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THE DEVELOPMENT OF IMPOSEX IN RELATION TO ORGANOTIN CONTAMINATION IN THE COMMON WHELK, *BUCCINUM UNDATUM*

Field observations in the Eastern Scheldt and experimental exposure in a laboratory study

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This report describes the results of a field study into organotin contamination in the Eastern Scheldt, commissioned by the Directorate-General for Environmental Protection (DGM) of the Ministry of Housing, Physical Planning and the Environment (case number 9512.0037), and the results of the third phase (up till May 1996) of a long term laboratory study to determine the causal relationship between tributyltin exposure and imposex development in the common whelk, *Buccinum undatum* (RIKZ reference number DG-502).

UITGEBREIDE SAMENVATTING

Dit rapport beschrijft de resultaten van twee studies naar de ontwikkeling van mannelijke geslachtskenmerken (imposex) in de wulk, *Buccinum undatum*, onder invloed van chronische blootstelling aan organotinverbindingen uit anti-fouling verven (butyltinverbindingen). Een veldstudie in de Oosterschelde werd uitgevoerd in opdracht van het Ministerie van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer, Directoraat-Generaal Milieubeheer, (zaaknummer 9512.0037) teneinde organotin concentraties vast te stellen in zowel wulken als hun voedsel (mosselen) en hun directe omgeving (sediment). In een laboratorium experiment (Rijksinstituut voor Kust en Zee projekt DG-502), werd bij langdurige blootstelling het oorzakelijke verband tussen tributyltinblootstelling en de ontwikkeling van mannelijke geslachtskenmerken onderzocht, waarvan hier de derde fase wordt gerapporteerd.

Veldstudie

Dit deel beschrijft de eerste studie naar imposex in de wulk, *Buccinum undatum*, en de mate van organotin verontreiniging in de Oosterschelde gedurende verschillende seizoenen in 1995. Zowel wulken, mosselen *(Mytilus edulis)* en sedimenten werden hiertoe geanalyseerd voor mono-, di- en tributyltin en fenyltin verbindingen. In tegenstelling tot de butyltin verbindingen worden fenyltin verbindingen vooral gebruikt als nematocide in de landbouw.
Trifenyltin (TPT) werd overal aangetroffen. In de wulk wel in 4 tot 100 keer hogere concentraties dan TBT. Voor zowel de butyltin als fenyltin verbindingen werden geen verschillen in concentraties aangetroffen tussen de geslachten, adulten en juvenielen, en tussen vrouwtjes met en zonder imposex. In sedimenten konden vrijwel geen organotin verbindingen worden aangetoond, waarschijnlijk als gevolg van de zandige structuur van het sediment. In de toekomst verdient het aanbeveling uitsluitend te meten in een van te voren gescheiden fijne fraktie van het sediment (bijvoorbeeld < 63 μm).
Dealkylering van tributyltin tot dibutyltin (DBT) en monobutyltin (MBT) lijkt sneller te verlopen in wulken dan in mosselen. Fenyltin concentraties zijn 4-10 maal hoger in wulken dan in mosselen. Biomagnificatie van butyltin verbindingen lijkt voor de wulk onwaarschijnlijk, voor fenyltin verbindingen kan dit niet worden uitgesloten.
Voor de organotinanalyses werden van wulken meerdere organen apart geanalyseerd. Organotinverbindingen (gebaseerd op versgewicht) werden in het digestieve kanaal in hogere concentraties aangetroffen dan in de voet. In september werd uitsluitend tributyltin (TBT) in sterk verhoogde concentraties aangetroffen in de zenuwknopen, wat van groot belang kan zijn in het kader van het mechanisme van imposex. TBT zou kunnen interfereren met neuropeptiden, wat tot de inductie van penisgroei en/of zaadleider zou kunnen leiden .
Bijna alle vrouwelijke wulken in de Oosterschelde (95%) vertoonden imposex-verschijnselen, meer dan de helft hiervan de meer gevorderde stadia. In februari werden eimassa's met zich ontwikkelende jonge wulkjes aangetroffen, hetgeen betekent dat voortplanting nog steeds plaatsheeft.

Laboratoriumstudie

Het laboratorium experiment werd uitgevoerd, teneinde het causale verband aan te tonen tussen TBT blootstelling en de ontwikkeling van mannelijke geslachtskenmerken. Zowel volwassen als zich ontwikkelende juveniele wulken werden bestudeerd om vast te stellen welk ontwikkelingsstadium van de wulk het gevoeligst is voor de beïnvloeding van de ontwikkeling van mannelijke geslachtskenmerken.
Volwassen vrouwtjes ontwikkelden ondanks 11 maanden blootstelling aan concentraties tot 1 μg tributyltinacetaat per liter (TBTAc/l) geen mannelijke geslachtskenmerken. Hun juvenielen vertoonden echter een sterk verhoogde mannelijke ontwikkeling. Na 8 maanden blootstelling aan nominale concentraties van 100 ng TBTAc/l (gelijkwaardig met 35 ng Sn/l), vertoonde 54% van de juvenielen mannelijke geslachtskenmerken, dit nam zelfs toe tot 100% na 14 maanden. In de hoogste blootstellingsgroep van 1 μg TBTAc/l vertoonde na 8 maanden 100% van de juvenielen al mannelijke kenmerken, bijna allemaal met een volledig ontwikkelde penis en zaadleider, sommigen hadden zelfs een dubbele penis. In de controlegroep en de laagst gedoseerde (10 ng TBTAc/l) groep convergeerden de percentages met mannelijke geslachtskenmerken naar 50% na twee jaar blootstelling. Dit wijst erop, dat deze geslachtsorganen dan in principe aanwezig zijn en na die tijd alleen nog grotere afmetingen bereiken.
Hoewel normaliter verwacht mag worden dat 50% van de juvenielen zich zal ontwikkelen tot man, leidt blootstelling van deze juveniele wulken aan con-

centraties van 100 ng TBTAc/l (vergelijkbaar met 35 ng Sn/l) tot een duidelijke inductie en versnelling van de ontwikkeling van mannelijke geslachtskenmerken op een dosis afhankelijke wijze, wanneer deze juvenielen worden blootgesteld in de eicapsule of net na (ei)uitkomst. Omdat de nominale concentraties niet werden bereikt in de experimentele aquaria in het eerste jaar doseren en de effekten al na 8 maanden (waarin de gemeten TBT concentraties lager dan 20 ng TBTAc/l waren) zichtbaar werden, mag een invloed op de sexuele ontwikkeling al bij concentraties van 17 ng TBT/l (7 ng Sn/l) worden verwacht.
De lichaamsgroei werd zelfs al geremd bij nog lagere concentraties dan waarbij de sexuele ontwikkeling werd beïnvloed. Een significant lagere lengte kon worden waargenomen bij concentraties $\geq$ 10 ng TBTAc/l ($\geq$ 4 ng Sn/l).
Een vaststelling van een NOEC en LOEC voor een effekt op de ontwikkeling van mannelijke geslachtsorganen in wulken resulteert in een NOEC van 8.3 ng TBT/l en een LOEC van 17 ng TBT/l. Echter bij 8.3 ng TBT/l is er in deze studie al wel een negatief effekt zichtbaar op de groei (en dus ontwikkeling) van de juveniele wulken.
Een dosis-afhankelijke toename in lichaamsgehalten van alle butyltin verbindingen kon worden waargenomen bij de volwassen vrouwelijke wulken gedurende de blootstellingsperiode.

Vergelijking van studies
Een combinatie van de lichaamsgehalten van de volwassen wulken uit het laboratorium experiment met lichaamsgehalten in wulken van de Oosterschelde, leidde tot een voorspelde TBT concentratie in de Oosterschelde van tussen 10 en 80 ng TBT/l. Zulke gehalten zijn ook daadwerkelijk gerapporteerd voor de jachthaven Colijnsplaat in 1990-1992 (gemiddeld 32-61 ng TBT/l). Deze concentraties zijn zeker in staat om de ontwikkeling van mannelijke geslachtsorganen te induceren in juveniele wulken in dit gebied.

