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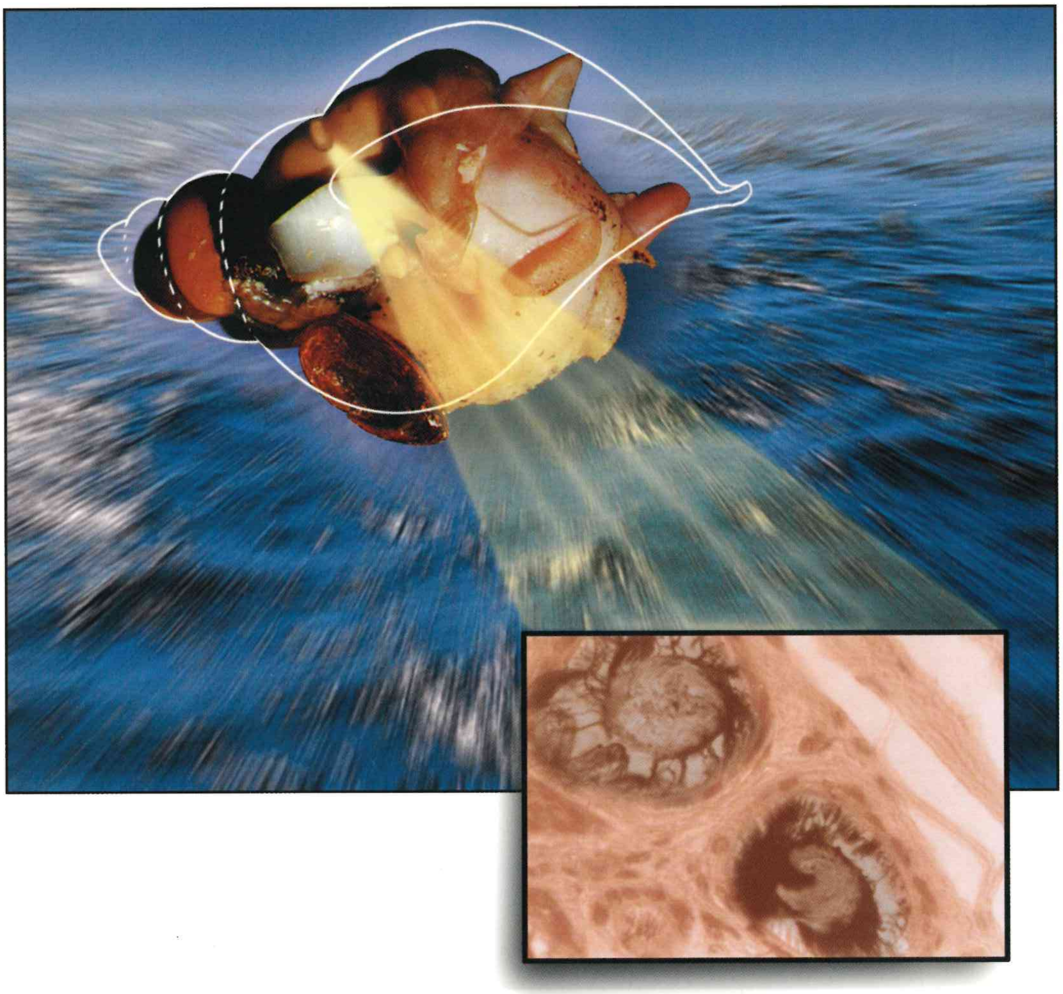

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The reported studies into organotin contamination and its effects were commissioned by the Directorate-General for Environmental Protection (DGM) of the Ministry of Housing, Physical Planning and the Environment (case number 9512.0037) and the Directorate-General for Public Works and Water Management, National Institute for Coastal and Marine Water Management (RIKZ) of the Ministry of Transport, Public Works and Water Management (reference DG-502).

UITGEBREIDE SAMENVATTING

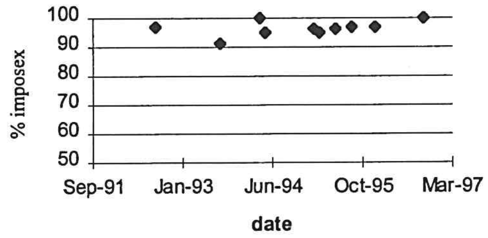
Dit rapport beschrijft de resultaten van studies naar de ontwikkeling en het voorkomen van mannelijke geslachtskenmerken (imposex) in de wulk, *Buccinum undatum*, onder invloed van (chronische) blootstelling aan organotinverbindingen uit anti-fouling verven (butyltinverbindingen).
Het onderzoek werd verricht, teneinde meer inzicht te krijgen in de effecten van organotin verontreiniging op het mariene milieu, welk mogelijk een relatie heeft met de achteruigang van de wulk in de Nederlandse kustwateren. Zowel veld- als laboratorium studies werden hiertoe uitgevoerd. Omdat de Oosterschelde het enige Nederlandse kustgebied is waar nog wulken voorkomen en sprake kon zijn van een substantiële organotinbelasting via recreatiescheepvaart (butyltinverbindingen) en landbouw (fenyltinverbindingen) was dit een geschikt onderzoeksgebied. Het optreden van mannelijke kenmerken in vrouwelijke wulken, imposex, (het meest gevoelige effect van tributyltin) werd hiertoe onderzocht. Omdat in de literatuur (hoewel tegenstrijdig) gerapporteerd wordt, dat trifenyltin eveneens in staat zou zijn imposex te veroorzaken, werden in de veldstudie tevens fenyltin verbindingen geanalyseerd. In het laboratorium werd een dosis-effect relatie tussen TBT en imposex onderzocht, waarbij het verkrijgen van meer kennis over de achterliggende toxicologische mechanismen eveneens een onderzoeksdoel was.

Veldstudies in de Oosterschelde (hoofdstuk 2) leverden een uitgebreid beeld op van de mogelijke stadia van imposex in volwassen wulken. Naast imposex stadia en percentages (1992-1996) zijn in 1995 wulken, hun voedsel (mosselen) en hun directe omgeving (sediment) onderzocht op organotinconcentraties. In enkele laboratoriumexperimenten (hoofdstuk 3) werd bij (langdurige) blootstelling het oorzakelijke verband tussen tributyltinblootstelling en de ontwikkeling van mannelijke geslachtskenmerken onderzocht. Onderzoek naar het toxicologisch mechanisme van imposex bestond uit histologisch onderzoek naar de geslachtsorganen van jonge en volwassen wulken met en zonder imposex (hoofdstuk 4) en uit onderzoek naar een mogelijke invloed van TBT op het cytochroom P450 enzymsysteem van wulken (hoofdstuk 5). Dit enzymsysteem diende eerst te worden aangetoond in deze mariene slakkensoort.

Veldstudies

Dit deel beschrijft de studies naar imposex (1992-1996) 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 tri- butyltin en fenyltin verbindingen. In tegenstelling tot de butyltin verbindingen worden fenyltin verbindingen vooral gebruikt als nematocide in de landbouw.
Bijna alle vrouwelijke wulken in de Oosterschelde (> 91%) vertoonden imposex-verschijnselen, meer dan de helft hiervan de meer gevorderde stadia. In mei 1994, februari en maart 1995 werden eimassa's met zich ontwikkelende jonge wulkjes aangetroffen, hetgeen betekent dat voortplanting wel plaatsheeft. Hoewel in 1990 een verbod op het gebruik van TBT bevattende verven voor schepen < 25 m van kracht werd, zijn de imposex percentages van de wulk in de jaren daarna niet veranderd (zie figuur A).

Organotin concentratie metingen van 1995 worden in Tabel A samengevat weergegeven.
Trifenyltin (TPT) werd in alle monsters 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, tussen adulten en juvenielen, en tussen vrouwtjes met en zonder imposex.



Figuur A. **Imposex percentages in de wulk, *Buccinum undatum*, gedurende vijf jaren in de Oosterschelde (Hammen).**

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).

Dibutyltin (DBT) en monobutyltin (MBT) komen in hogere concentraties voor dan TBT in wulken vergeleken met mosselen, waar TBT de hoogste concentraties vertoont. Dit wijst op metabolisatie (dealkylering) in wulken. Biomagnificatie van butyltin verbindingen lijkt voor de wulk onwaarschijnlijk. Voor fenyltinverbindingen kan dit niet worden uitgesloten op grond van de concentraties, welke 4-10 maal hoger zijn in wulken dan in mosselen en vanwege de schijnbaar geringe metabolisatie van de fenyltins. Trifenyltin is in de mossel en de wulk de belangrijkste fenyltinverbinding. Voor de organotinanalyses werden van wulken meerdere organen apart geanalyseerd. Organotinverbindingen (versgewicht) werden in het digestieve kanaal in hogere concentraties aangetroffen dan in de voet. In september 1995 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 een ontwikkeling van een zaadleider zou kunnen leiden .

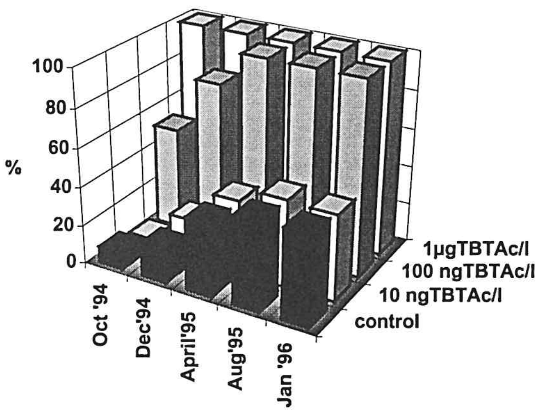
Tabel A. **Gemiddelde organotin concentraties in wulken (*Buccinum undatum*) mosselen (*Mytilus edulis*) en sediment van de Oosterschelde in 1995.**

	maand	TBT	DBT	MBT	TPT	DPT	MPT
				(ngSn/g versgewicht)			
	feb	2.40	4.63	3.17	20.3	13.3	1.90
wulk	juni	1.14	11.9	5.99	27.0	3.34	5.91
	sept	0.66	2.25	1.76	17.8	3.27	3.09
				(ngSn/g versgewicht)			
	feb	12	4.0	3.7	5.8	<8.5	0.3
mossel	juni	4.3	16.7	4.2	1.95	0.45	0.38
	sept	12.6	4.3	2.55	1.85	<0.6	<0.2
				(ngSn/kg drooggewicht)			
	feb	0.8	<0.4	<1.1	0.4	<1.3	<0.4
sediment	juni	1.15	1.3	3.4	<0.4	<0.8	<0.2
	sept	0.2	<0.3	<0.8	<0.3	<1.1	<1.1

Laboratoriumstudies

Het eerste 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 nominale concentraties tot 1 µg tributyltinacetaat per liter (TBTAc/l) geen mannelijke geslachts-kenmerken. Hun juvenielen vertoonden echter een sterk verhoogde mannelijke ontwikkeling. Na 8 maanden blootstelling aan nominale concentraties van 100 ng TBTAc/l (gelijkwaardig met 83 ng TBT/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 (figuur B). Dit wijst erop, dat de geslachtsorganen dan in principe aanwezig zijn en in de tijd alleen nog zullen groeien.



