no genetic basis for sex determination (Vitturi, pers. comm., 1995).

B. undatum with imposex showed no sterilization effects, unlike the direct adverse reproductive effects observed in e.g. *N. lapillus* and *O. erinacea* (Oehlmann 1994; Gibbs, 1996). Adult female *B. undatum* with the heaviest imposex stage still produced eggs. Therefore, population dynamics are probably not directly affected due to the observed male sexual organs in the females. The consequences for reproduction and hence population development can be quite different for the various species, although similar male sexual characteristics are formed. However, the observed growth retardation in *B. undatum*, but also in mussel larvae and other bivalves (Lapota *et al.*, 1993) due to exposure to TBT indirectly affects reproduction of these species when juveniles take longer to mature.

TBT pollution can be bio-monitored by using the imposex response of gastropod species (Davies *et al.*, 1997b). To study TBT pollution at open sea using subtidal species, often bottom trawling or diving is used for the collection of the individuals. This is much more intensive than the collection of intertidal specimens by hand at low tides. However, sexual development in the common whelk can be investigated in a non-invasive manner, since the whelk extends out of the shell when exposed to air, thereby revealing the presence or absence of male sexual characteristics. After examination, the animal can be returned to its original habitat.

Although in the Netherlands legislative measures were taken to reduce the contamination with TBT of mainly coastal waters in 1990 (implementation of Directive 89/677/EC), the expected improvement of the water quality could not be observed in the Eastern Scheldt area in the period 1992-1995, since the incidence of imposex in *B. undatum* remained > 90% and in 1995 the majority of the females showed the most advanced stages of imposex. Bio-effective TBT levels in the water as calculated from body burdens of whelks and mussels in 1995 matched the measured (water) monitoring data in the area (A. de Jong, pers. comm., 1996).

Beam trawl fishery is also known to affect benthic ecosystems including many non-target organisms (Lindeboom & de Groot, 1998). Previously, whelks were considered to be highly resistant to trawling effects and were even thought to profit from the discards (Fonds, 1994; Evans *et al.*, 1996). The research described in *Chapter 6* of this thesis showed however, that damage and mortality by the trawling gear can be considerable, despite the thick shell of *B. undatum*. When the effects on whelk population development are compared, the effect of beam-trawl fishery is considered dominant over the effect of TBT-exposure, although both constitute a threat to the common whelk.

The common whelk and its shell have a special role in the ecosystem. The whelk is a predator, but is also preyed upon by larger fishes, crabs and lobsters. The shell of living animals is a hard substrate for hydroids, sponges and sea-anemones, whereas the empty shell provides housing and protection for adult hermit crabs (*Pagurus bernhardus*). The indicated population effects described for *B. undatum* in this thesis will evidently and consequently affect other species as well.

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SAMENVATTING EN SLOTOPMERKINGEN

Het onderzoek beschreven in dit proefschrift behelst de waarschijnlijk bekendste en meest onderzochte slak uit de open Noordzee, waarvan echter een beperkte hoeveelheid informatie bekend was. Met het huidige onderzoek is meer inzicht verkregen in deze lang-levende, benthische open-zee soort, waarvan het bestaan wordt bedreigd door menselijk handelen. Dit onderzoek beschrijft tot nu toe als enige laboratorium blootstellingsstudies met *Buccinum undatum* in verschillende ontwikkelingsstadia.

Samenvatting van resultaten

Laboratorium opkweek en onderhoud (Hoofdstuk 2)

De inspanningen voor het optimaliseren van de omstandigheden voor het blootstellen van volwassen wulken in het laboratorium, leidden tot een succesvolle methode voor het laten reproduceren van *B. undatum* in gevangenschap en het opkweken van het voortgebrachte nageslacht. Hierdoor waren juveniele wulken met een bekende achtergrond beschikbaar voor de blootstellingsstudies, teneinde de doelstellingen van de studies te realiseren.

Laboratorium blootstellingsstudies (Hoofdstuk 3)

Imposex werd geïnduceerd in juveniele wulken bij concentraties > 7 ngSn/l, waaruit bleek dat *B. undatum* minder gevoelig lijkt dan *N. lapillus*. Aan de andere kant kan hieruit worden opgemaakt, dat de TBT verontreiniging van de Noordzee aanzienlijk kan zijn.