Belang voor het beleid
Tributyltin en trifenyltin hebben veelal vergelijkbare toxische werkingen en toxische concentratieniveaus (Lowest Observed Effect Concentrations, LOEC) in een veelheid aan soorten (Fent, 1996). Veel van deze toxische effekten treden op bij concentraties > 100 ng/l. Echter, voor de meest gevoelige toxische effekten, namelijk de inductie van imposex in gastropoden en de groeireductie en schelpverdikkingen in oesters en oesterlarven, is alleen voor TBT een oorzaak-effekt relatie vastgesteld.
Voor TBT lijkt de voorgestelde MTR (maximaal toelaatbare risiconiveau) door Crommentuijn *et al.* (concept 1996) een realistische maximale waarde. Onze studies toonden aan, dat 17 ng TBT/l de ontwikkeling van mannelijke geslachtorganen induceerde, wat dus erg dicht ligt bij deze MTR waarde (11 ng TBT/l). Toepassing van deze waarde zou betekenen, dat er absoluut geen veiligheidsmarge wordt gehanteerd met betrekking tot het genoemde effekt. Literatuurgegevens suggereren echter, dat in de purperslak met deze MTR zeker nog imposex zal worden opgewekt, omdat dit in laboratoriumstudies al bij concentraties van 2-3 ng TBT/l plaatsvond. Deze resultaten lijken niet meegenomen in de berekening van de voorgestelde MTR door Crommentuijn *et al.*. Aanbevolen wordt deze resultaten wel mee te nemen bij de definitieve afronding van het pesticiden rapport.
Horiguchi *et al.*(1994) hebben in een recente studie vastgesteld dat in *Thais clavigera* en *T. bronni* imposex kan worden geïnduceerd na blootstelling aan TPT. Dit is echter in tegenspraak met wat Bryan *et al.* (1988) hebben geconcludeerd. Zij vonden nl. geen toename van imposex in *Nucella lapillus* na blootstelling aan TPT. Dit geeft aan, dat in de literatuur geen duidelijkheid bestaat omtrent de potentie van TPT imposex te veroorzaken.
De voorgestelde MTR voor TPT van 5 ng TPT/l kan echter (nog) niet tot een veilige waarde worden gerekend met betrekking tot bovengenoemde effekten, vanwege het ontbreken van dosis-effekt studies en lijkt daarom enigszins speculatief. Mocht TPT deze effekten niet induceren, dan is deze waarde wellicht te streng.
Stäb *et al.* (1996) toonden echter aan, dat TPT in alle zoetwater systemen in Nederland voorkomt, vaak zelfs in hogere concentraties dan TBT, behalve in wateren met een hoge (recreatie) scheepvaart. Dit wijst erop, dat TPT misschien beschouwd moet worden als een nog groter probleem dan TBT.

Toekomstig onderzoek
Binnen projekt DG502 wordt verder onderzoek verricht naar het fysiologisch mechanisme van imposex. Histologisch onderzoek zal hierbij een grote rol spelen. Eveneens zal worden onderzocht of het gebruikte Waddenzee water verstorend kan heb-

ben gewerkt op de bestudeerde effekten door in een tweede blootstellingsexperiment kunstmatig zeewater te gebruiken.

Aanvullend op het genoemde huidige contract zijn uit de behaalde resultaten de volgende suggesties naar voren gekomen :

*Om aan te kunnen geven of de voorgestelde MTR voor TPT van 5 ng/l een realistische waarde is of een veel te strenge norm, is het van essentieel belang de potentie van TPT bij het veroorzaken van imposex in gastropoden als mogelijk gevoeligste effekt te bestuderen.

*De specifiek hoge TBT concentraties, die in de neurale ganglia (vergelijkbaar met hersenen) alleen in september werden waargenomen, moeten worden bevestigd. De rol bij imposexontwikkeling is nog onduidelijk, wellicht interfereert TBT met de regulatie van neurohormonen juist aan het begin van de reproductieve periode. Een meer gedetailleerde studie naar maandelijkse (/seizoens) variaties in TBT concentraties in dit orgaan is daarom gewenst. Indien uitsluitend TBT selektief accumuleert, verklaart dit wellicht waarom andere organotinverbindingen geen rol spelen bij de imposex ontwikkeling.

*Organotinverbindingen in sedimenten waren als gevolg van het zandige karakter niet of nauwelijks detecteerbaar. In de toekomst is het analyseren van alleen de fijne fractie aan te bevelen.

EXECUTIVE SUMMARY

This report describes the results of two studies that concerned the development of male sexual characteristics (imposex) in the common whelk *(Buccinum undatum)* under the influence of the chronic exposure to organotin compounds originating from antifouling paints (butyltin compounds). A field study, commissioned by the Directorate-General for Environmental Protection (DGM) of the Ministry of Housing, Physical Planning and the Environment (case number 9512.0037), was carried out in the Eastern Scheldt in order to determine the concentrations of butyltin and phenyltin compounds in whelks, their food (mussels) and their habitat (sediment). A laboratory study (National Institute for Coastal and Marine Water Management (RIKZ) project DG-502) was performed to investigate the long-term causal dose-effect relationship between tributyltin (TBT) and the development of male sexual characteristics.

Field study
A one year study was performed to investigate the imposex incidence in the common whelk, *Buccinum undatum*, in relation to organotin contamination in the Eastern Scheldt. Whelks, mussels *(Mytilus edulis)*, and sediments were analysed for mono-, di- and tri- butyltin and phenyltin compounds. Phenyltin compounds are, in contrast to butyltin compounds, primarily used in agriculture.
Triphenyltin (TPT) was detected in whelks in concentrations up to two orders of magnitude higher than tributyltin (TBT). No differences in organotin concentrations could be observed between adult and juvenile whelks, nor between the sexes, nor between females with and without imposex. In total sediments, all organotins were mostly below their detection limit. In future, it seems preferable to analyse only the fine fraction (< 63 μm) of the sediments .
Dealkylation of tributyltin to dibutyltin (DBT) and monobutyltin (MBT) seems much more effective in whelks than in mussels. In contrast, concentrations of triphenyltin and its metabolites diphenyltin (DPT) and monophenyltin (MPT) were 4-10 fold higher in whelks than in mussels. Biomagnification of butyltin compounds is therefore unlikely to occur in whelks, but for phenyltin compounds it cannot be excluded.
For organotin analysis, animals were separated in two parts in February; in June and September three organs were analysed. The organotin concentrations (based on wet weight) were always higher in the digestive gland than in the foot (muscle tissue). Surprisingly high concentrations of only tributyltin (TBT) were found in the neural ganglia (the "brain" of the whelk) in September but not in June. This could be very important in view of mechanistical aspects concerning the induction of imposex. Possibly, TBT interferes with neuropeptides, which could lead to the induction of development of a penis and a *vas deferens*.
Nearly all females (95 %) in the Eastern Scheldt showed male sexual characteristics of which more than 50% had the most advanced stages of imposex. However, egg masses with developing young whelks were still found, indicating that reproduction is still occurring in the Eastern Scheldt.

Laboratory study
Laboratory experiments with common whelks were conducted to investigate the cause-effect relationship between TBT and the development of male sexual characteristics. Adult and developing juvenile whelks were exposed to determine the most sensitive life stages with respect to imposex development.
Adult females did not develop male sexual characteristics despite an 11 month exposure period to concentrations up to 1 μg tributyltin acetate per litre (TBTAc/l). In contrast, their offspring showed a strongly increased male development. After 8 months of exposure to a nominal dose of only 100 ng TBTAc/l (equivalent to 35 ng Sn/l), 54% of the juveniles showed male sexual characteristics, which increased over time to 100% after 14 months. In the highest dose of 1 μg TBTAc/l, 100% of the juveniles already showed male sexual characteristics after 8 months. Moreover, nearly all animals showed the most advanced stages of penis development. Some of the animals even developed a double penis. In the control group and lowest dose (10 ng TBTAc/l) group, percentages with male sexual characteristics converged to 50% after two years of exposure, indicating that sexual organs were basically present and will only show a further increase in size after this period.
Although it is the normal situation that 50% of the juveniles will eventually develop as males, exposure to nominal TBT concentrations of 100 ng TBTAc/l (equivalent to 35 ng Sn/l), clearly induced and accelerated the development of male sexual

organs in a dose-dependent manner, when exposed *in ovo* or just after hatching. Because nominal TBT concentrations were not reached in the experimental aquaria within the first year of exposure and effects were already observed after 8 months, when the measured TBT levels in solution had not exceeded 20 ng TBTAc/l, an influence on the development of male sexual characteristics can be expected already at concentrations of 17 ng TBT/l (equivalent to 7 ng Sn/l).

Growth rates were affected at an even lower dose as sexual development. A significantly reduced growth of the juveniles was observed, when these animals were exposed to nominal doses ≥ 10 ng TBTAc/l (equivalent to ≥ 4 ng Sn/l).

From these results concerning the effect of TBT on the development of male sexual characteristics, a NOEC and LOEC of 8.3 and 17 ng TBT/l respectively, can be determined. However, a concentration of 8.3 ng TBT/l has an effect on the growth (and thus development) on these juveniles.

Concentrations of butyltin compounds were only measured in adult animals. A dose-dependent increase in body burdens of all butyltin compounds was observed over time.

Combination of both studies

Combination of the results of the body burdens of TBT and its metabolites DBT and MBT of adult females from the field and the laboratory study, led to an expected TBT concentration range in the Eastern Scheldt of 10-80 ng TBT/l. For the marina at Colijnsplaat during 1990-1992, average concentrations of 32-61 ng TBT/l were indeed reported. The results of our laboratory study showed that these concentrations are well capable to influence the development of male sexual characteristics in juvenile whelks.

Policy implications

Tributyltin and triphenyltin show similar Lowest Observed Effect Concentration (LOEC) levels for a large number of toxic effects in a multitude of species (Fent, 1996). Most of the observed toxic effects occur at concentration levels ≥ 100 ng/l. However, a cause-effect relationship has been established only for TBT with respect to the most sensitive toxic effects, i.e. the induction of imposex in gastropods and shell thickening and growth of oyster larvae.

For TBT, the proposed MTR (Maximum Permissible Concentration) by Crommentuijn *et al.*, (1996) seems to be a realistic maximum level for the common whelk. In our studies, a concentration of around 17 ng TBT/l caused an induction of the development of male sexual characteristics, which is very close to the MTR (11 ng TBT/l). Thus, application of this value means there is no safety margin at all with respect to the induction of this masculinisation effect in common whelks. However, literature data show that this level will certainly cause imposex in the dogwhelk. For the dogwhelk levels of 2-3 ng TBT/l are already responsible for this masculinisation effect. These results are not taken into account in determining the proposed MTR and it is recommended to do so in the final report on pesticides.