Figuur B. **Geslachtsontwikkeling, uitgedrukt als het percentage met mannelijke kenmerken van juveniele wulken, *Buccinum undatum*, gedurende de eerste twee levensjaren na blootstelling aan nominale TBTAc concentraties.**

Hoewel normaliter verwacht mag worden dat 50% van de juvenielen zich zal ontwikkelen tot man, leidt blootstelling van deze juveniele wulken aan nominale concentraties van 100 ng TBTAc/l (vergelijkbaar met 83 ng TBT/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 en/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 waren, mag een invloed op de sexuele ontwikkeling al bij concentraties van 17 ng TBT/l (7 ng Sn/l) worden verwacht.

De lichaamsgroei leek geremd te worden bij lagere concentraties dan waarbij de sexuele ontwikkeling werd beïnvloed. Een significant kleinere lengte kon worden waargenomen bij concentraties ≥ 10 ng TBTAc/l (≥ 4 ng Sn/l).

Een dosis afhankelijke toename in lichaamsgehalten van alle butyltin verbindingen kon worden waargenomen bij de volwassen wulken gedurende de blootstellingsperiode.

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.

Tabel B. NOEC en LOEC waarden voor TBT en wulken.

LEVENSSTADIUM	STOF	CONC. ng/l	EFFECT NOEC	LOEC	BLOOTSTELLINGS-DUUR (MND)
adult	TBT	830	imposex		9
juveniel	TBT	8.3	imposex		23
juveniel	TBT	17		imposex	8
juveniel	TBT	8.3		groeiremming	18

In het tweede laboratorium experiment werd onderzocht of blootstelling aan TBT tijdens de ontwikkeling in het ei tot een verhoogde mannelijke ontwikkeling zou leiden. Hier werden vier eimassa's verdeeld over twee groepen en twee maanden ofwel gehouden in Waddenzeewater ofwel blootgesteld aan nominaal 1 µgTBTAc/l. Op het moment dat de eerste juveniele wulkjes uit het ei kwamen werd een van de twee eimassa's overgeplaatst naar de andere behandeling. Overplaatsing naar en uitkomen in 1 µgTBTAc/l bleek voor beide groepen letaal. Alle wulkjes stierven binnen een aantal dagen na uitkomst. De andere wulkjes ontwikkelden zich in Waddenzee water gelijkwaardig en na 1.5 jaar was nauwelijks verhoging van het percentage met mannelijke geslachtsorganen waar te nemen in de groep die tijdens de eifase blootgesteld is geweest.
Hieruit blijkt het belang van blootstelling via de waterfase direct na ei-uitkomst en de beschermende werking van de eimassa in een verontreinigde omgeving.

Het derde experiment betrof twee jaar oude wulken, die gedurende drie maanden werden blootgesteld aan ofwel Waddenzee water ofwel nominaal 100 ngTBTAc/l. Dit werd gedaan teneinde een leeftijdafhankelijke gevoeligheid voor TBT te kunnen onderscheiden. In beide groepen werd in deze korte tijdsperiode geen ontwikkeling van mannelijke kenmerken waargenomen.
Wulken die hun geslachtsorganen al gevormd hebben lijken minder gevoelig, maar dit moet nog uitgebreider onderzocht worden.

Mechanistische studies
Aangezien uiterlijke kenmerken geen volledig uitsluitsel geven over het functioneren van het organisme, werden volwassen en juveniele wulken gefixeerd en gesneden voor orgaanstruktuur en functioneel onderzoek.
Histologische studies toonden aan, dat het vrijwel onmogelijk is wulkjes te sexen voordat ze een lengte van circa 4 cm hebben bereikt (2.5-3 jaar oud). In juvenielen was (nog) geen gonadevorming. Sterilisatie van wulken als gevolg van mechanische afsluiting van de genitale opening door de zaadleider treedt in wulken niet op, noch een ontwikkeling van mannelijk weefsel in het ovarium weefsel van Oosterschelde vrouwtjes met de meest gevorderde stadia van imposex. Een invloed op de fertiliteit van de vrouwtjes met imposex kon in dit onderzoek niet worden vastgesteld. In deze studie zijn geen extra sexuele kenmerken waargenomen, naast die die met het oog al waren waargenomen.
Gezien het belang van het cytochroom P450 enzymsysteem bij de omzetting van zowel endogene (hormonen) als exogene stoffen (TBT), werd onderzocht of TBT invloed had op dit enzymsysteem door microsomale fracties van de wulk *in vitro* bloot te stellen. Als positieve controle is een microsomale fractie van de schar meegenomen, omdat hierin al is vastgesteld dat TBT een

inactiverende invloed heeft op dit enzym. In zowel het digestieve kanaal, als in de nier van de wulk kon CYP450 worden aangetoond. Zowel bij de schar als bij de wulk werd aangetoond, dat TBT CYP450 inactiveert door de actieve vorm om te zetten in de inactieve CYP420 vorm. Bij alleen de schar kon een type I bindingsspectrum worden gevonden, wat wijst op een binding van TBT aan het eiwitgedeelte van het CYP450 molekuul. In de wulk lijkt TBT andere onderdelen van het enzymsysteem te inactiveren. Bij beide studies zijn hoge TBT concentraties gebruikt, het is de vraag of deze in de organismen zullen voorkomen. Verder onderzoek zal meer duidelijkheid moeten verschaffen over de fysiologische betekenis van deze inactivatie.

Vergelijking van studies
Een combinatie van de lichaamsgehalten van de volwassen wulken uit dit laboratorium experiment met lichaamsgehalten in wulken van de Oosterschelde, leidde tot een onverwacht hoge voorspelde TBT concentratie in de Oosterschelde van tussen 10 en 80 ng TBT/l. Op basis van concentratiemetingen in mosselen en literatuurgegevens (Evers *et al.*, 1995) over bioconcentratiefactoren (BCF's) van TBT in mosselen werd op deze manier eveneens een voorspelling gedaan over te verwachten TBT concentraties. In Tabel C staan de gegevens vermeld, waarbij een minimale en maximale waterconcentratie werden berekend.

Tabel C. **Omgerekende TBT waterconcentraties in de Oosterschelde op basis van concentraties in mosselen, *Mytilus edulis*, en BCF gegevens uit de literatuur.**

	BCF	BCF	Conc	Conc	Conc	Conc
	droog	nat	mossel	mossel	Water	Water
		(f=*0.18)	ngSn/g WW	ngSn/kg WW	ng Sn/l	ng TBT/l
min	1000	180	4.3	4300	0.40	0.97
max	60000	10811	12.6	12600	69.93	170.63

De range is breder (1-170 ngTBT/l) dan de voorspelde range op basis van wulken (10-80 ng TBT/l) en ontkracht die waarden dus zeker niet. Deze concentraties werden ook daadwerkelijk gerapporteerd voor de jachthaven Colijnsplaat in 1990-1992, waar gemiddelde waarden van 32-61 ng TBT/l werden gemeten. Deze jachthaven wordt weliswaar als (punt)bron van organotinverbindingen gezien, maar de concentraties zijn zeker in staat om de ontwikkeling van mannelijke geslachtsorganen te induceren in juveniele wulken. Concentratiemetingen in de waterfase in de gehele Oosterschelde zouden uitsluitsel kunnen geven over of de voorspelde TBT concentraties te hoog zijn.

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 het 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). Echter, bij 8.3 ngTBT/l is in deze studie al wel een negatief effect zichtbaar op de groei (en dus ontwikkeling) van de juveniele wulken. Toepassing van deze MTR zou betekenen, dat absoluut geen veiligheidsmarge wordt gehanteerd met betrekking tot de genoemde effekten. 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 aangaande effekten bij gastropoden lijken niet meegenomen in

de berekening van de voorgestelde MTR door Crommentuijn *et al.*. Aanbevolen wordt deze resultaten wel degelijk 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 *N. 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. 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 beschouwd kan worden als een groter probleem dan TBT in het zoete water. Gezien de hogere TPT dan TBT concentraties in de wulken van de Oosterschelde (deze studie) en van de Noordzee (Ten Hallers-Tjabbes & Van Hattum, 1995) geldt dit wellicht ook voor het zoute milieu.
Het beleid is momenteel sterk gefocused op TBT en mogelijkheden tot uitbanning van ervan. Uit het onderzoek komt naar voren dat dit terecht is. Maar het is niet uitgesloten dat andere stoffen, bijvoorbeeld TPT, ook een rol spelen bij de inductie van imposex in gastropoden. Indien uit een dosis-effect relatie zou blijken, dat TPT inderdaad imposex kan induceren, zou dit een reden zijn het toelatingsbeleid voor TPT te herzien.
Hoewel de resultaten van het verrichte histologisch onderzoek geen strukturele en functionele afwijkingen vertoonden bij vrouwtjes met de meest gevorderde stadia van imposex, kon met deze studie geen invloed op de fertiliteit van de vrouwtjes met imposex worden vastgesteld.