Dit onderzoek heeft duidelijke verschillen aangetoond in de ontwikkelings/ leeftijds-gerelateerde verschillen in gevoeligheid voor de ontwikkeling van imposex. Zich ontwikkelende juveniele wulken veranderden door TBT blootstelling, terwijl volwassen vrouwelijke wulken na 11 maanden blootstelling geen waarneembare afwijkingen vertoonden. Bijna geslachtsrijpe wulken ontwikkelden imposex op een andere manier dan juveniele wulken welk net uit de eicapsule werden blootgesteld. Dit wijst op een gevoeligheid, waar het ontwikkelingsstadium van het dier bepalend is voor de wijze waarop en welke mannelijke geslachtsorganen gevormd worden.

Blootstelling van ei-massa's met daarin ontwikkelende wulken (*in ovo*) aan TBT leidde niet tot een verhoogd percentage wulken met mannelijke geslachtskenmerken.

Twee jaar oude wulken hadden (nog) geen gonaden ontwikkeld. De formatie van een penis en zaadleider in aan TBT blootgestelde juvenielen kan daarom niet worden gerelateerd aan, noch worden beïnvloed door steroid hormonen welke in de gonaden geproduceerd worden. Dit wijst op een ander, mogelijk neuro-endocrienologisch mechanisme.

TBT remt de cytochroom P-450 enzym activiteit in de wulk. Indien bepaalde cytochroom P-450 iso-enzymen betrokken zouden zijn in steroid hormoon metabolisme of synthese, zou TBT deze processen kunnen beïnvloeden.

Hoewel TBT blootstelling leidde tot imposex, werd sterilisatie niet waargenomen. Consequenties voor de reproductie van populaties *B. undatum* door (fysieke) sterilisatie van vrouwtjes, zoals waargenomen in o.a. *N. lapillus*, zijn niet waarschijnlijk. Echter, er zijn indicaties dat TBT de groei van juvenielen remt, waardoor indirect een effect op reproductie ontstaat, wanneer deze juvenielen pas op latere leeftijd geslachtsrijp worden.

Naast een penis- en/of zaadleiderontwikkeling in vrouwelijke dieren waren geen andere mannelijke geslachtkenmerken gevormd na twee jaar blootstelling aan TBT. Histologisch onderzoek toonde aan dat de weefsels en organen op dezelfde manier waren samengesteld en gevormd zoals bij (normale) mannelijke exemplaren. Juveniele wulken ontwikkelden eerst de secundaire geslachtskenmerken alvorens de differentiatie van primaire geslachtsorganen te beginnen.

Dit onderzoek heeft bijgedragen aan de besluitvorming van het Marine Environmental Protection Committee (MEPC) van de International Maritime Organization (IMO) omtrent het verbod op het toepassen van TBT in anti-fouling verven vanaf 1 januari 2003 en het verbod op de aanwezigheid ervan vanaf 1 januari 2008 (bijeenkomst MEPC 42, 1998).

Methode voor de identificatie van de mannelijke sexuele ontwikkeling in B. undatum (Hoofdstukken 2, 3, 4 & 5)

Voor het uitvoeren van zowel de laboratorium- als veldstudies diende een niet-invasieve methode voor het vaststellen van mannelijke sexuele ontwikkeling of imposex ontwikkeld te worden. Deze methode is voorgesteld in de OSPAR-SIME (Oslo and Paris commissions-Substances in the Marine Environment) workshop van de JAMP (Joint Assessment Monitoring Programme) als richtlijn voor het uitvoeren van TBT-specifieke biologische effect monitoring in open-zee soorten (Davies *et al.*, 1997b).

Zowel de veld- als de laboratoriumstudies hebben geresulteerd in een uitvoerig overzicht van de mogelijke stadia van mannelijke sexuele ontwikkeling en/of imposex in *B. undatum*.

Onderzoek naar de struktuur en weefsel samenstelling van de penis en de zaadleider in vrouwelijke dieren met imposex en bij zich mannelijk ontwikkelende dieren gaf eenzelfde beeld als die van de strukturen die bij volwassen mannetjes werd waargenomen. Echter bij volwassen vrouwtjes met imposex was de penis altijd kleiner dan die van een volwassen mannetje. Andere sexuele organen dan die reeds zichtbaar met het oog (binoculair) werden niet waargenomen, wat de methode uit de eerste paragraaf valideerde.

Imposex en organotin gehalten in de Oosterschelde (Hoofdstukken 4 en 5)

Onderzoek in de periode 1992-1995 toonde aan dat het verbod op het gebruik van anti-fouling verven op basis van TBT voor schepen kleiner dan 25 m lengte uit 1990 niet heeft geleid tot een afname in de imposex incidentie in de Oosterschelde. Meer dan 90% van de vrouwelijke slakken vertoonde imposex, waarvan de meerderheid de meest vergevorderde stadia tot dusverre bekend voor *B. undatum* vertoonde.