A recent experimental study (Horiguchi *et al.*, 1994) also showed the induction of imposex in *Thais clavigera* and *T. bronni* by TPT. This is in contrast with a previous study (Bryan *et al.*, 1988), where TPT did not result in an increase of imposex in *N. lapillus*. So, the literature on TPT causing imposex is not conclusive.

Therefore the proposed MTR for TPT (5 ng TPT/l) seems speculative with respect to the most sensitive effects mentioned above and the value may be too strict if TPT does not induce these effects.

On the other hand, research of *Stäb et al.*(1996) showed that TPT is present in all freshwater systems in the Netherlands in concentrations usually exceeding those of TBT, except in areas of high boating activity. This indicates that TPT probably should even be considered a bigger problem than TBT.

Future research

In the next phase of project DG-502 the fysiological mechanism for the induction of imposex will be studied. Histological research shall be an important tool. Also, a second TBT exposure experiment will be conducted with artificial sea water in order to study a possible interfering effect of Wadden Sea water.

In addition to the project mentioned, the findings from this study result in the following suggestions :

- *In order to determine whether the proposed MTR level for TPT of 5 ng/l is realistic or too strict, it is essential to study the ability of this compound to interfere with the most sensitive effects with respect to TBT. Thus, it is nescessary to study whether TPT can also act as an inducer of imposex in gastropods.
- *The high concentration of only TBT in neural ganglia (representing the brain of the animal), as

was found in September, needs confirmation. Its role in imposex development, possibly as an agent, interfering with the regulation of neuro-hormones at the start of the reproductive period, is not well understood. A more detailed study into monthly differences in TBT concentrations in this organ is therefore desirable. If only TBT shows this selective accumulation, it might explain why other organotin compounds are not involved in the process of imposex development.

* To investigate organotin contamination in sediments, we would suggest only to study the small fraction. Due to the sandy nature of the sediment in the Eastern Scheldt, organotins were mostly below their detection limit.

CHAPTER 1

GENERAL INTRODUCTION

Marine pollution by organotin compounds has become of great concern due to the toxic effects of these biocides on non-target marine organisms. Tributyltin (TBT) and triphenyltin (TPT) are the most toxic organotin biocides. TBT is virtually exclusively used in anti-fouling paints, whereas TPT is used mainly as a nematocide in agriculture, but is also added in minor amounts to anti-fouling paints (up to 10%; Fent, 1996).
The leachates of these TBT based paints are now known to have deleterious effects on many non-target organisms. Neogastropod molluscs appear to be the most sensitive non-target organisms, concentration levels of 1-2 ng $Sn.l^{-1}$[*see note below] already cause morphological changes in reproductive organs of female dog-whelks, *Nucella lapillus*, which ultimately leads to sterility (Gibbs *et al.*, 1988) : Females start growing male sexual characteristics next to their own reproductive organs. This superimposition of male characters on female gastropods is termed imposex (Smith, 1971). In the most severe stages of imposex in the dog-whelk, the opening of the oviduct becomes blocked by an overgrowth of spermduct tissue. In this way egg capsules can no longer be released and the unfertilised egg masses accumulate in the capsule gland. Accumulation of such egg masses eventually can cause a rupture of the wall of this gland, leading to the death of the animal (Gibbs & Bryan, 1986).
For more than a decade it was thought, that the occurrence of imposex was restricted to areas in the immediate vicinity of the coast line close to marinas and harbours. However, also in the open North Sea imposex occurs in the common whelk, *Buccinum undatum*, and the frequency correlated very well to shipping traffic intensities (ten Hallers-Tjabbes *et al.*, 1994). During the last five years, a number of publications have shown that imposex is a global phenomenon, 72 species belonging to 49 genera of mesogastropods and neogastropods, suffer from imposex all over the world. However, not all species show the same development (Fioroni *et al.*, 1991) or have the same sensitivity. Thus, the consequences for the reproduction process may well be different.
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. Recently an experimental study with TPT (Horiguchi *et al.* 1994) also showed the induction of imposex in *Thais clavigera* and *T. bronni.* This is in contrast with a previous study (Bryan *et al.*, 1988) where TPT did not result in an increase of imposex in *N. lapillus*. So, literature on TPT causing imposex is not conclusive.

The Eastern Scheldt is an area which receives a considerable amount of organotins. TBT as well as TPT are used for human activities in the surroundings of the Eastern Scheldt. TBT is still illegally used in the anti-fouling paints for leisure boats, as the occurrence of an annual spring peak in TBT concentrations near marinas clearly indicate (Ritsema, 1994; Smedes, 1994). TPT is used in the potato culture, which is an important economic sector in the area surrounding the Eastern Scheldt. Several years ago, *N. lapillus* showed a high percentage of imposex and a number of females were sterile. They were expected to become extinct in this area. *B. undatum* in the Eastern Scheldt are known to suffer from imposex, but very few organotin concentrations have been reported so far (Mensink *et al.*, 1996).

[* TBT concentrations expressed as ng $Sn.l^{-1}$ can be converted to TBT by multiplying by 2.44].

CHAPTER 2

LEVELS OF ORGANOTIN COMPOUNDS AND THE OCCURRENCE OF IMPOSEX IN THE EASTERN SCHELDT (THE NETHERLANDS)

1. INTRODUCTION

Imposex is a phenomenon, which is found along many coastal areas not only in Europe, but all over the world. Many gastropod species suffer from this masculinisation effect in females, which can cause a decline in population density or even cause the local disappearance of certain species. Indirectly it can have effects on other organisms as well. The hermit crab, *Pagurus bernhardus*, is in its adult stage dependent on shells from bigger snail species, like the common whelk. Is there a decline in snail abundance, hermit crabs will have more difficulty in finding a suitable protection against their enemies. Imposex is caused by tributyltin in several species like *N. lapillus* and *H. reticulata.*

The Eastern Scheldt is an area likely to be affected by organotins. Not only a (high) TBT input caused by high leisure boating activity, but also an important TPT input due to its use in potato culture can be expected. *N. lapillus* were thought to become extinct in this area in 1988 (Mertens & van Zwol, 1988). Oysters in the same area showed shell deformaties, also caused by butyltin compounds (van Zwol & Mertens, 1988). Almost all 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.*, 1996). The state of the dog-whelk population and the occurrence of imposex in common whelks in the Eastern Scheldt led us to investigate the levels of organotin contamination in whelks, their food (mussels) and their habitat (sediment) during 1995.

2. METHODS

Cruises were made with the RV Navicula (NIOZ). Specimens of *Buccinum undatum* were caught at the location 'Hammen' in the Eastern Scheldt with a 4 m beam trawl. The mesh size of the trawl net was 6 cm. Several days were used to study a sufficient number of individuals and to prepare samples for tissue analyses of butyltin and phenyltin compounds. The length of the shell was measured for all animals.

Determination of organotin compounds

a. Preparation of samples

For organotin analysis, samples were prepared by dissecting several organs for individual and pooled samples. The digestive gland, foot and ganglia were analysed seperately in selected samples, next to whole body organotin analysis. Animals were divided into adults and juveniles and according to stages of imposex.

Samples of the sediment and mussels, *Mytilus edulis*, were taken in order to determine organotin concentrations in the habitat and food of *B. undatum.* Sediment samples were taken with a van Veen sampler in the same fishing lane as where the whelks were collected. Five samples were homogenized en divided in two subsamples. *M. edulis* were also caught in the same area as the sediment samples and were not collected from commercially fished musselbeds. Mussels were pooled before analysis, n = 5 in February, in June and September twelve mussels were pooled before homogenisation and analysis.

b. Analysis of organotin compounds

Organotin concentrations in *B. undatum* and *M. edulis* tissue have been determined with GC-MS after pentylation of the organotin compounds. Extraction, derivatisation and clean-up procedures have been described extensively elsewhere (Stäb *et al.*, 1994). In short, this 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., activated 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, in some cases GC-MS-MS was used. Quantitation ions are indicated in Table 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 $Sn.g^{-1}$ tissue fresh or dry weight.

Sediment samples (10 g fresh weight) first were acidified with HCl to pH 2. Then the same steps as described for biological tissues (see above) were performed.

c. Quality control of organotin analysis

Quality control included analysis of procedural blanks, control samples, spiking experiments and the application of internal standards. All data were corrected for overall recovery. Detection limits were determined based on the variability of signal-to-noise ratios in the chromatogram (see Table 1a,b). Variability within and between sample series was determined by using internal control samples (*Dreissena* homogenate and harbour dredge spoil, see Table 1a,b). The reproducibility of TBT and TPT were 21% and 23% respectively, for DBT 35% (highest) and for MPT 13% (lowest). To date, no suitable certified reference material for organotin analysis in animal tissue is commercially available.

Table 1.a Detection limits and reproducibility- animal tissue

	Quantitation mass (m/z) GC-MS-MS	Detection limit[a] Sn ng/g (dry wt.)	Sn ng/g (wt wt.)	Variation[b] coefficient MO-94/OT	Concentration wet wt. Sn ng/g 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*

a : 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.