Aanbevelingen voor toekomstig onderzoek
Uit de gerapporteerde studies zijn 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, is het van essentieel belang de potentie van TPT inzake de gevoeligste effekten (met betrekking tot TBT) te bestuderen. Daarom is het nodig de rol van TPT bij het veroorzaken van imposex in gastropoden te bestuderen.
- De specifiek hoge TBT concentraties, die in de neurale ganglia (vergelijkbaar met hersenen) alleen in september 1995 werden waargenomen, moeten worden bevestigd. De rol in 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 alleen TBT selektief accumuleert, verklaart het wellicht, waarom andere organotinverbindingen geen rol spelen bij de imposex ontwikkeling.
- Organotinverbindingen in sedimenten waren, als gevolg van het zandige karakter ervan, niet of nauwelijks detecteerbaar. In de toekomst is het analyseren van alleen de fijne fractie niet alleen aan te bevelen, maar tevens noodzakelijk in verband met de normstelling welk voornamelijk op de waterbodems betrekking heeft.
- De in het laboratorium (al dan niet blootgesteld) opgegroeide wulken moeten gevolgd blijven worden, teneinde te zien of ze in staat zijn te reproduceren. Hierdoor kan duidelijk worden of TBT blootstelling een effect heeft op de vruchtbaarheid van deze diersoort.
- In een heel andere proefopzet zou de invloed van imposex op de fertiliteit van de vrouwelijke wulken en tevens op de staat van de wulkenpopulaties onderzocht moeten worden.
- De invloed van TBT op het cytochroom P450 enzymsysteem dient verder uitgezocht te worden voor meer inzicht in het fysiologisch mechanisme verantwoordelijk voor imposex.

EXECUTIVE SUMMARY

In this report the development and occurrence of male sexual characteristics (imposex) in the common whelk, *Buccinum undatum*, under the influence of (chronic) exposure to organotin compounds originating from antifouling paints (butyltin compounds) is described.
The main objective of this study was to obtain more information about the effects of organotin compounds on the marine environment, which possibly relates to the reported decline of *B. undatum* in Dutch coastal waters. Laboratory and field studies were conducted for that purpose. The only coastal area in the Netherlands where *B. undatum* is known to be present is the Eastern Scheldt. This area is likely to be contaminated by organotins from leisure boating activities (butyltins) and agricultural use (phenyltins). The development of male sexual characteristics in female gastropods (imposex), the most sensitive effect of exposure to tributyltin (TBT), was investigated in *B. undatum*. Because literature is not conclusive on the potency of triphenyltin (TPT) in developing imposex in gastropods, phenyltin compounds were also analysed in the field study. In the laboratory studies, a dose-dependent (cause-effect) relationship between TBT and imposex in *B. undatum* was investigated. Another goal of the laboratory experiments was to obtain more insight into the toxicological mechanism leading to imposex.

Field studies in the Eastern Scheldt (Chapter 2) resulted in a comprehensive overview on possible stages of imposex in adult common whelks. During several seasons in 1995, organotin concentrations were determined in common whelks, their food (mussels) and their environment (sediment).
In the laboratory studies a cause-effect relationship between exposure to tributyltin and the development of male sexual organs was established (Chapter 3). To study the toxicological mechanism leading to imposex, tissues of juvenile and adult whelks with and without imposex were histologically examined (Chapter 4) and a possible effect on the cytochrome P450 enzyme system of whelks was tested (Chapter 5). This enzyme system first had to be demonstrated in these marine snails.

Field studies
This part describes the studies into imposex (1992-1996) in the common whelk, *B. undatum*, and into the level of organotin contamination in the Eastern Scheldt during several seasons in 1995. Common whelks, mussels (*Mytilus edulis*) and sediment were therefore analysed for mono-, di- and tri butyltin and phenyltin compounds. In contrast to butyltin compounds, phenyltin compounds are primarily used in agriculture as nematocide.
Nearly all female common whelks (> 91%) from the Eastern Scheldt showed imposex, more than half of them the advanced stages. In May 1994, February and March 1995 egg masses with developing young whelks were observed, which indicates that reproduction takes place. Despite the ban in 1990 on the use of TBT based antifouling paints for ships < 25 m in length, percentages of imposex did not decrease in the Eastern Scheldt in the following years (Figure A).
In Table A the average organotin concentrations from 1995 are shown. Triphenyltin (TPT) was detected in all animal samples, in the common whelk 4-100 times higher than tributyltin (TBT) levels. No differences in butyltin and phenyltin levels were observed between the sexes, between females with and without imposex and between juveniles and adults. In total sediments the organotins were often below the level of detection. In future it is advised only to analyse the fine fraction (< 63 μm). Dibutyltin (DBT) and monobutyltin (MBT) levels in common whelks are higher than TBT as opposed to in mussels, where TBT shows the highest levels. This points to metabolism (dealkylation) of TBT in common whelks, biomagnification of butyltins seems therefore unlikely.

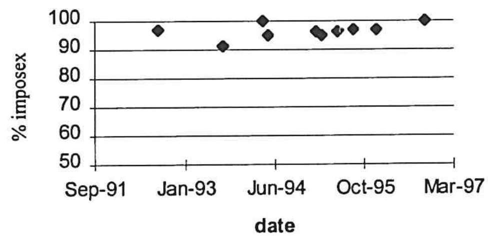


Figure A. **Percentages of imposex in the common whelk, *Buccinum undatum*, during five years in the Eastern Scheldt (Hammen).**

For phenyltins this can not be excluded, because levels are 4-10 times higher in common whelks compared to mussels and metabolism seems slow or little. TPT is the most important phenyltin in common whelks and in mussels. To determine organotin levels in common whelks, several organs were sampled and analysed seperately. In the digestive gland, organotin concentrations (wet weight) were always higher than in the foot of the animals. In the neural ganglia ('brain') in september 1995 unexpected high TBT concentrations were found, which could be important in view of the mechanism of induction of imposex. TBT might interfere with neuropeptides which could result in the development of a penis and a sperm duct.

Table A. **Average whole body organotin concentrations in common whelks (*Buccinum undatum*), mussels (*Mytilus edulis*) and sediment from the Eastern Scheldt in 1995.**

	month	TBT	DBT	MBT	TPT	DPT	MPT
				(ngSn/g wet weight)			
	Feb	2.40	4.63	3.17	20.3	13.3	1.90
whelk	June	1.14	11.9	5.99	27.0	3.34	5.91
	Sept	0.66	2.25	1.76	17.8	3.27	3.09
				(ngSn/g wet weight)			
	Feb	12	4.0	3.7	5.8	<8.5	0.3
mussel	June	4.3	16.7	4.2	1.95	0.45	0.38
	Sept	12.6	4.3	2.55	1.85	<0.6	<0.2
				(ngSn/kg dry weight)			
	Feb	0.8	<0.4	<1.1	0.4	<1.3	<0.4
sediment	June	1.15	1.3	3.4	<0.4	<0.8	<0.2
	Sept	0.2	<0.3	<0.8	<0.3	<1.1	<1.1

Laboratory studies

The first experiment was conducted to investigate a cause-effect relationship between TBT exposure and the development of male sexual organs. Adult and juvenile common whelks were exposed to study a possible difference in sensitivity concerning imposex for these life stages. Adult females did not develop male sexual characteristics despite the exposure to up to nominally 1 µg tributyltin acetate (TBTAc)/l for 11 months. However, their offspring showed a strong increased male development. After 8 months of exposure to a nominal concentration of 100 ngTBTAc/l (equivalent to 83 ngTBT/l), 54% of the juveniles showed male sexual development which increased over time to 100% after 14 months. All juveniles in the highest dose (nominally 1 µgTBTAc/l) group showed male sexual characteristics after 8 months. Moreover, by that time nearly all of them showed the most advanced stages of male sexual development. Some of these juveniles even developed a double penis. In the reference group and the lowest dose (nominally 10 ng TBTAc/l) group percentages with male sexual characteristics converged to 50% after two years of exposure (Figure B). This indicates that by then, sexual organs are basically present and will only grow after this period.

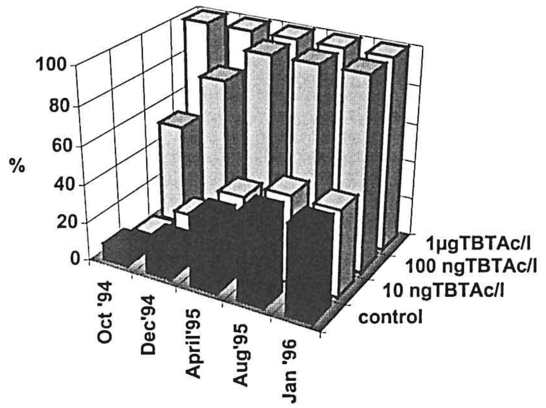


Figure B. **Sexual development, expressed as the percentage of juvenile *B. undatum* with male sexual characteristics after exposure to nominal TBTAc concentrations during the first two years of development.**

Although it is expected that 50% of the juveniles will eventually develop as males, exposure to nominal concentrations of 100 ng TBTAc/l clearly induced and accelerated the development of male sexual characteristics in a dose-dependent manner, when exposed *in ovo* and/or just after hatching. Because nominal concentrations were not reached in the experimental aquaria within the first year of exposure and effects were already observed after 8 months when measured (actual) TBT concentrations were below 20 ngTBTAc/l, an influence on the development of male sexual organs can be expected at concentrations of 17 ngTBT/l (equivalent to 7 ngSn/l).
Growth seemed to be reduced at an even lower dose than where sexual development was affected. Juveniles from the TBT exposed groups were significantly smaller than reference juveniles.
Body burdens of organotin concentrations were only determined in adult animals. A dose-dependent increase in all butyltin compounds was observed over time.
From these results concerning the effect of TBT on the development of male sexual characteristics, a No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) of 8.3 and 17 ngTBT/l respectively were deduced. However, a concentration of 8.3 ngTBT/l has an effect on the growth (and thus development) of these animals.