Analyses van gehele wulken en mosselen uit de Oosterschelde in 1995 toonden gehalten van TBT < 17 ngSn/g versgewicht.

Hoge gehalten aan specifiek TBT werden in september 1995 in de neurale ganglia van wulken gemeten. Deze overtroffen de gehalten van alle andere gemeten organotins en dit kan relevant zijn voor het mechanisme van de inductie van imposex.

In totale sedimenten kon TBT nauwelijks worden aangetoond (≤ 1.4 ng Sn/g dw). Het wordt aanbevolen alleen TBT te meten in de fracties van de sedimenten met een kleine korrelgrootte.

Er waren geen significante verschillen te onderscheiden tussen organotin gehaltes en verschillende groepen wulken, te weten tussen mannelijke en vrouwelijke wulken, tussen volwassen en juveniele wulken en tussen vrouwtjes met vergevorderde stadia van imposex en vrouwtjes zonder imposex kenmerken of met een lichte vorm van imposex. Echter in september 1995 werd een significante afname in organotin gehalten in wulken gemeten.

Over het algemeen waren de TPT gehalten hoger dan de TBT gehalten in wulken van de Oosterschelde, maar in mosselen werd het omgekeerde gevonden.

De gehalten aan DBT en MBT waren hoger dan TBT in wulken, wat wijst op biotransformatie. In mosselen overschreed het gehalte TBT vaak die van de metabolieten.

TPT gehalten in wulken waren verhoogd ten opzichte van die in mosselen, wat wijst op biomagnificatie.

Consequenties van boomkorvisserij voor Buccinum undatum (Hoofdstuk 6)

Boomkorvisserij resulteerde in verschillende vormen van schelpschade. In wulken gevist met aasvallen werd significant minder schelpschade geregistreerd dan wulken gevist met boomkorren. De meerderheid van alle geviste wulken vertoonde herstelkenmerken van eerdere schelpbeschadigingen.

Wulken gevist met aasvallen overleefden in een significant hoger percentage de zes weken herstel periode in het laboratorium vergeleken met wulken gevist met een 12m boomkor (>95% vs 40%). Boomkorvisserij wordt geacht populaties van wulken in de Noordzee negatief te beïnvloeden.

Slotopmerkingen

Voor de eerste maal zijn laboratorium studies uitgevoerd met de commercieel beviste, lang-levende, open-zee wulk, *Buccinum undatum*. De geslaagde pogingen wulken in het laboratorium te houden, de mogelijkheid ze te laten reproduceren en te laten opgroeien in het laboratorium maakten het mogelijk langdurige experimenten uit te voeren met wulken in verschillende ontwikkelingsstadia waarvan de levensgeschiedenis grotendeels bekend was. Dit in tegenstelling tot de vele veldstudies naar imposex met voornamelijk slakken uit het getijde gebied (kust). De laboratorium blootstellingsstudies toonden onomstotelijk aan, dat TBT imposex induceert in *B. undatum* op een dosis afhankelijke wijze. Dit was ook bekend voor *Nucella lapillus, Ocenebra erinacea* en *Hinia reticulata* (Oehlmann, 1994; Gibbs, 1996; Stroben, 1994). Hoewel deze vermannelijking, waarbij een penis en zaadleider gevormd worden bij van oorsprong vrouwelijke dieren, reeds 10 jaar bekend is, was er slechts een zeer beperkte hoeveelheid informatie beschikbaar over de ondergedoken (sub-getijde) soorten. Onderzoeken naar deze open-zee soorten geven daarom nieuwe inzichten betreffende de verontreiniging door TBT afkomstig van de oceaan bevarende grotere schepen en de daaraan geassocieerde effecten.

Hoewel de wulk niet de meest gevoelige soort is gebleken voor wat betreft het ontstaan van imposex na TBT blootstelling en gezien het feit dat imposex in *B. undatum* alom wordt gevonden in de Noordzee (ten Hallers-Tjabbes *et al.*, 1994; Ide *et al.*, 1997; Nicholson & Evans, 1997; *Hoofdstuk 6*), kan worden verondersteld dat de TBT verontreiniging van de Noordzee aanzienlijk is.