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

Table 1.b Detection limits and reproducibility- sediment

	Detection limit sediment[a] Sn ng/g (dry wt.)	Sn ng/g (wt wt.)	Variation coefficient[b] 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%*

a : 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.

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

Determination of imposex

Since the animals crawl out of their shell when exposed to air, thereby exposing the penis and/or *vas deferens* (sperm duct) development, *B. undatum* could generally be sexed without being sacrified. 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. According to the size and shape of the penis homologue, 3 stages of imposex were discriminated (Fig.1).

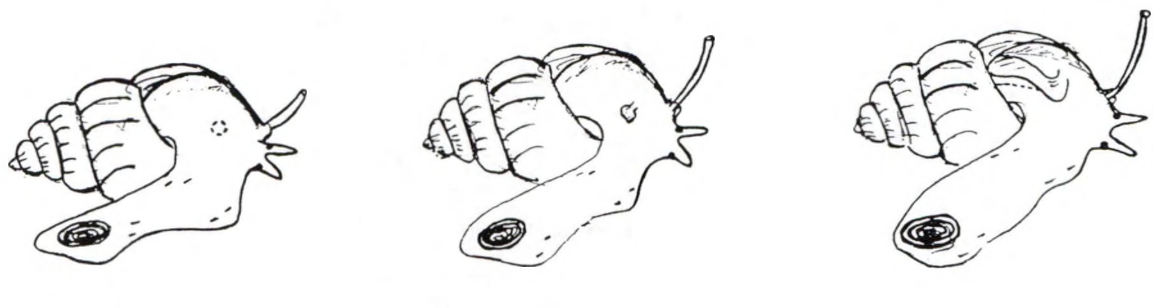
Stage 1 resembles the presence of a small knob at the site where males have their penis.

Stage 2 shows a differently shaped small structure.

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.

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, ovarium and bursa in females were checked as well as the testis and seminal vesicle in males. The length of the shell was measured and the shape of the penis homologue was recorded.



stage 1 stage 2 stage 3+

Figure 1. Determination of imposex stages in female *Buccinum undatum*.

3. RESULTS

Organotin concentrations

Tissue organotin concentrations are given in Tables 2, 3 and 4. The digestive gland and the foot of some female *B. undatum* were analyzed seperately in some samples. Ganglia were always pooled as well as the mussels to obtain enough tissue for organotin analysis.

In general, TBT whole body concentrations in *B. undatum* are low. In June, DBT levels in the digestive gland are at a maximum and higher than concentrations of MBT, which could be an indication for an enhanced environmental TBT concentration.

TPT was generally present in the highest concentrations. Low concentrations can be found in the foot of the animals (up to 11 Sn ng/g wet wt.), highest are found in the digestive gland of the animals (up to 250 Sn ng/g wet wt.) in February.

In September whole body organotin concentrations seemed to have dropped compared to February and June (Table 2b). In the ganglia and foot of the animals, however, TBT concentrations are much higher (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). Metabolites of TPT were usually around or below the detection limit, indicating that metabolism of this compound is slower than that of TBT, or that its metabolites are excreted extremely rapidly.

During this one year study no systematic differences in organotin levels were found between sexes. Thus, males and females are apparently exposed to the same organotin concentrations and have a comparable organotin metabolism. Also, no differences in concentrations were observed between females without imposex and females showing advanced stages 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.

In mussels, TBT is always found in higher concentrations than TPT. Organotin concentrations in the sandy sediments were low and phenyltins could hardly be detected in the samples taken. Sediment characteristics are given in appendix 1.

The TBT/DBT ratio(*) in *B. undatum* is in general < 1 as is the TBT/MBT ratio. In the edible mussel, M. edulis, these ratios are often > 1. For phenyltins, the TPT/DPT and TPT/MPT ratios are in the same order of magnitude and > 1 for both species. This indicates these two species have a different butyltin metabolism, whereas phenyltin metabolism seems comparable. In mussel tissue the TBT/TPT ratio is about an order of magnitude higher (>1) than the whole body ratio in *B. undatum* (0.1).

Occurrence of imposex

In Table 5 and 6 the results for *B. undatum* are given. The imposex frequency is constantly very high and almost constant (> 95%). Only a few females did not have any male sexual characteristic. 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 were observed, some even with a double penis.

No indications for sterility of common whelks have been obtained during the present study. In February more than twenty egg masses with developing young *B. undatum* were found, indicating that reproduction is still taking place in the Eastern Scheldt. In June only several empty egg masses were observed. No occluded genital pores were found.

[*For determining organotin ratios, values below detection limits were replaced by values half of the reported detection limit].

Table 2a. **Whole body organotin concentrations (Sn ng/g 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*

Table 2b. **Average (± SD) whole body organotin concentrations (Sn ng/g wet weight) of *B. undatum* per month.**

month	n	TBT	DBT	MBT	TPT	DPT	MPT
February	3	*2.40*	*4.63*	*3.17*	*20.3*	*13.3*	*1.90*
(± SD)		*0.72*	*0.50*	*0.60*	*3.51*	*11.9*	*1.01*
June	7	*1.14*	*11.9*	*5.99*	*27.0*	*3.34*	*5.91*
(± SD)		*0.66*	*6.08*	*2.41*	*8.00*	*1.71*	*9.76*
September	7	*0.66*	*2.25*	*1.76*	*17.8*	*3.27*	*3.09*
(± SD)		*0.79*	*0.93*	*1.36*	*5.40*	*1.96*	*0.73*

Table 3. **Tissue organotin concentrations (Sn ng/g 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*

Table 4. Organotin concentrations in mussels, *M. edulis*, (Sn ng/g wet wt.) and sediment (Sn ng/kg dry wt.) from the Eastern Scheldt in February, June and September 1995.

sample	month	TBT	DBT	MBT	TPT	DPT	MPT
	Feb	*12*	*4.0*	*3.7*	*5.8*	*<8.5*	*0.3*
mussels	June	*5.6*	*9.4*	*3.0*	*2.3*	*0.4*	*0.5*
whole body	June	*<6.0*	*24*	*5.4*	*1.6*	*0.5*	*<0.5*
	Sept	*8.1*	*4.6*	*4.6*	*2.0*	*<0.6*	*<0.1*
	Sept	*17*	*4.0*	*<1.0*	*1.7*	*<0.5*	*<0.2*
	Feb	*0.5*	*<0.1*	*<0.4*	*0.6*	*<0.5*	*<0.1*
	Feb	*1.1*	*<0.4*	*<1.1*	*<0.4*	*<1.3*	*<0.4*
sediment	June	*1.4*	*2.4*	*6.6*	*<0.3*	*<0.6*	*<0.1*
	June	*<1.8*	*<0.4*	*<0.3*	*<0.4*	*<0.8*	*<0.2*
	Sept	*<0.1*	*<0.2*	*<0.5*	*<0.2*	*<0.7*	*<0.2*
	Sept	*0.4*	*<0.3*	*<0.8*	*<0.3*	*<1.1*	*<1.1*

Table 5. **Number of investigated *B. undatum*, male: female ratio, average length 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 6. **Stages of imposex development in female *B. undatum* in February, June and September 1995 in the Eastern Scheldt (expressed as percentage of total number of females investigated).**

month	number of females	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*

4. DISCUSSION

In this study we investigated the organotin contamination and imposex incidence during a one year period in the marine predatory gastropod, *B. undatum*, in the Eastern Scheldt, a marine estuarine area with high leisure boating activity, many marinas and agriculture in the surrounding land.

Tissue organotin concentrations in *B. undatum* showed a different pattern from water. Although in the water TBT was the dominant butyltin compound (Ritsema, 1994), in tissue DBT > MBT > TBT. High metabolite levels were measured in the digestive gland and were one to two orders of magnitude higher in the digestive gland than in the foot of the same animals, indicating the digestive gland is an important organ for the biotransformation of TBT as also has been observed in the dog-whelk (Bryan *et al.*, 1993). No differences in organotin levels were observed between males and females and between females with advanced stages of imposex and those without imposex. Females and males seem to be exposed to organotins to the same extent and do not differ in organotin metabolism. The possibility that imposex in most of the females observed is a result of butyltin exposure in the past is likely regarding the comparable organotin levels in females with and without imposex. However, tissue concentrations inducing imposex in *B. undatum* have not been established so far. Juvenile whelks do not show any differences in organotin body burden compared to adults and juvenile females also showed imposex. So, no conclusions concerning the potency of organotin body burdens to induce imposex can be drawn from these findings.

In September the high concentrations of exclusively TBT in the ganglia of *B. undatum* are remarkable. Bryan *et al.* (1993) found high TBT levels in nervous tissue in *N. lapillus* and they speculated on the role of TBT and neurohormones in the induction of imposex. Féral & LeGall (1983) concluded that TBT affected cerebropleural ganglia in *Ocenebra erinacea* (a related species) causing a release of neural factors (hormones) inducing penis growth. In this study, the high affinity of the ganglia only for TBT (no other organotin compound is enhanced) seemed to occur only at the start of the reproductive period and could point to a seasonal sensitivity at a critical moment. The importance of these findings will have to be further elucidated. These measurements should be repeated around the critical period, to obtain more insight in this process.