Table B. **NOEC and LOEC values for TBT and common whelks.**

LIFE STAGE	COMPOUND	CONC. ng/l	EFFECT NOEC	EFFECT LOEC	EXPOSURE TIME (months)
adult	TBT	830	imposex		9
juvenile	TBT	8.3	imposex		23
juvenile	TBT	17		imposex	8
juvenile	TBT	8.3		reduced growth	18

In the second experiment we investigated the effect of exposure to TBT during the developemnt *in ovo* on male sexual development. Four egg-masses were used; two were kept in Wadden Sea water and two were exposed to nominally 1 µg TBTAc/l for two months. When the first juveniles were about to emerge/emerged from the eggs, one of the two egg-masses was transferred to the other treatment. Transfer to and hatching in 1 µg TBTAc/l proved lethal for juveniles from both egg-masses; all juvenile common whelks died within several days after emergence. The juvenile whelks emerged and developed equally in Wadden Sea water. The group which has been exposed during the development *in ovo* showed a slightly higher percentage of animals with male sexual characteristics after 18 months, but this percentage was still < 50%. This shows the importance of aqueous exposure to TBT and the protection of the egg-mass in a heavily contaminated environment.
In the third experiment, two year old female common whelks were experimentally exposed for three months to Wadden Sea water or a nominal dose of 100 ngTBTAc/l. The aim of this study was to investigate an age dependent sensitivity for TBT. In both groups no male sexual development was observed in this short time span. Common whelks which have already formed their sexual organs seem less sensitive, but this needs to be investigated more thoroughly.

Studies into the mechanism of imposex
Because external characteristics give insufficient information about the health of the animal, adult and juvenile common whelks were sampled and fixed for (organ) structural and functional analysis.
Histological studies showed the difficulties in sexing the juveniles when they are smaller than about 4 cm. (2.5-3 years old). Gonadal development in juveniles was absent. Sterilisation due to the mechanical blockage of the female genital opening by sperm duct tissue was not observed in common whelks. Testis development in ovary tissue of females from the Eastern Scheldt (most advanced stages of imposex) was also not observed. An influence of imposex on the fertility of females could not be investigated in this study. No other sexual characteristics than those already visible with the eye were found.
The cytochrome P450 (CYP450) enzyme system is known in mammals and fish to be involved in the transformation of both endogenous (hormones) and exogenous (TBT) compounds. Therefore microsomal fractions of common whelks were analysed for the presence of this enzyme system and exposed to TBT to investigate a possible influence of TBT on this enzyme system. Microsomal fractions of dab, *Limanda limanda*, were used as a positive control, because in fish liver microsomes an inhibition of CYP450 by TBT was already observed. In the digestive gland and the kidney of common whelks CYP450 was present. In dab and the common whelk an inactivation of CYP450 was observed from the subsequent formation of CYP420 (inactive CYP450) after TBT addition. In dab a type 1 bindingspectrum was observed, which points to binding of TBT to the protein part of the CYP450 molecule in this species. In common whelks such binding was not observed. The TBT concentrations used were high, it is questionable whether these concentrations will occur *in vivo* in the organisms investigated. Further research should clearify the physiological meaning of this apparent inactivation.

Combination of studies
A combination of body burdens of adult common whelks from the laboratory study and from the field study, led to a (high) predicted TBT concentration range of 10-80 ng TBT/l in the Eastern Scheldt. On the basis of body burdens of mussels and bioconcentration factors (BCF's) for mussels reported in literature (Evers *et al.*, 1995), another prediction was made on prevailing TBT concentrations in the Eastern Scheldt. This resulted in a minimum-maximum concentration range of 1-170 ng TBT/l, which is wider than the prediction made from common whelks. In Table C results are listed with minimal and maximal values.

Table C. **Calculated TBT concentrations in the Eastern Scheldt on the basis of concentrations from mussels, *Mytilus edulis*, and BCF data from literature.**

	BCF dry	BCF wet (f=*0.18)	Conc mussel ngSn/g WW	Conc mussel ngSn/kg WW	Conc Water ng Sn/l	Conc Water ng TBT/l
min	1000	180	4.3	4300	0.40	0.97
max	60000	10811	12.6	12600	69.93	170.63

For the marina at Colijnsplaat during 1990-1992, average concentrations of 32-61 ngTBT/l were indeed reported. Although this marina is considered a point source for butyltin compounds, laboratory data show that these concentrations are well capable to induce male sexual development in juvenile common whelks. Analysis of water samples (further) away from an assumed source will give more insight into the TBT distribution in and contamination of the Eastern Scheldt.

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 (Maximal Permissible Risk concentration) seems to be a realistic maximum level for the common whelk. In our studies, a concentration of around 17 ngTBT/l caused an induction of the development of male sexual characteristics, which is very close to the MTR (11 ngTBT/l). A concentration of 8.3 ngTBT/l results, however, in a reduced growth (and development) in juvenile common whelks. Thus, application of this value means there is no safety margin at all with respect to the induction of the effects mentioned in common whelks. However, literature data show that this level will certainly cause imposex in the dogwhelk. For the dogwhelk levels of 2-3 ngTBT/l are already responsible for this masculinisation effect. These results from gastropods are not taken into account in determining the proposed MTR and it 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, because of the absence of dose-effect studies.

On the other hand, research of Stäb *et al.*(1996) showed that TPT is present in all fresh water systems in the Netherlands in concentrations usually exceeding those of TBT, except in areas of high boating activity. This indicates that TPT may even be considered a bigger problem than TBT in fresh water systems in the Netherlands. Considering the TPT levels which are higher than TBT levels in common whelks from the Eastern Scheldt (this study) and from the North Sea (Ten Hallers-Tjabbes & van Hattum, 1995), this may also count for the marine environment.

At present policy is primarily focused on phasing out TBT. From these studies this seems correct, but it can not be excluded that other compounds, possibly TPT, can also induce imposex in gastropods. If it is shown from laboratory studies that TPT indeed induces imposex, this would justify a revision of the acceptance of this organotin.

Although results from the histological study showed no structural and functional aberrations in female sexual organs from females with imposex, an influence of TBT on the fertility of these females could not be studied.

Future research

From the reported studies the following suggestions are made :

- In order to determine whether the proposed MTR level for TPT of 5 ng/l is realistic, 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 1995, needs confirmation. Its role in imposex development, possibly as an agent which interferes with the regulation of neurohormones 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 proces of imposex development.
- Due to the sandy nature of the sediment in the Eastern Scheldt, organotins were mostly below their detection limit. To investigate organotin contamination in sediments in future, analysing the small fraction is not only recommended, but even necessary in view of policy making which relates to sediments.
- Juveniles which hatched in the laboratory and continue to develop should be followed in their development to study their ability to reproduce. This way, more information is obtained about the ability of TBT to affect the fertility of common whelks.
- A new experimental set up is necessary to study the effect of imposex on the fertility of adult female whelks and on the status of populations of common whelks.
- To study the physiological mechanism responsible for imposex development more attention should be paid to the influence of TBT on the cytochrome P450 enzyme system.

CHAPTER 1

GENERAL INTRODUCTION

Marine pollution by organotin compounds has become a matter 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 in the marine environment 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] 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 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.
[* TBT concentrations expressed as ng $Sn.l^{-1}$ can be converted to TBT by multiplying by 2.44].

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 potato culture, which is an important economic sector in the area surrounding the Eastern Scheldt. Several years ago, *N. lapillus* from this area showed a high percentage of imposex and a number of females were sterile. Based on the severity of the imposex stages observed, they were expected to become extinct in this area (Mertens & van Zwol, 1988).

CHAPTER 2

ASSESSMENT AND OCCURRENCE OF IMPOSEX IN THE COMMON WHELK, *BUCCINUM UNDATUM*, AND LEVELS OF ORGANOTIN COMPOUNDS IN THE EASTERN SCHELDT

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 such as *N. lapillus* and *H. reticulata* (Bryan & Gibbs, 1988 and Stroben, 1994).
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. 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. In the same area where *Nucella* was affected by imposex, oysters showed shell deformaties, also caused by butyltin compounds (van Zwol & Mertens, 1988).
The common whelk, *Buccinum undatum*, is a benthic predator that lives subtidally at the seabed. It preys predominantly on live benthic bivalve molluscs and (fresh) 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.*, 1996). 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).
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 cruises in 1995, samples were taken for the determination of tissue concentrations of butyltin and phenyltin compounds in whelks, their food (mussels) and their habitat (sediment).

2. 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 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 a longer cruise (several days) allowed to study more individuals and to prepare samples for tissue analyses of butyltin and phenyltin compounds.

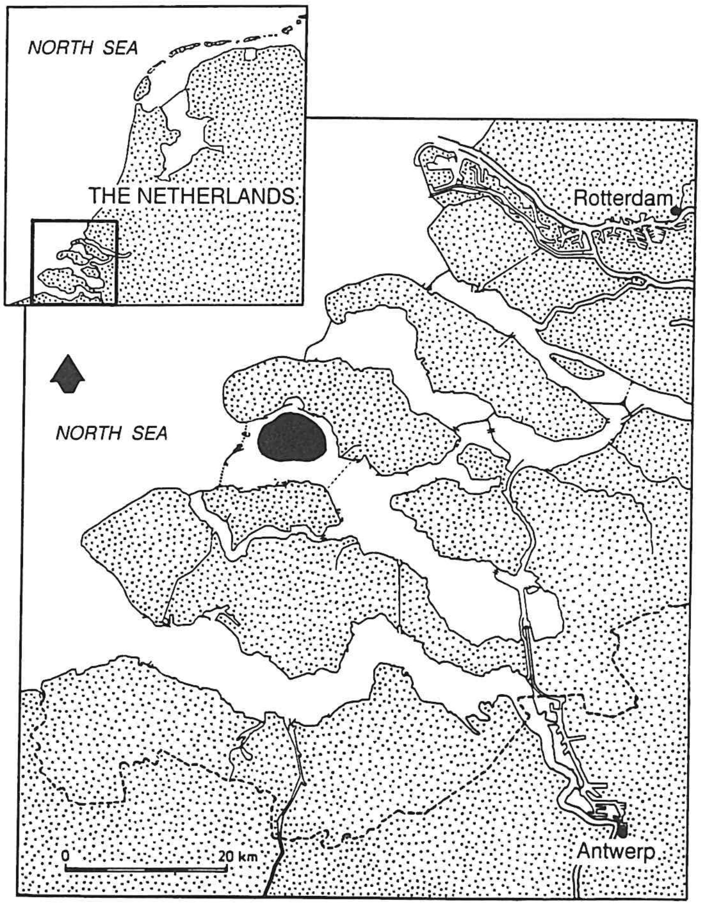


Figure 1. Sampling location in the Eastern Scheldt.

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.2).
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.