Juveniele en bijna-geslachtsrijpe wulken vertoonden mannelijke sexuele ontwikkeling na blootstelling aan TBT, volwassen vrouwtjes ontwikkelden deze kenmerken echter niet. Dit toont aan, dat een leeftijd afhankelijkheid bestaat in de gevoeligheid voor de vorming van mannelijke sexuele kenmerken. Hoewel in het algemeen juvenielen gevoeliger bleken dan volwassenen (bijv. Gibbs *et al.*, 1988; Stroben, 1994; Gibbs, 1996; Davies *et al.*, 1997) is slechts voor *Littorina littorea* bekend dat bij volwasen vrouwtjes de morfogenetische verandering o.i.v. TBT (intersex) niet voorkomt (Bauer *et al.*, 1995). De algemeen aanvaarde hypothese, dat de inductie van imposex een gevolg is van verhoogde testosteron spiegels, veroorzaakt door inhibitie van aromatase (Bettin *et al.*, 1996; Matthiessen & Gibbs, 1998) wordt in twijfel getrokken vanwege het feit, dat steroide geslachtshormonen geproduceerd zouden worden in de gonaden, welk in de onderzochte juveniele wulken nog niet waren gevormd.

Aromatase is een specifiek iso-enzym van het cytochroom P450 enzymsysteem, welk de reactie van mannelijke (waaronder testosteron) naar vrouwelijke (oestradiol) steroide hormonen catalyseert. Deze biochemische reactie komt algemeen voor in alle vertebraten, maar een verklaring waarom slechts in mariene gastropoden imposex geïnduceerd zou kunnen worden door een invloed op dit enzym, is vooralsnog niet voorhanden. De hypothese dat een verhoogd androgeen gehalte imposex veroorzaakt verdient meer aandacht. 17-β oestradiol gehalten of de verhouding testosteron/17-β oestradiol uit TBT blootstellingsstudies met *H. reticulata* verschilden tussen blootgestelde en controle slakken niet significant (Bettin *et al.*, 1996). Van TBT is bekend, dat het de activiteit van vele iso-enzymen behorende tot het cytochroom P450 enzymsysteem remt *in vitro* in verschillende aquatische organismen, waaronder *B. undatum*. Een dergelijke remming kan daarom meerdere fysiologische processen verstoren welk zijn gerelateerd aan de functie van de cytochroom P450 iso-enzymen.

Op dit moment wordt onderzocht of in *B. undatum* geslachtshormonen aan te tonen zijn en zo ja, welke dit dan zijn. Helaas is weinig bekend over productie en functie van geslachtshormonen in (mariene) gastropoden. Nader onderzoek betreffende de (neuro-)endocrinologie van deze taxonomische groep zal leiden tot een beter begrip van het mechanisme verantwoordelijk voor de mannelijke sexuele ontwikkeling. Dit is ook daarom belangrijk, omdat in het algemeen in gonochoristische gastropoden (waaronder *Buccinidae*) een genetische basis voor de geslachtsbepaling ontbreekt (Vitturi, pers. mededeling, 1995).

B. undatum met imposex vertoonden geen sterilisatie kenmerken, dit in tegenstelling tot de nadelige gevolgen zoals waargenomen bij o.a. *N. lapillus* en *O.*

erinacea (Oehlmann, 1994; Gibbs, 1996). Volwassen vrouwelijke *B. undatum* met de zwaarste imposex stadia produceerden nog steeds eipakketten. Een directe invloed van de waargenomen mannelijke geslachtsorganen op de populatie-ontwikkeling lijkt daardoor niet erg waarschijnlijk. De consequenties voor de voortplanting en daarmee de populatie-ontwikkeling kunnen per soort verschillen, hoewel dezelfde mannelijke sexuele kenmerken worden gevormd. Echter, de waargenomen groei-remming bij *B. undatum* alsmede bij mossellarven en andere tweekleppigen (Lapota *et al.*, 1993) als gevolg van blootstelling aan TBT kan indirect wel de voortplanting bedreigen, indien het langer duurt voordat juvenielen van deze soorten geslachtsrijp worden.

TBT verontreiniging kan met behulp van de imposex reactie van mariene gastropoden ('biomonitoring') in de gaten worden gehouden (Davies *et al.*, 1997b). Voor het verkrijgen van informatie over de TBT verontreiniging van de open zee wordt veelal gebruikt gemaakt van sleepnetten over de bodem of er moet worden gedoken voor het verzamelen van individuen van de zeebodem. Dit is vele malen intensiever dan het verzamelen met de hand van drooggevallen individuen bij eb. Echter, de bestudering van de eventuele mannelijke sexuele ontwikkeling in de wulk kan geschieden op een niet-invasieve wijze, omdat de wulk zich voor een groot deel buiten de schelp uitstrekt, indien ondersteboven geplaatst en blootgesteld aan lucht. Hierbij kunnen de aan- of afwezigheid van mannelijke sexuele organen op eenvoudige wijze worden geregistreerd. Na bestudering kunnen de wulken dan worden teruggeplaatst in hun originele habitat.