In this study, phenyltin levels during one year in the marine environment are reported for the first time. Phenyltin compounds in whole body analysis as well as in seperate organs of whelks showed TPT levels well above those of the degradation products DPT and MPT. These TPT levels could point to a constant input into the area or indicate that biotransformation is slower for phenyltin than for butyltin compounds, or that metabolites formed are excreted extremely rapidly. Horiguchi *et al.* (1994) also found TPT to be dominant over its metabolites in *Thais clavigera* and *T. bronni*, but phenyltin concentrations reported were much higher (up to 1800 ng/g wet wt) than the concentrations in this study. TPT concentrations reported here are comparable with values reported for the Western Scheldt and North Sea (Crijns *et al.* 1992), but no data on other phenyltin compounds in the marine environment are available. Further studies on the kinetics of these organotin compounds are needed to elucidate the bioaccumulation processes determining the organotin load of the organisms and their environment.

The imposex incidence reported here is high (> 95%) and is not different from earlier findings in this area, although this time a far greater number of females could be investigated (Mensink *et al.*, 1996). ten Hallers-Tjabbes *et al.* (1994) only found an imposex incidence of 100 % in the North Sea in 1991 at one location in the "Eurogully"; the entrance for larger ships to the harbour of Rotterdam. All other stations during North Sea cruises showed an incidence < 30 %. Another case of imposex in *B. undatum* has been reported for the White Sea in the area of the Solevetski Isles, where only 19 females were investigated, 15 of which (79%) showed penis homologues (Kantor, 1984).

The male : female ratio is 1, as was found in reference (no observed imposex) populations from the central North Sea (ten Hallers-Tjabbes, 1979 and ten Hallers-Tjabbes *et al.*, 1996). 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 due to the rupture of the egg capsule gland of females resulting in the presence a surplus of males.

In the Eastern Scheldt we found all stages of impo-

sex but not a single female with an occluded genital pore. 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. In February quite a number of egg masses were caught with developing young whelks. 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.
Although in 1990 the use of TBT containing antifouling paints for ships and vessels up to 25 m in length was banned, TBT concentrations in water near marinas in the Eastern Scheldt 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. The reported threshold concentration for the induction of imposex in *N. lapillus* (1 ng/l) was always exceeded. DBT (mean 20-40 ng/l) and MBT (mean 4-9 ng/l) were also measured in the waterphase, but always at lower concentrations than TBT. Also spring peaks in TBT concentrations occurred, coinciding with the launching of freshly painted boats (Ritsema, 1994 and Smedes, 1994). At present no data on aqueous concentrations of phenyltin compounds in the Eastern Scheldt are available.
A comparison of the effectiveness of several organotin compounds, with respect to the induction of imposex was made by Bryan *et al.* (1988). TPT alone did not induce imposex in *N. lapillus*. Horiguchi *et al.*, (1994), however, state that injection of TPT had an inducing and promoting effect on the development of imposex in *Thais clavigera* and *T. bronni*. An exposure to a combination of organotins was never tested in both studies. Thus, it remains to be investigated, whether TPT has an inducing and/or additive effect on imposex.

5. CONCLUSIONS

Of butyltin compounds, the order of concentrations in whole body homogenate of whelks was DBT > MBT > TBT. TBT was often just above the limit of detection except in September in the neural ganglia, representing the brain of these animals. Here, only TBT could be detected and even the TBT/TPT ratio was > 1. 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.

Phenyltins were present in all animal samples. TPT clearly showed the highest levels, followed by its metabolites DPT and MPT, both in comparable amounts. TPT concentrations in whole body homogenates were 4-100 times higher than those of TBT.

No structural difference in organotin contamination was found between:

I. the sexes
II. different stages of imposex
III. adult and juvenile animals

Mussels, *Mytilus edulis*, representing the most important food source for the whelks, showed a different pattern in butyltin ratios from *B. undatum*. In mussels TBT > DBT > MBT. Phenyltin ratios were comparable to 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 suggest a possible biomagnification.

In total sediment samples organotins were usually below their detection limit. Because of this, we would suggest only to analyse the fraction < 63 µm in future research.

The percentage of females showing imposex was high (> 95%) and adult as well as juvenile females showed male sexual characteristics. This did not change throughout the year.

No sterile females were observed although more than 50% showed stage 3 or 3+ of imposex. Egg capsules with developing young animals clearly showed reproduction still takes place in the Eastern Scheldt population.

CHAPTER 3

TRIBUTYLTIN EXPOSURE IN LABORATORY EXPERIMENTS

1. INTRODUCTION

Common whelks (*Buccinum undatum*) from the open North Sea show imposex; the development of male sexual characteristics in female prosobranch gastropods. Since a correlation with shipping intensities could be established, tributyltin (TBT), the active biocide in anti-fouling paints, was expected to be the cause. Laboratory experiments with the dog-whelk (*Nucella lapillus*) and *Hinia reticulata*, which live in the immediate vicinity of the coast-line, have clearly showed that TBT causes imposex in these species, already at concentrations as low as 1 ng Sn/l. Because TBT concentration levels were assumed to be lower in the open North Sea, common whelks were expected to be at least as sensitive to TBT as *N. lapillus* and *H. reticulata*.
To study the supposed cause-effect relationship for *B. undatum*, adult and juvenile common whelks were experimentally exposed to TBT. Juvenile *B. undatum* were produced by the experimental adults and were already exposed *in ovo* or just after hatching to test whether life-stage is an important parameter for the sensitivity with respect to imposex development.
Since whelks crawl out of their shell when exposed to air, the developmental stages of male sexual characteristics could be recorded over time, since animals did not have to be sacrificed.

2. METHODS

Origin of animals
Adult *B. undatum* were collected from the North Sea near the Dogger Bank in September 1993. At this station (54°29'N; 4°00'E) no imposex had been found during former cruises.
Two months after the start of the experiment and the production of egg-masses by the adult whelks (February 1994), young whelks hatched and were also exposed.

Experimental conditions
TBT was administered to the experimental aquaria by dissolving tributyltinacetate (Aldrich) in demineralised water (stock solutions); these solutions were diluted 1 : 100 with Wadden Sea water in a mixing chamber to nominal concentrations of 1µg 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 the second week of December 1993.
Experimental conditions were a waterflow of 80 l/day per aquarium, a 12h light-12h darkness regime and an air temperature of 12°C. Each experimental aquarium contained five female animals and one male. During the acclimation period, egg masses were produced in all experimental groups in November/December 1993.
Fresh mussel meat (*Mytilus edulis*) was fed *ad libitum* once a week. To make the meat easily available to the animals, the shells of the mussels were seperated just before offering them to the whelks.

Analysis of butyltin compounds
To determine the actual butyltin concentrations, 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). Water samples were acidified with 30 % HCl (1 ml per litre) (Merck, Germany) before storage at 4°C. Analyses were conducted at the RIKZ (Rijkswaterstaat) laboratory in Haren.
Butyltin concentrations in animals were measured by combined gas-chromatography and mass-spectrometry with ion trap detection (GC/ITD-MS) as already has been described in Chapter 2. Analyses were carried out at the IES (Free University) in Amsterdam.

Determination of sexual development
The animals could be sexed by exposing them to air; since they react by crawling out of their shells, thereby showing some of their sexual organs. The differing stages of sexual development of a penis were characterised as follows: (see also figure 1, Chapter 2)
0 : no male sexual characteristics
1 : small round bud at the site of a where males grow a penis
2 : enlargement to various shaped structures
3 : development of a curved penis similar to adult males

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

Statistical testing

Statistical analysis of the data 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 in length, also log(P/1-P) transformation of the data was used.

Calculation factors for TBTAc, TBT and Sn concentrations

Because often in literature TBT concentrations (in ng/l) are not reported uniformly, here calculation factors are given for comparison of concentrations expressed as ng TBTAc/l, ng TBT/l and ng Sn/l.

ng TBTAc/l → ng Sn/l, values should be divided by 2.94
ng TBT/l → ng Sn/l, values should be divided by 2.44
ng TBTAc/l → ng TBT/l, values should be multiplied by 0.83

3. RESULTS

TBT exposed adult females did not show any signs of imposex during an 11 months period of exposure, although a dose-dependent increase in body burden of TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) over time was observed (Table 7).

Table 7. Whole body butyltin concentrations (in ng Sn/g wet wt.) of experimentally exposed common whelks, *B. undatum*. Analysis were 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				<1, <1, 8	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

In contrast to the adult specimens, their offspring showed marked differences in sexual development (fig.2), when exposed in the same manner as their parents. The control group and the low dose (10 ng TBTAc/l) group showed a similar penis development. After two years of exposure the male : female ratio converged to 1. This points to a life stage where the sexual organs are basically present and where there is only a further growth of these organs (figure 3a-d).
When exposed to a 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. 3c). After 8 months 34 % of the animals showed the first stage of penis development. Nearly all animals showed a small structure or a penishomologue 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*.

The highest dose (1 µg TBTAc/l) showed virtually only fully developed stages in all exposed animals. After 10 months of exposure even animals with a double penis were found in this group as well as other aberrations like 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 had a penis. 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. Almost all animals showed a penis and a *vas deferens*. Only one whelk showed the formation of only a *vas deferens*.

One-way ANOVA on the percentages with male sexual characteristics showed a significant difference between groups after 8 months ($F = 41.3$, $P = 0.002$) and 10 months ($F = 636.3$, $P < 0.001$) of exposure. A post-hoc comparison between the control group and the 100 ng TBTAc/l group also showed significantly ($F = 11.7$, $P = 0.027$ and $F = 210.4$, $P = 0.001$) higher frequencies of animals with male sexual characteristics in the exposed group after 8 and 10 months respectively.