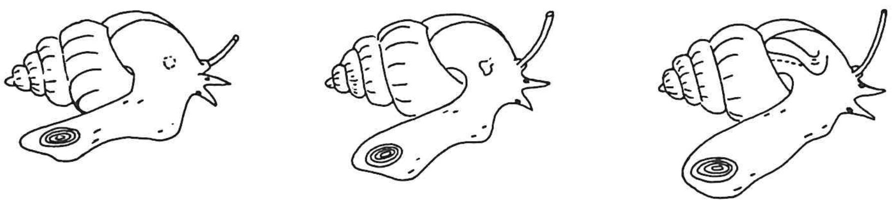


Figure 2. Determination of imposex stages in female *Buccinum undatum*.

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.

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 trajectory where the whelks were collected. Five samples were homogenized and divided into two subsamples. *M. edulis* were also caught in the sampling area and were not collected from commercially fished musselbeds. Mussels were pooled before analysis, n = 5 in February 1995, in June and September 1995 twelve mussels were pooled before homogenisation and analysis.

b. Analysis of organotin compounds

Organotin concentrations in *B. undatum* and *M. edulis* tissue were 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 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 with 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 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.

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)

3. 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, three in March, five in June and September 1995, three in January 1996 but none in October 1996 (Table 2). 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 (Fig.3). The penis homologue of such animals was always among the largest observed in these pseudohermaphrodites. In 1995 and 1996 the percentage of females with a large penis (about 2 cm) and some with vas deferens was higher than 55% (Table 3). Also females with abnormal penis structures were observed, some even with a double penis. 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. No occluded genital pores were found.

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

Table 2. Number of investigated *B. undatum*, male: female ratio, average length and imposex frequency in the Eastern Scheldt.

month	number of individuals	average length (cm) ± SD	males (n)	females (n)	m/f ratio	imposex %
September 1992	63	7.56 ± 0.45	34	29	1.2	97
September 1993	56	7.45 ± 0.85	45	11	4.1	91
April 1994	17	6.78 ± 0.68	12	5	2.4	100
May 1994	74	7.03 ± 0.58	53	21	2.5	95
February 1995	220	6.76 ± 0.89	114	106	1.1	96
March 1995	101	7.18 ± 0.59	46	55	0.84	95
June 1995	208	7.02 ± 1.00	94	114	0.82	96
September 1995	345	6.66 ± 1.26	178	167	1.1	97
January 1996	222	7.20 ± 0.58	132	90	1.5	97
October 1996	64	7.25 ± 0.73	35	29	1.2	100

Table 3. Stages of imposex development in female *B. undatum* in 1995 & 1996 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 1995	106		4	12	17	2	39	26
March 1995	55		5	4	29	0	49	13
June 1995	114		4	6	22	3	41	25
September 1995	167		3	24	17	0.6	39	16
January 1996	90		3	4	16	0	69	8
October 1996	29		0	21	14	7	24	34

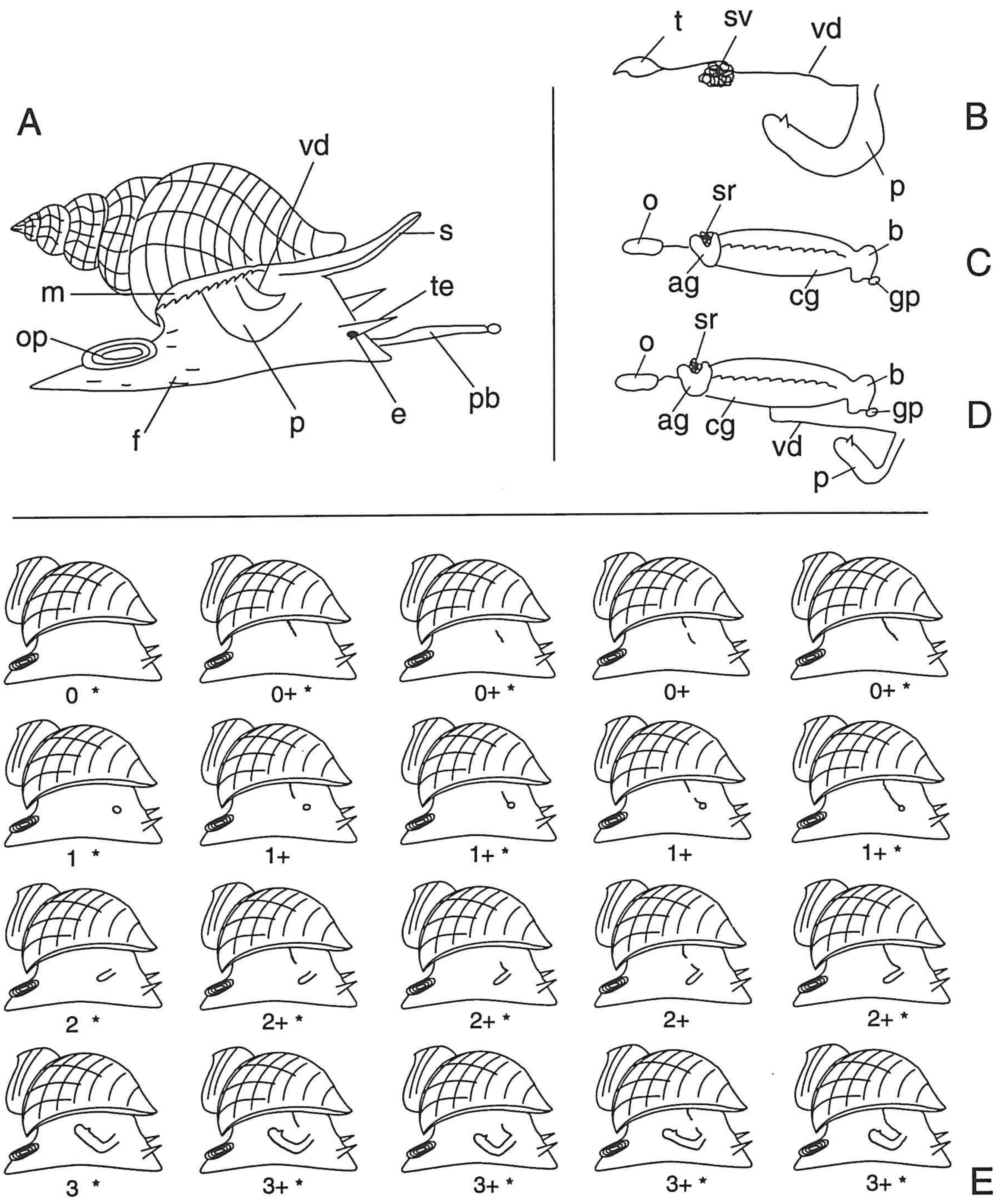


Figure 3. **A**: Normal male *B. undatum*, **B**: Simplified genital duct of a male *B. undatum,* **C**: Simplified genital duct of a normal female *B. undatum*, **D**: Simplified genital duct of an imposex female *B. undatum* stage 3+. Abbreviations : m = mantle, op = operculum, f = foot, p = penis, e = eye, te = tentacle, pb = proboscis, s = siphon, vd = vas deferens, t = testis, sv = seminal vesicle, o = ovary, sr = seminal receptacle, ag = albumin gland, cg = capsule gland, b = bursa copulatrix, gp = genital pore, **E**: Scheme of possible imposex stages for *B. undatum*. The observed stages from field and laboratory studies are indicated with *.

Organotin concentrations

Tissue organotin concentrations are given in Tables 4, 5 and 6. The digestive gland and the foot of some female *B. undatum* were analysed 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 1995, 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.), the highest concentrations are found in the digestive gland of the animals (up to 250 Sn ng/g wet wt.) in February 1995.

In September 1995 whole body organotin concentrations seemed to have dropped compared to February and June (Table 4b). 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 (1995), 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 of these animals are different, the disposition of these xenobiotic compounds is very much alike.

Table 4a. **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 4b. **Average (± SD) whole body organotin concentrations (Sn ng/g wet weight) of *B. undatum* per month in 1995.**

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*

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).

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

Table 5. **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 6. **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*

4. DISCUSSION

In this study we investigated the imposex incidence and organotin contamination 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.

The imposex incidence reported here is high (> 90%), although ten Hallers-Tjabbes *et al.* (1994) found an imposex incidence of 100 % (n = 6 females) in the North Sea in 1991 at a location directly north of the 'Euro Channel', 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 %. Other cases of imposex in *B. undatum* are reported for the White Sea in the area of the Solevetski isles, where 15 out of 19 females showed penis homologues (Kantor, 1984). A very recent study in the North Sea and Irish Sea also showed imposex in *B. undatum* even at stations at the open sea (Ide *et al.*, 1996).
Although in 1993 (n=56) and 1994 (n=91) the male to female ratio is higher than 1, results from 1992 (n=63), 1995 (n=874) and 1996 (n=286) did not show a real 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.*, 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 and consequently result in the presence of more males than females. In the Eastern Scheldt we have observed many 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.
The known stages of imposex in *B. undatum* are shown in figure 3. 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 ≥ 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*. Ide *et al.* (1996) showed a number of possible stages for imposex development in *B. undatum*, but this scheme is not complete. In our studies we found also stage 3c (according to Stroben *et al.*, 1992), where the vas deferens is not complete, whilst a penis with penis duct is already present. Ide *et al.* (1996) do find stage 4+ (not sterile) to be the final stage, which is in accordance with our findings.
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.

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. This was also found in the study of Ide *et al.* (1996), where TBT concentrations did not correlate with the imposex stage.
In the present study, 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 1995 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 exclusively for TBT (no other organotin compound is enhanced) seemed to occur only at the start of the reproductive period, strongly indicating 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/different 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), whereas no other 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.
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 water phase, but always at lower concentrations than TBT. Also spring peaks 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.
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

- The percentage of females showing imposex was high (> 91%) and adult as well as juvenile females showed male sexual characteristics. This did not change throughout the years after the ban (1990) on the use of TBT containing anti-fouling paints on ships < 25m length.
- 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.
- 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 compared to those in the common whelk.
- Phenyltin whole body concentrations, however, were 5-10 fold lower than 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.

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. The active biocide in anti-fouling paints, tributyltin (TBT), was expected to be the cause, since a correlation with shipping intensities was established (Ten Hallers-Tjabbes et al., 1994). 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 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 in three experiments. In the first experiment common whelks were exposed to three different TBT doses. Adult *B. undatum* reproduced and their developing juveniles (*in ovo*) were also exposed to test whether life-stage is an important parameter with respect to imposex development. Animals did not have to be sacrificed, since whelks crawl out of their shell when exposed to air and therefore the developmental stages of male sexual characteristics could be recorded over time nicely.