Hoewel in Nederland reeds in 1990 wettelijke maatregelen werden getroffen voornamelijk ter reductie van de verontreiniging met TBT van de kustwateren (implementatie van Richtlijn 89/677 van de Europese Gemeenschap), is de verwachte verbetering van de oppervlaktewater kwaliteit uitgebleven in de Oosterschelde in de periode tussen 1992-1995. De incidentie van imposex in *B. undatum* bleef > 90% en in 1995 bezat de meerderheid van de vrouwelijke exemplaren de meest vergevorderde stadia van imposex. Bio-effectieve TBT gehalten in het water konden worden berekend uit de lichaamsgehalten van wulken en mosselen in 1995 en deze kwamen overeen met monitoring gegevens van TBT van het water uit nabijgelegen gedeelten van de Oosterschelde (A. de Jong, pers. mededeling, 1996).

Van boomkorvisserij is tevens bekend dat het bentische ecosystemen aantast, waaronder vele niet-doelorganismen (Lindeboom & de Groot, 1998). Voorheen werden wulken geacht zeer goed bestand te zijn tegen effecten van het vissen met een boomkor en tevens te profiteren van de voor visserij nutteloze, teruggegooide restanten (Fonds, 1994; Evans *et al.*, 1996). Het onderzoek zoals beschreven in *Hoofdstuk 6* van dit proefschrift leverde echter een ander beeld op. Schade en mortaliteit als gevolg van de boomkor vistuigen kunnen aanzienlijk zijn, ondanks de robuuste schelp van *B. undatum*. Indien de effecten op de ontwikkeling van wulken populaties worden vergeleken, kan het effect van de boomkorvisserij dominant worden beschouwd over het effect van TBT, hoewel beide een bedreiging voor de wulk vormen.

De wulk en haar schelp nemen in het ecosysteem een speciale plaats in. De wulk is een predator, maar wordt zelf bejaagd door grotere vissoorten, krabben en kreeften. De schelp van levende individuen vormt een hard substraat voor o.a. poliepen, sponzen en zee-anemonen, terwijl de lege schelp een huis en bescherming biedt voor volwassen heremietkreeften (*Pagurus bernhardus*). De in dit proefschrift beschreven populatie-effecten voor *B. undatum* zullen daarom ook effect kunnen hebben voor andere soorten.

ABBREVIATIONS

ANOVA	Analysis of Variance
CNS	Central Nervous System
CYP420	'Inactive' cytochrome P450 enzymes (absorbance at 420 nm)
CYP450	Cytochrome P450 enzymes (absorbance at 450 nm)
DBT	Dibutyltin
DPT	Diphenyltin
GC	Gas-Chromatography
IMO	International Maritime Organisation
ITD	Ion Trap Detection
K_{ow}	Octanol-water partition coefficient
LOD	Limit of Determination
MBT	Monobutyltin
MEPC	Marine Environmental Protection Committee
MPT	Monophenyltin
MS	Mass-Spectrometry
OT	Organotin
RPSI	Relative Penis Size Index
SD	Standard Deviation
SIM	Single Ion Monitoring
TBT	Tributyltin
TBTAc	Tributyltinacetate
TPT	Triphenyltin
VDSI	Vas Deferens Sequence Index