In August 1995, a significant difference in growth between the experimental groups ($F = 17.4$, $P < 0.001$) was observed. The average length in the control group is significantly higher than in the TBT exposed groups ($F = 49.3$, $P < 0.001$). Surprisingly, TBT exposed groups did not differ from each other. The lowest dose group was not significantly different from the highest dose group ($F = 0.17$, $P = 0.68$). The different weights have not yet been tested on significance. Because there is a strong relationship between length and weight, weights probably will differ significantly also (figures 4 & 5).

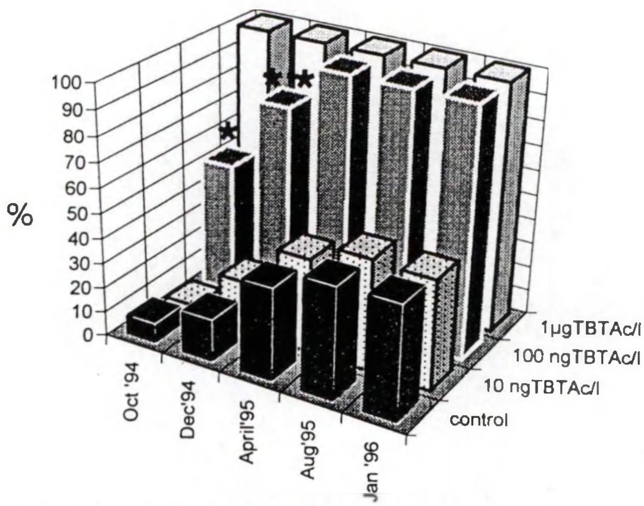


Figure 2. Sexual development, expressed as the percentage with male sexual characteristics, of juvenile *B. undatum* during the first two years.
* *means significanly higher than the control group (P< 0.05).*
** *means significantly higher than the control group (P = 0.001)*

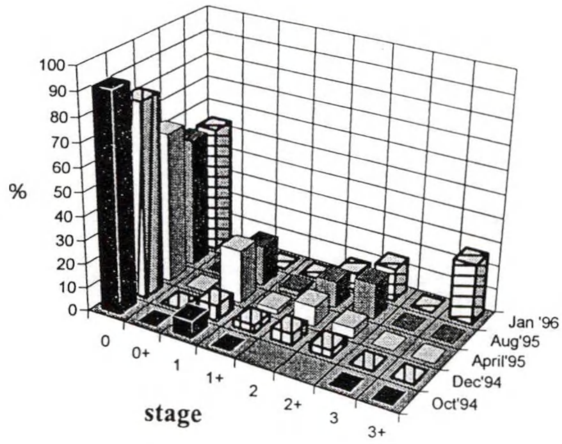


Fig. 3a. Control group.

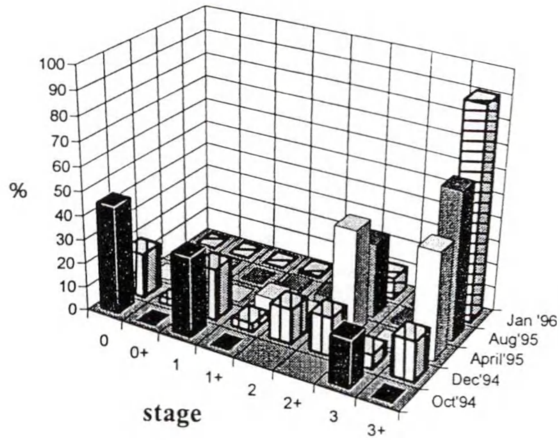


Fig. 3c. Dose : 100 ng TBTAc/l.

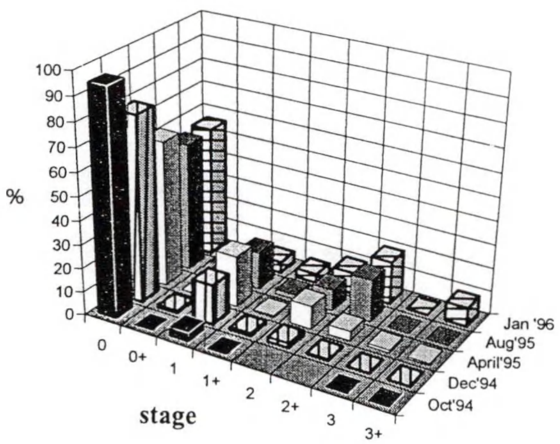


Fig. 3b. Dose : 10 ng TBTAc/l

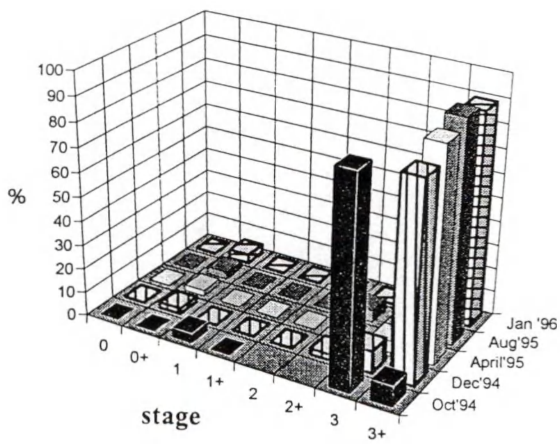


Fig. 3d. Dose : 1 µg TBTAc/l

Figure 3a-d. Stages of the development of male sexual characteristics in juvenile *B. undatum* for different TBT doses during the first two years.

Actual butyltin concentrations in water varied for the different groups. In the beginning of the experiment both the reference and low dose group sometimes showed detectable (1-10 ng/l) levels of TBT. During the experiment, these concentrations reached at the most TBT levels equivalent to 30 ng TBTAc.l^{-1} for the control group and 40 ng TBTAc.l^{-1} for the low dose group (fig. 6a,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^{-1} after 11 months. After one year concentrations increased to the nominal dose (fig 6c). In the highest dose actual concentrations ranged from 35 to about 2000 ng TBTAc.l^{-1}(fig. 6d). The apparent TBT contamination of 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).

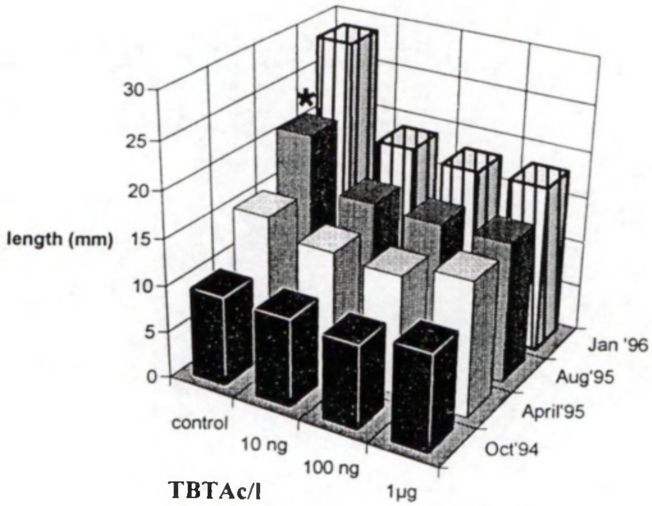


Figure 4. Average length for the experimental groups.
** means significantly higher than TBT exposed groups (P< 0.001).*

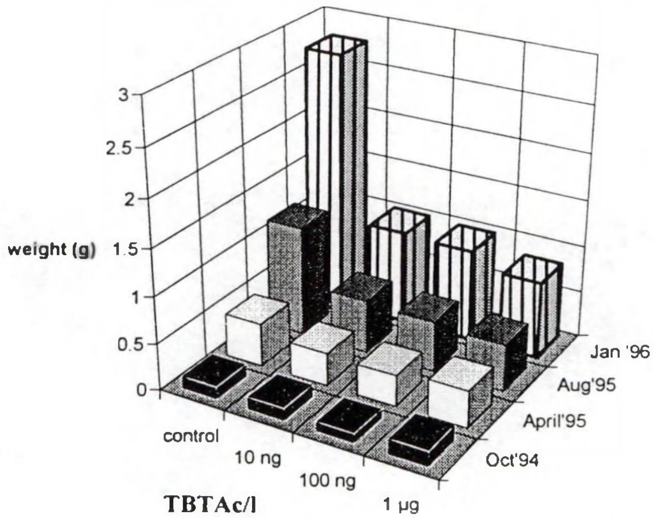


Figure 5. Average weight for the experimental groups.

So, in the low and medium dosed groups actual TBT concentrations were often below nominal concentrations especially in the beginning of the experiment, probably due to adsorption and metabolism, since analysis of stock solutions showed that these were prepared correctly.
In figures 7a-d, actual DBT concentrations are given. With increasing TBT doses, DBT and MBT (fig.8 a-d) levels also increased. In the reference group and lowest dose group, DBT concentrations were often higher than TBT levels, whilst MBT levels were comparable to TBT levels.
The highest dose showed much higher TBT concentrations than DBT and MBT.

Survival percentages were 66 % for the low dose group, 74 % for the medium dose group and 60 % 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 % of the initial number of whelks survived in this group.