A second experiment was conducted to test whether exposure to a high TBT concentration *in ovo* already has an influence on the development of juvenile whelks.

To test whether sensitivity towards TBT exposure differs in different life stages, two year old female whelks were also exposed in a third experiment.

2. METHODS

Origin of animals

Experiment 1

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.

In every experimental tank during the acclimation period, adult females produced egg masses in all groups. In February 1994, two months after the start of the experiment (TBT administration) and the production of egg-masses by the adult whelks, the young whelks hatched.

Experiment 2

The egg-masses used in this *in ovo* experiment were produced by female whelks from the Dogger Bank, collected in September 1994.

Experiment 3

Other not experimentally used adults from *Experiment 1* also produced egg-masses in storage tanks and their juveniles were reared under the same physical and nutritional conditions as described below for the experiments (except now the aquaria only received Wadden Sea water) for two years before they were used in the exposure experiment.

Experimental conditions

Experiment 1

TBT was administered to the experimental aquaria by dissolving tributyltinacetate (TBTAc, > 97.5% purity, Aldrich) in demineralised water (stock solutions); these solutions were diluted 1 : 100 with Wadden Sea water 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 the second week of December 1993.

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.

Experiment 2

Two egg masses were exposed for two months (December 1994-February 1995) 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. When the first juvenile whelks emerged from the egg-masses a translocation was done. One egg-mass of the reference group was transferred to the high TBT dose group and an egg-mass of the high TBT dosed group was transferred to the reference group.

Experiment 3

In July 1996, two and a half year old females from laboratory reared common whelks 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.

In all experiments, a waterflow of 80 l/day per aquarium, a 12h light-12h darkness regime and an air temperature of 12°C was applied. 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 from the first experiment 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 divided by 1.20

3. RESULTS

Experiment1

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).

In contrast to the adult specimens, their offspring showed marked differences in sexual development (fig.4), when exposed in the same manner as their parents. . The juveniles from the control group and the low dose (10 ng TBTAc/l) group showed comparable penis development. After two years of exposure the male : female ratio (based on the animals with male sexual characteristics) converged to 1. This points to a life stage where the sexual organs are basically present and only a further growth of these organs will occur in the future (figure 5a-d).

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
TBT	control	<2*, <2	<2, <2			<2
	10				<2, <2, <2	4, <2, <2, <2, <2
	100	<2	<2, <2		<2	3, <2
	1000	<2, 6	8	32		140, 107, 68
DBT	control	<1, <1	<1, <1			6
	10				<1, <1, 8	8, 8, <1, <1, <1
	100	<1	<1, <1		<1	25, 22
	1000	<1, 14	26	95		503, 536, 509
MBT	control	<1, <1	<1, <1			3
	10				2, 3, <1	6, <1, <1, <1, <1
	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.

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. 4c). After 8 months, when actual TBT concentrations had not exceeded 20 ngTBTAc/l (fig. 8c), 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*.

After 8 months, the highest dose (1 µg TBTAc/l) showed virtually only fully developed stages of penises 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 some of 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*.

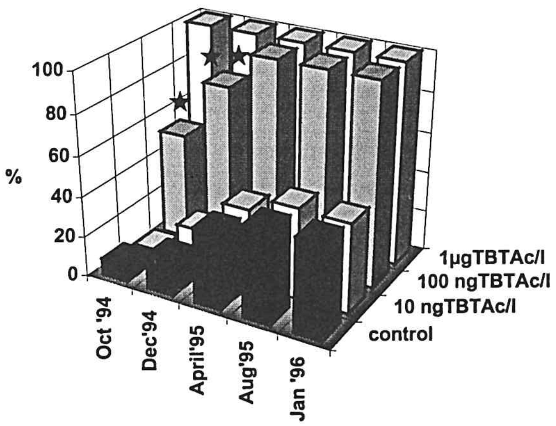


Figure 4. Sexual development, expressed as the percentage with male sexual characteristics, of juvenile *B. undatum* during the first two years.
* *means significantly higher than the control group (P < 0.05).*
** *means significantly higher than the control group (P = 0.001)*

One-way ANOVA on the percentages of juveniles 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 too (figures 6 & 7).

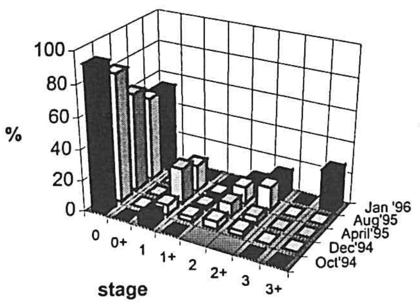


Fig. 5a. Control group.

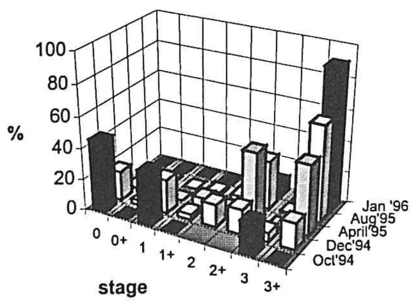


Fig. 5c. Dose : 100 ng TBTAc/l.

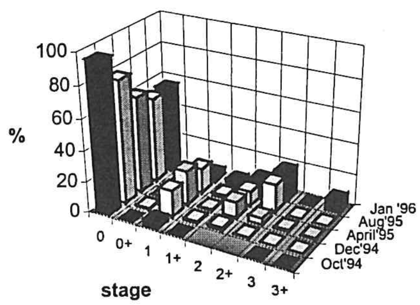


Fig. 5b. Dose : 10 ng TBTAc/l

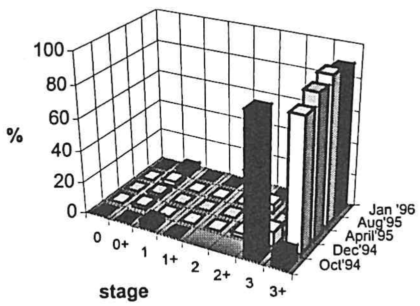


Fig. 5d. Dose : 1 μg TBTAc/l

Figure 5a-d. Stages of the development of male sexual characteristics in juvenile *B. undatum* for different TBT doses during the first two years.

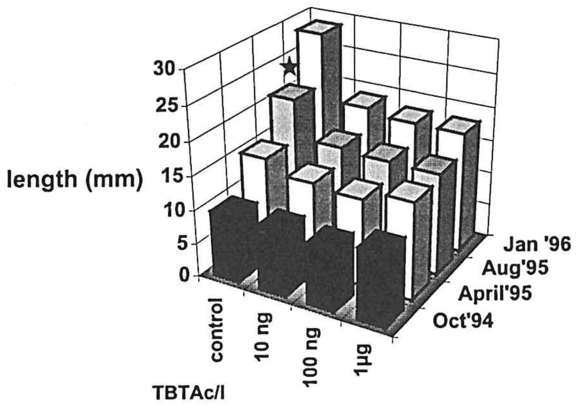


Figure 6. Average length for the experimental groups.

** means significantly higher than TBT exposed groups (P< 0.001).*

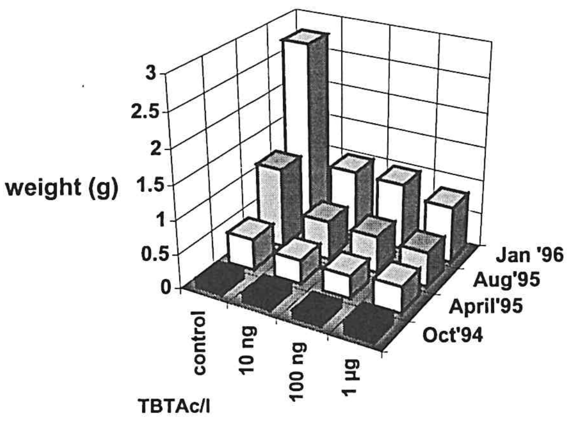


Figure 7. Average weight for the experimental groups.

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 $ng.l^{-1}$) levels of TBT. During the experiment, these concentrations reached TBT levels equivalent to 30 ng $TBTAc.l^{-1}$ at the most for the control group and 40 ng $TBTAc.l^{-1}$ for the low dose group (fig. 8a,b). The concentrations in the medium dose group ranged from below detection limit to 20 ng $TBTAc.l^{-1}$ in the first 40 weeks to 40 $ng.l^{-1}$ after 11 months. After one year concentrations increased to the nominal dose (fig. 8c). In the highest dose actual concentrations ranged from 35 to about 2000 ng $TBTAc.l^{-1}$(fig. 8d). The apparent TBT, DBT and MBT 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).

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 9a-d, actual DBT concentrations are given. With increasing TBT doses, DBT and MBT (fig.10a-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.

After two years of exposure 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.