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CURRICULUM VITAE

Bernhard Peter Mensink werd geboren op 15 januari 1968 te Den Haag. In 1986 behaalde hij het VWO diploma aan het Aloysius College ook te Den Haag en begon met de studie Milieuhygiëne aan de Landbouwuniversiteit Wageningen. Tijdens de doctoraalfase verrichtte hij onderzoek onder begeleiding van de vakgroep Natuurbeheer/Hydrobiologie (Prof. dr W.J. Wolff) op het Instituut voor Bos- en Natuuronderzoek (IBN-DLO) op Texel (dr N. Dankers; dr ir A.G. Brinkman). Erop volgde onderzoek zowel bij de vakgroep Toxicologie (Prof. dr J.H. Koeman; dr A.J. Murk) alsmede bij de N.V. KEMA (dr H.A. Jenner) te Arnhem. Aansluitend werd een stageperiode doorgebracht bij ICI Agrochemicals, Jealott's Hill Research Station, in het Verenigd Koninkrijk (dr D. Riley; M. Hamer), waarna hij afstudeerde in augustus 1992. Via een korte periode bij de N.V. KEMA startte hij met het promotie-onderzoek eind 1992 bij de afdeling Beleidsgericht Wetenschappelijk Onderzoek NIOZ (BeWON), later binnen de afdeling Mariene Biogeochemie en Toxicologie (MBT) van het Nederlands Instituut voor Onderzoek der Zee op Texel. Hier werd het in dit proefschrift beschreven onderzoek uitgevoerd onder begeleiding van dr J.P. Boon, dr C.C. ten Hallers-Tjabbes en Prof. dr J.H. Koeman (Toxicologie, LUW). Naast het verrichten van onderzoek heeft hij enkele modulen van de postdoctorale opleiding Toxicologie gevolgd en werd onderwijs gegeven aan doctoraalstudenten van de Landbouwuniversiteit, aan cursisten van de postdoctorale opleiding Toxicologie en aan cursisten binnen de onderzoeksschool Milieuchemie & Toxicologie. Op uitnodiging van Rijkswaterstaat werd in november 1997 als expert op het gebied van imposex praktijkles gegeven aan een internationaal gezelschap in een workshop georganiseerd door de 'OSPAR working group on concentrations, trends and effects of substances in the marine environment (SIME)'. Sinds 1 juni 1998 is hij werkzaam als 'Registration Officer' bij de technische afdeling van Zeneca Agro te Ridderkerk.

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"MEN HEEFT DIT LIGHAAM, ZOO ALS HET LEDIG AAN DE STRANDEN SPOELT, *VESICARIA MARINA* OF *ALCYONIUM* GENOEMD, EN HET MEESTAL AANGEZIEN VOOR EEN SPONSAGTIG ZEE-GEWAS. HET IS EIGENLYK DE BAARMOEDER OF HET EIJERNEST VAN DEN GEMEENEN KINKHOORN, WELKEN, ZOO IK MY NIET BEDRIEGE, DE ZEEUWEN *WULK* NOEMEN. ....
...... BIJ GEZONDHEID HOOP IK NADER ONDERZOEK OP DE BYZONDERHEDEN VAN DIT ZEE-PRODUCT TE DOEN, EN EINDIGE VOOR TEGENWOORDIG MET AANTEMERKEN, DAT HET ONVERGEEFLYK IS, DAT ONZE INLANDSE LIEFHEBBERS MET AL WAT VREEMD IS EN UIT DE ANDERE WERELDDEELEN KOMT ZOO ONBEGRYPELYK VEEL OP HEBBEN, EN GROOTE KOSTEN MAAKEN TOT HET VERZAMELEN VAN NATUURLYKE ZELDZAAMHEDEN EN LIGCHAMEN, WELKEN ZY DOORGAANDS MAAR ALLEEN IN 'T UITERLYKE KENNEN; TERWYL HUN EIGEN LAND EN DE WATEREN HUNNER ZEE EN RIVIEREN EENE MENIGTE VAN DINGEN OPLEVEREN, DIE IN FRAAIHEID VOOR GEENE VREEMDE ZAAKEN WYKEN, EN INZONDERHEID DEEZEN VOORRANG HEBBEN, DAT MEN ZE VAN NABY KAN ONDERZOEKEN. ALS IETS RAARS BESCHOUWT MEN DAT EIJERNEST VAN EENE SLEK, HET WELK DE HEER M. LISTER OP DE 6DE PLAAT VAN ZYNE *EXERCITATIO ANATOMICA* VERTOONT, EN MEN HEEFT ONDERTUSSEN IN HET VOORWERP, VAN 'T WELK HIER GEHANDELD IS, EEN VERSCHYNSEL, WAAR OVER MEN ZIG RUIM ZOO VEEL VERWONDEREN MOET".

UIT : BESCHRYVING VAN HET EIJERNEST DER ZEESLEK WELKE WULK GENOEMD WORD.
UITGEZOGTE VERHANDELINGEN UIT DE NIEUWSTE WERKEN VAN DE SOCIETEITEN DER WETENSCHAPPEN IN EUROPA EN VAN ANDERE GELEERDE MANNEN, MET NAAUWKEURIGE AFBEELDINGEN EERSTE DEEL, TE AMSTERDAM, BY F. HOUTTUYN 1757.