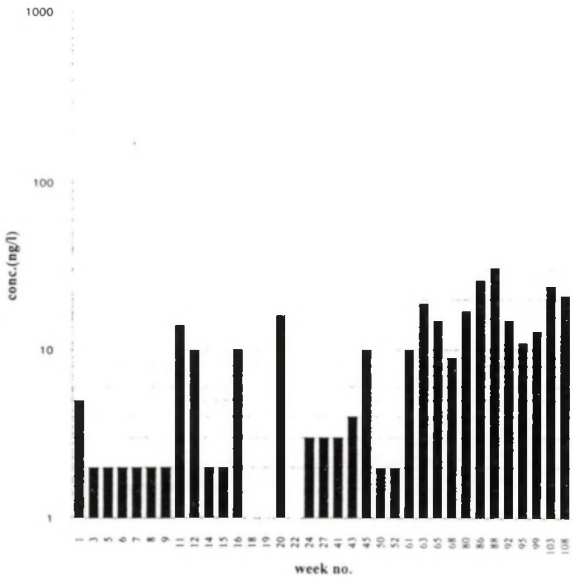


Fig. 6a. Control group

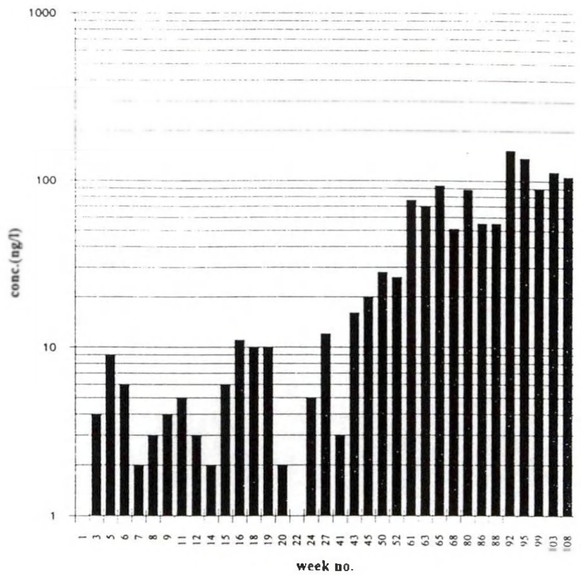


Fig. 6c. Dose : 100 ng TBTAc/l

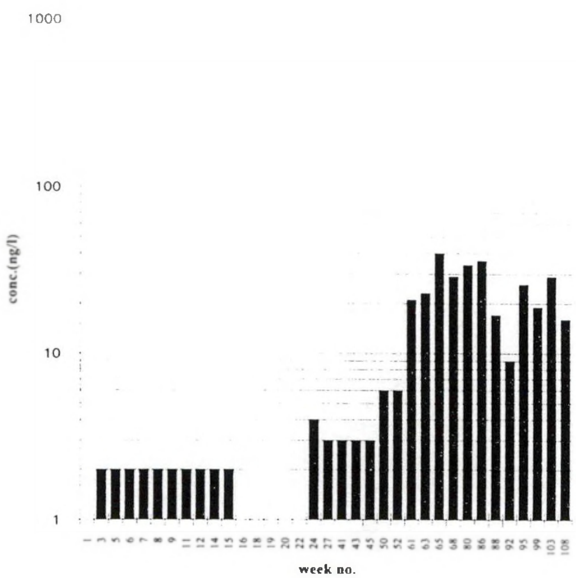


Fig. 6b. Dose : 10 ng TBTAc/l

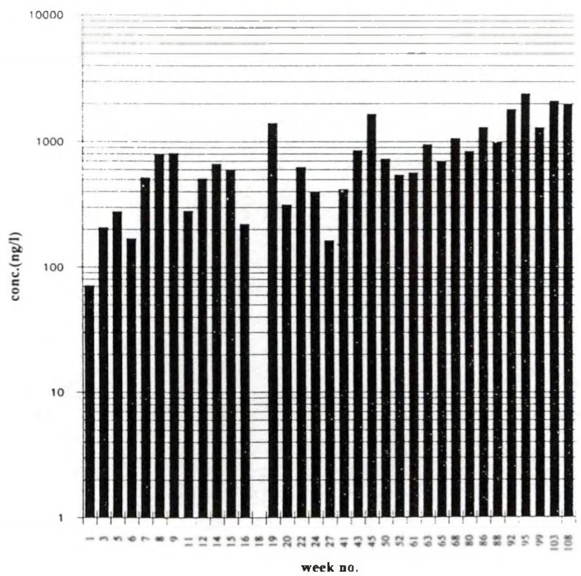


Fig. 6d. Dose : 1 µg TBTAc/l

Figure 6a-d. Actual TBTAc concentrations (in ng/l) for the TBT exposed groups during the experimental period.