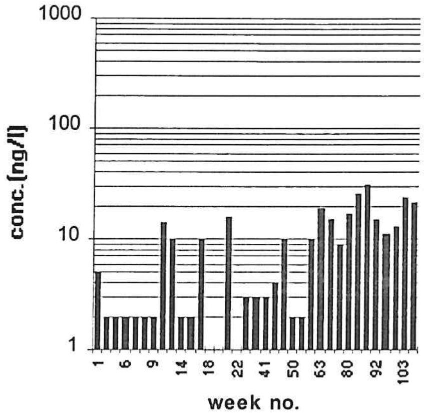


Fig. 8a. Control group

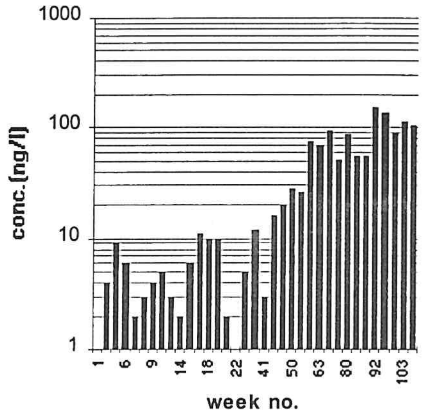


Fig. 8c. Dose : 100 ng TBTAc/l

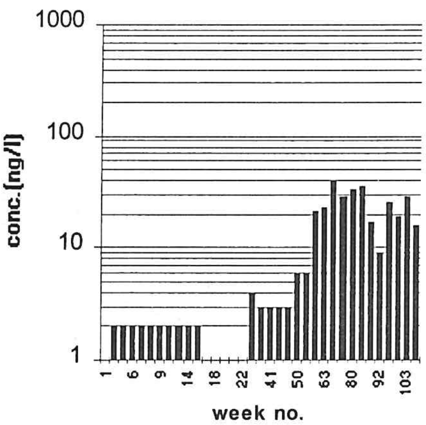


Fig. 8b. Dose : 10 ng TBTAc/l

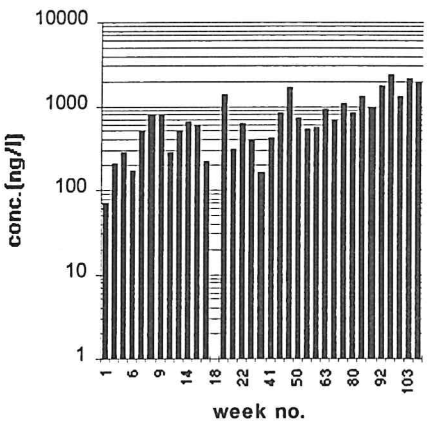


Fig. 8d. Dose : 1 µg TBTAc/l

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

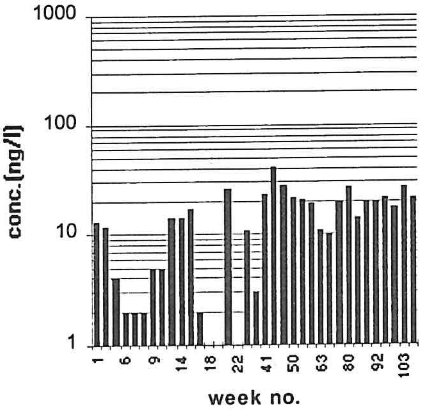


Fig. 9a. Control group

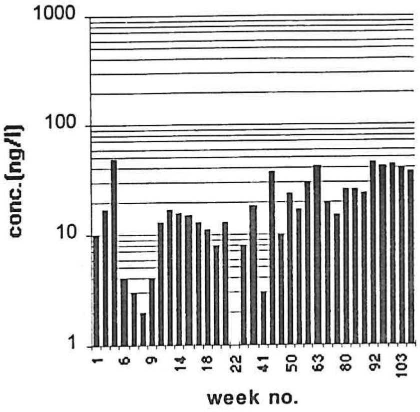


Fig. 9c. Dose : 100 ng TBTAc/l

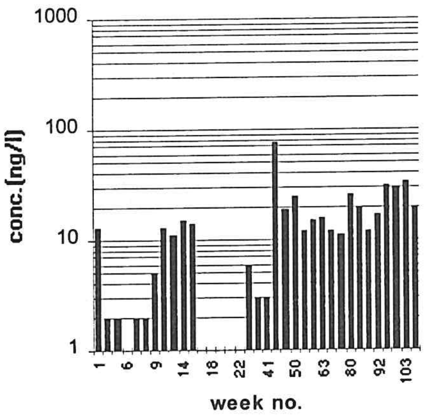


Fig. 9b. Dose : 10 ng TBTAc/l

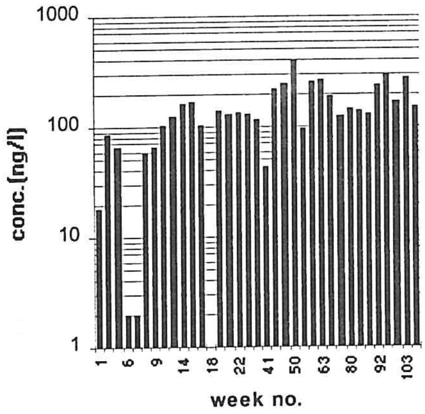


Fig. 9d. Dose : 1 µg TBTAc/l

Figure 9a-d. Actual DBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

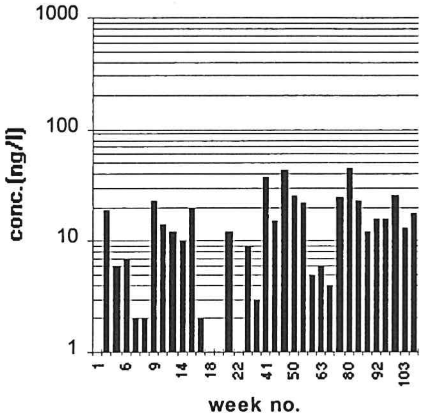


Fig. 10a. Control group

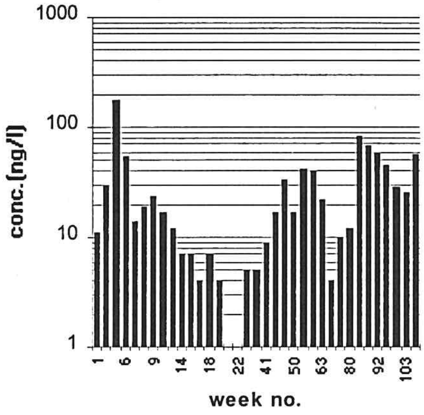


Fig. 10c. Dose : 100 ng TBTAc/l

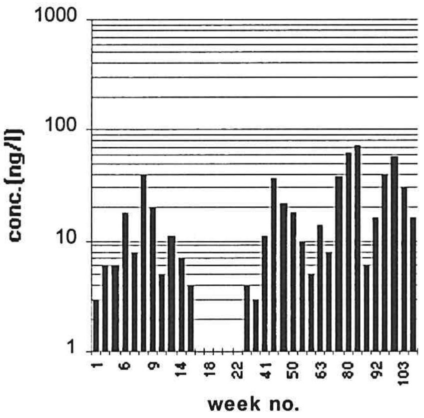


Fig. 10b. Dose : 10 ng TBTAc/l

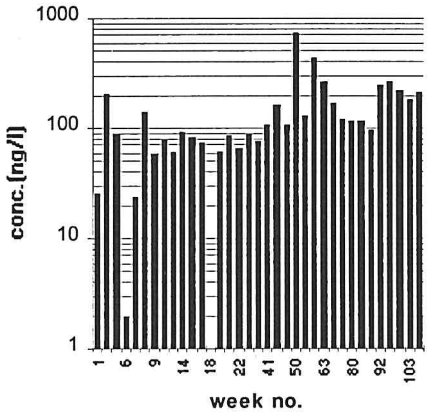


Fig. 10d. Dose : 1 µg TBTAc/l

Figure 10a-d. Actual MBT concentrations (in ng/l) for the TBT exposed groups during the experimental period.

Experiment 2

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

The juveniles (n =31) from the egg-mass which had been transferred to the reference treatment developed normally compared to the juveniles (n = 64) from the reference group, except there was a slightly higher percentage of animals with male sexual characteristics in the *in ovo* exposed group (figures 11 & 12) during all examinations. Mortality in the two groups was equal (6.5%).

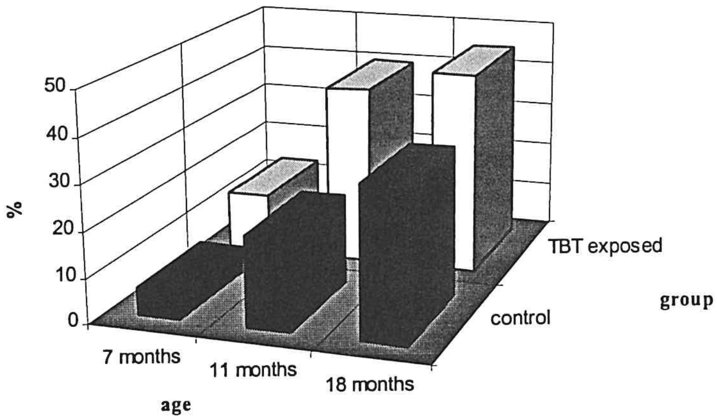


Figure 11. Percentage of juveniles with male sexual characteristics

The juveniles exposed *in ovo* were on average always the largest and heaviest, but the differences do not seem statistically different (Fig.13).

At all moments of sampling an increase in the percentage of animals with male sexual characteristics was observed in both groups. The last sampling date, when the animals were 1.5 years 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 seems no evidence for a substantial masculinization effect due to exposure to a high TBT (720-1800 ng TBTAc/l) dose *in ovo*, nor an effect on the development of these animals.

Experiment 3

In this experiment no induction of imposex could be observed over a 3 months period of TBT exposure (nominal concentration 100 ngTBTAc/l). 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. The control group showed a slightly higher increase in length (Δl control = 2.00 mm), whilst the TBT exposed group only increased 0.88 mm in length. The difference in average length is not significant. Mortality in the control group and in the TBT exposed group were 10% and 3% respectively.

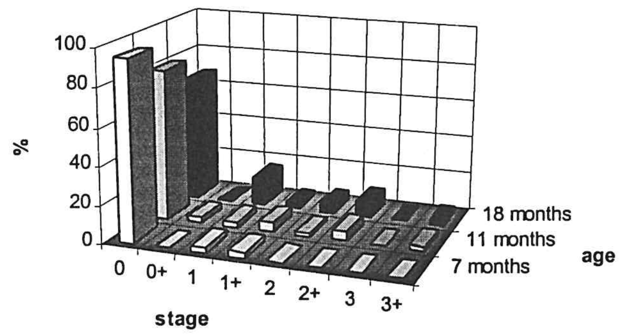


Figure 12a. Sexual development in the reference group.

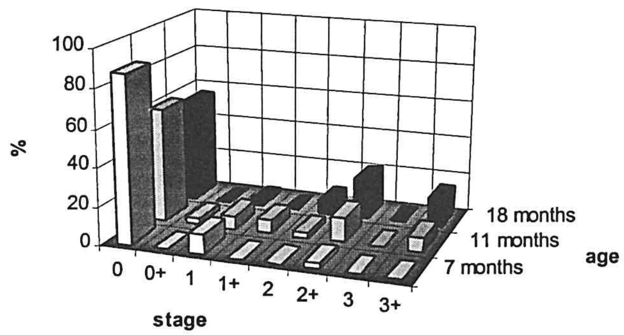


Figure 12b. Sexual development in the *in ovo* TBT exposed group.