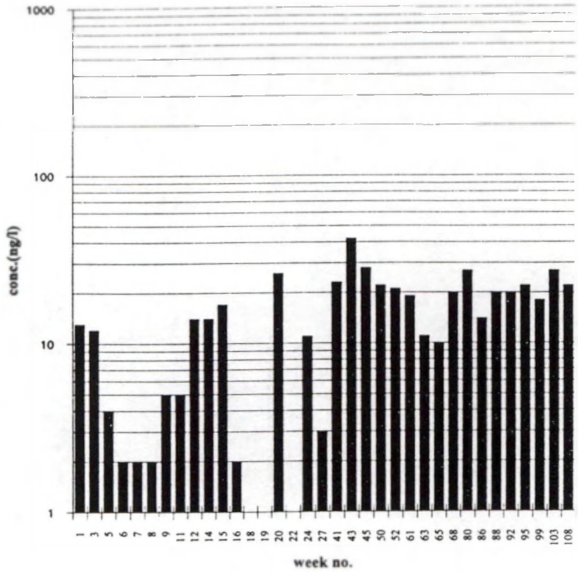


Fig. 7a. Control group

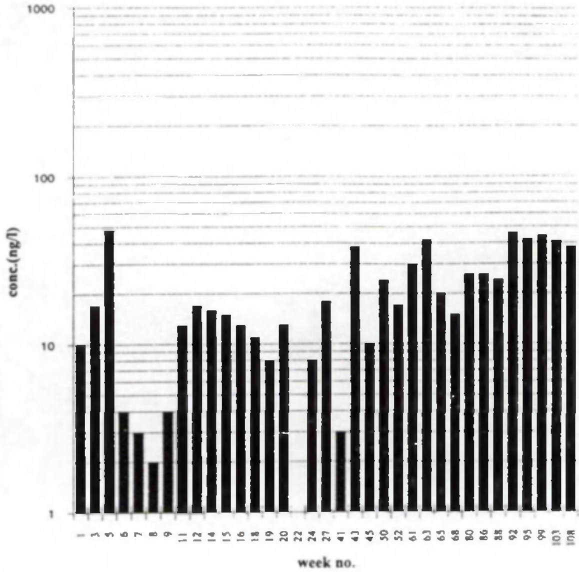


Fig. 7c. Dose : 100 ng TBTAc/l

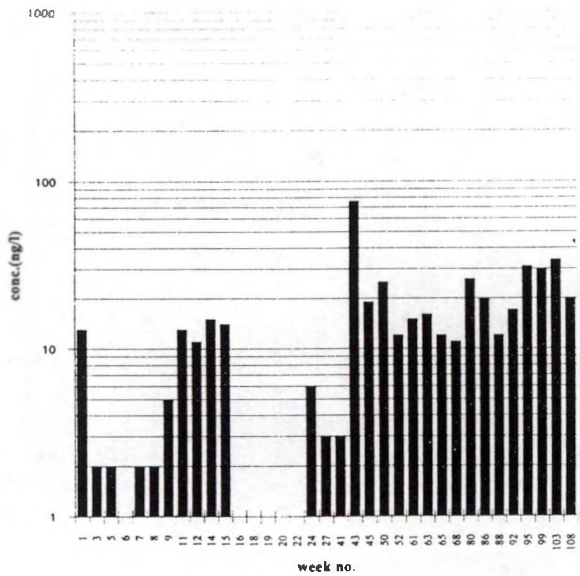


Fig. 7b. Dose : 10 ng TBTAc/l

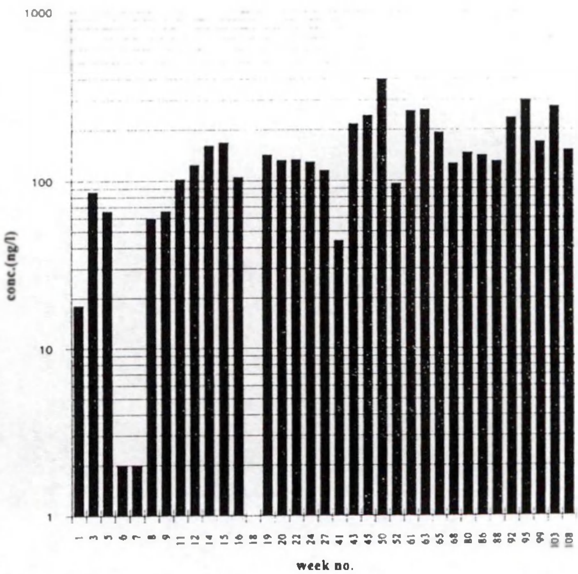


Fig. 7d. Dose : 1 μg TBTAc/l

Figure 7a-d. Actual DBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

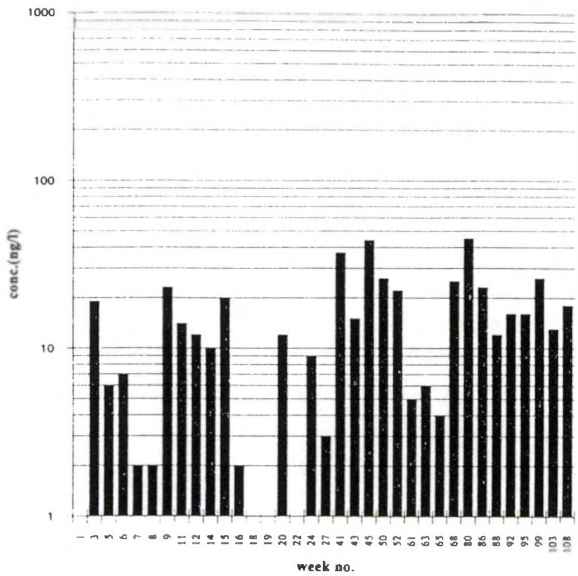


Fig. 8a. Control group

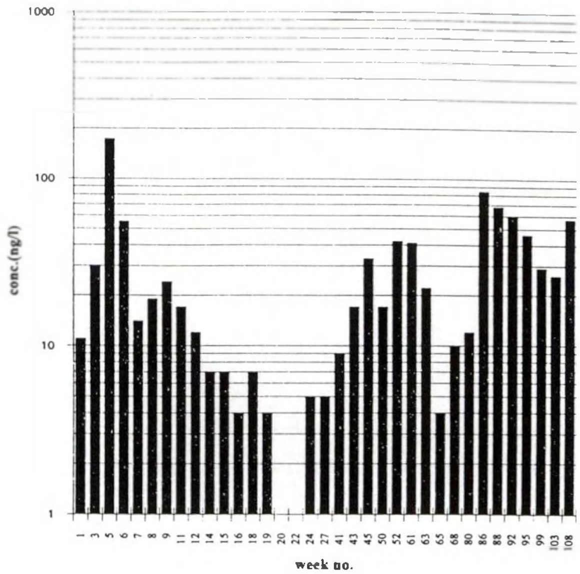


Fig. 8c. Dose : 100 ng TBTAc/l

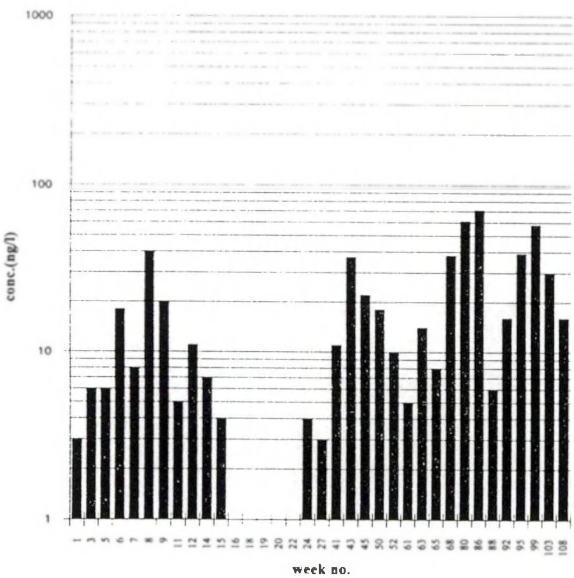


Fig. 8b. Dose : 10 ng TBTAc/l

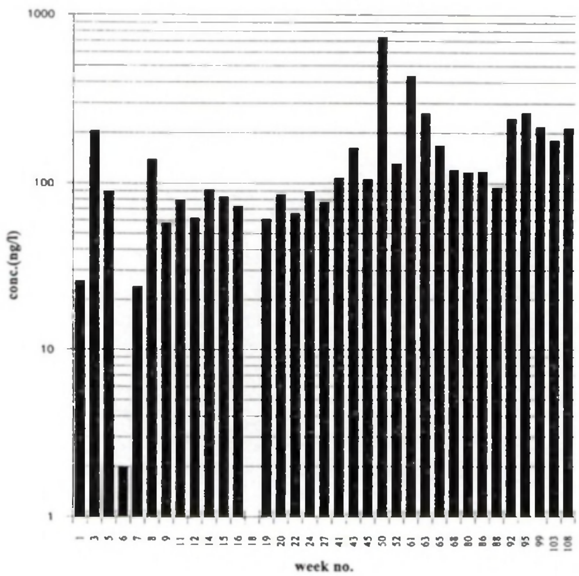


Fig. 8d. Dose : 1 µg TBTAc/l

Figure 8a-d. Actual MBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

4. DISCUSSION

In this research we investigated the effects of different doses of tributyltin on the common whelk. Adult as well as juvenile whelks were experimentally exposed to nominal concentrations of 10, 100 and 1000 ng TBTAc/l.

Although whelks were expected to be as sensitive as *N. lapillus* and *H. reticulata*, we could only find an influence of TBT on the development of male reproductive organs in juvenile common whelks, but not in adult females. Most likely, adult females are less sensitive to TBT exposure than juveniles, because they already have a fully developed genital system. These young animals develop their sexual organs in their first life stages and any interfering agent may cause a different development. TBT has been proven to cause an increase in testosterone levels in exposed gastropods, which leads to the formation of male sexual characteristics in female specimens of *N. lapillus* and *H. reticulata* (Spooner, 1991 and Oehlmann *et al.*, 1993). The same mechanism might also explain the increase of the percentage of male sexual characteristics in juvenile *B. undatum*.

Although in *N. lapillus* this development already starts at concentrations of 2-3 ng TBT/l*(see note below) (Gibbs & Bryan, 1994), in this study after 8 months of exposure, we observed a significant increase in the percentage of animals with male characteristics at (actual) concentrations of ≤ 17 ng TBT/l*. 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. 6c.).

The use of continuous flow conditions makes it very difficult to obtain and maintain the nominal TBT concentrations. Especially the lower doses are hard to obtain.

The exposure to TBT also seems to affect the growth of the juveniles. A significantly smaller length was observed at a dose of 10 ng TBTAc/l. However, there was no observed dose-dependent effect. For the weight no statistical tests were conducted yet, but the differences observed in length seem to be much more pronounced in the weigths of the groups.

[*concentrations expressed as ng TBTAc/l and ng Sn/l are converted to ng TBT/l according to the calculation factors given in methods].

We cannot exclude the possibility of a population density effect being responsible for the effects observed. However, if there was such an effect, we would expect aquaria containing less animals to have a greater average length (and weight) than 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, a density dependent effect is not expected to be responsible for the differences in length observed.

In the adult females, a dose dependent increase in butyltin body burden was observed. In the control group only low concentrations of DBT (6 ng Sn/g WW.) and MBT (3 ng Sn/g WW.) could be detected, but TBT concentrations were below the detection limit (< 2 ng Sn/g WW). In the low dose group a similar pattern 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.

If we assume equilibrium conditions after 11 months of exposure, in the medium dose group 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 showing average values of 0.66-2.40, 2.25-11.9 and 1.76-5.99 ng Sn/g for TBT, DBT and MBT repectively. Namely, butyltin body burdens of these 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. This could point to a TBT concentration between 10 and 80 ng TBT/l in the Eastern Scheldt. This is 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 there is dredging activity (3 times/year), which very likely reduces the TBT concentration in the water phase (Ritsema, 1994). These concentration levels will certainly cause a masculinisation effect in developing juvenile whelks.

5. CONCLUSIONS

Although it is expected that 50% of young whelks will eventually develop as normal males, exposure to nominal doses > 10 ng TBTAc/l (equivalent to ≈ 3.5 ng Sn/l) induced and accelerated the development of male sexual characteristics in a dose-dependent manner, when *B. undatum* were exposed *in ovo* or just after hatching.

Exposure to concentrations ≥ 10 ng TBTAc/l lead to reduced growth in juvenile common whelks.

Adults seem to be much less sensitive to TBT than juveniles.

The laboratory experiments show, that TBT levels encountered in the Eastern Scheldt can cause a masculinisation effect in developing juvenile whelks living in this area.

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Appendix 1. Sediment characteristics

Sedimentkarakteristieken

Experiment / field codes	Date	IVM code	Dr wt. %	Org-C %	CaCO3 %	Lutum	Korrelgrootte (cum. %) <2 micr.	<8	<16	<20	<63	<200	<2000
Sediment-1	febr. 95	370S040	69.35%	0.55	9.0	11.0	5.1	11.0	14.7	16.5	22.8	55.9	100
Sediment-2	febr. 95	370S041	69.57%	0.63	8.7	10.5	4.9	10.5	14.0	15.8	21.7	54.9	100
Binnen Hammen - 1	8 june 95	370SL025		0.48	11.9	13.6	5.4	13.6	18.3	20.5	28.2	70.6	100
Binnen Hammen - 2	8 june 95	370SL026		0.74	10.4	13.8	6.5	13.8	18.6	20.9	28.7	71.8	100
Sediment I	07/09/95	370S038	66.17%	0.60	11.2	12.0	5.7	12.0	16.0	18.0	26.3	87.3	100
Sediment II	07/09/95	370S039	66.74%	0.55	11.6	12.3	5.9	12.3	16.3	18.3	27.0	87.6	100

CONTENTS