Figure 12 a-b. Stages of the development of male sexual characteristics in juvenile *B. undatum* for different treated groups *in ovo*.

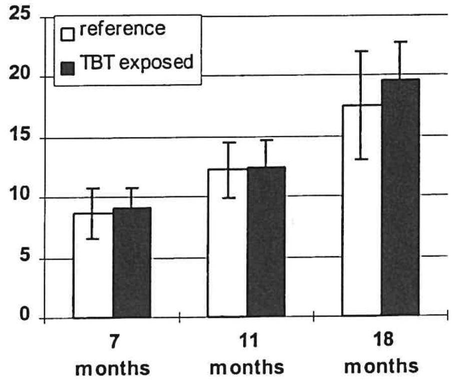


Figure 13. Average length in mm (± SD) for the two groups juvenile *B. undatum*.

4. DISCUSSION

Experiment 1

In this study 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 agent which interferes in this development 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. 7c.).

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

The exposure to TBT also seems to affect the growth of juveniles. A significantly lesser 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.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.
[*concentrations expressed as ng TBTAc/l and ng Sn/l are converted to ng TBT/l according to the calculation factors given in **Methods**].

In the adult common whelks, 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.
Based on the physical-chemical properties of the butyltins concerned, theoretically one would expect TBT to show the highest body burdens. In water concentrations of TBT>DBT≥MBT and the $\log K_{ow}$ value for TBT is two orders of magnitude higher than the $\log K_{ow}$ value for DBT, which consequently should result in a much higher TBT body burden 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 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 respectively. 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. The concentrations observed 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 there is dredging activity (3 times/year), which very likely reduces the TBT concentration in the water phase (Ritsema, 1994). These levels will certainly cause a masculinisation effect in developing juvenile whelks.

Experiment 2
In this 1.5 year 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, a TBT dose of 1000 ngTBTAc/l when animals just emerge from the eggs proved lethal irrespective (of a possible exposure during) the development within 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 *Experiment 1* (47%)

after the same experimental period. The difference in percentage of juveniles with male sexual characteristics between the two groups observed in this second experiment, can be considered to be a variation between egg masses. In *Experiment 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 when juveniles emerge from the eggs, where they were 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 emerged 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 or little imposex occurs. Disposition of TBT in these egg-masses seems therefore unlikely.

Experiment 3
In this experiment adolescent female common whelks were exposed to nominal concentrations of 100 ngTBTAc/l. During the 3 months of exposure no development of male sexual characteristics was observed. It is possible, that actual concentrations were not high enough to induce imposex in *B. undatum*, but a nominal concentration of 100 ng TBTAc/l certainly induces imposex already in 1 or 2 months in *Nucella lapillus* (Bryan *et al.*, 1988). It is more likely that these adolescent females are not as sensitive as in the juvenile stage where all sexual organs still had to be developed. It should be kept in mind that the exposure time has been only three months.
Although not substantial, a slightly decreased growth was observed in the exposed group compared to the control group. A longer exposure time might indicate whether this is an exposure (TBT) effect.

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* and just after hatching.
- Exposure to nominal concentrations ≥ 10 ng TBTAc/l lead to reduced growth in juvenile common whelks.
- Adults seem to be 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.
- 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 emergence from the eggs. The egg environment can therefore be considered a protective environment.
- Exposure for three months to a nominal concentration of 100 ngTBTAc/l does not cause imposex in adolescent (2.5 years old) female common whelks.

CHAPTER 4

HISTOLOGICAL STUDY OF THE REPRODUCTIVE ORGANS OF THE COMMON WHELK.

1. INTRODUCTION

Exposure to tributyltin causes imposex in many gastropods all over the world. However, responses of species can be different and will also depend on the prevailing TBT concentration. In *Nucella lapillus* structural abnormalities lead to sterility due to a mechanical blockage of the genital pore. Also, oogenesis was suppressed at TBT levels above 3-5 ng Sn/l to be supplanted by spermatogenesis leading to sperm production (Gibbs *et al.*, 1988). In *Hinia reticulata* imposex does not lead to sterility, even when a penis and a sperm duct are completely developed. The sperm duct does not block the vaginal opening, and thus copulation and disposition of egg masses is still possible (Stroben *et al.*, 1992).
In the common whelk, *Buccinum undatum*, no mechanical sterilisation has been found either. The most advanced stage of imposex development is represented by the presence of a penis and a sperm duct which ends in the capsule gland. Thus, the male sexual characteristics do not lead to a mechanical sterilisation as in *N. lapillus*.
The goal of the present study (chapter) was to investigate the development of imposex phenomena and their influence on the reproductive status in the common whelk in detail using histological techniques.

2. MATERIALS AND METHODS

Origin of the animals
Field samples
Adult females (>6 cm), adolescent (4-6 cm) whelks and juveniles (3-4 cm) from the Dogger Bank (North Sea) were used as reference animals. Here, no imposex had been found during former cruises. Females showing imposex were collected at the Eastern Scheldt (Hammen). In this coastal area, more than 90 % of the females show imposex of which over 50% show the more advanced stages of imposex.

Experimentally exposed animals
Juveniles (1-4 cm) from the reference group and exposed groups of *Experiment 1* (previous chapter) were used to determine the development of genital ducts and thus their sexe. These juveniles were 2 years old when used in this histological study.
The adolescent animals were used as a transitional stage where all reproductive organs were present but not fully developed/matured. This facilitated the interpretation of tissue structures of juveniles.
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 the adult and adolescent animals, only the reproductive organs were investigated. The juveniles were sampled as a whole. Tissues were fixed in Bouin's fluid. Dehydration was performed using increasing concentrations of ethanol, isopropanol and xylene. Dehydrated samples were embedded 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, cat.no. 1.07961). Sections were examined using a Carl Zeiss microscope (nr. 65266).

3. RESULTS

Although two juvenile whelks from the North Sea showed initial penis formation, no gonadal differentiation could be observed. Development of the seminal vesicle was already in progress, as the presence and development of a convoluted duct in the inner curve of the digestive gland clearly indicates. There was a clear *vas deferens* development, which ranged 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* did not differ between the exposure groups with respect to the structures observed. Reproductive organs other than penis and *vas deferens* could not be observed. In the highest dose group abnormal penises were characterised by either an additional penis formation, which was similar in structure and tissue as the single penis or by an extra tissue formation on or close to the base of the penis. Sometimes excrescences on penis tissue were found. Differences in penis size for juvenile males or juvenile females with imposex could not be observed. No additional sexual characteristics have been discovered than those already macroscopically visible and used for classification (Fig. 14a-d).

The studied adolescent whelks had partially developed their gonads. Some females as well as males showed immature gonads which could be recognised from immature oocytes and the highly proliferative testis tissue. The developing gonads were small compared to the much greater digestive gland. In males a seminal vesicle is present, but the size is much smaller than in adult males and the lumen is still 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 fertilised.
In these adolescent female whelks, the absence of male sexual characteristics was histologically confirmed. No penis growth nor sperm duct development could be observed.
The ovaries of adult females 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. 15a+b). Testis tissue development could not be observed even in females with the most advanced imposex stage (3+) from the Eastern Scheldt.

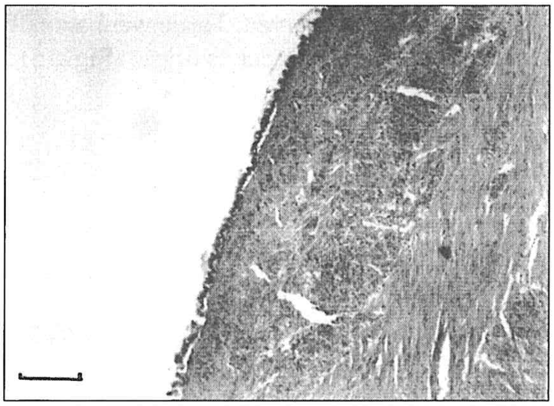


Figure 14a. Imposex stage 0

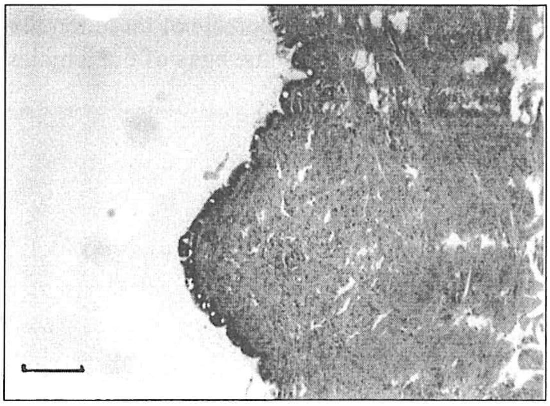


Figure 14b. Imposex stage 1

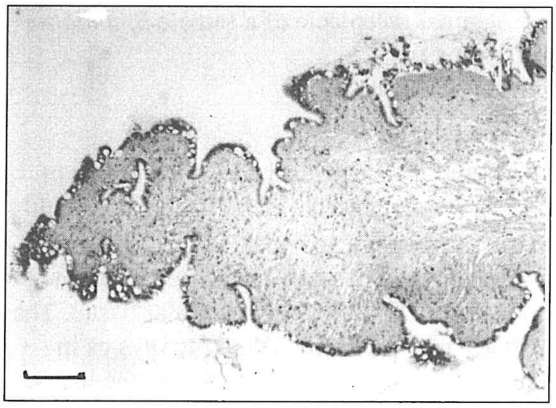


Figure 14c. Imposex stage 2

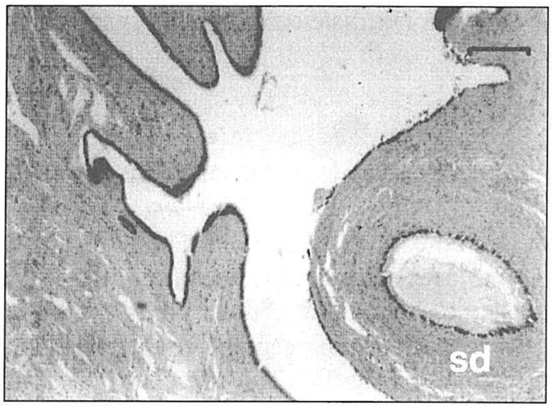


Figure 14d. Imposex stage 3+

Figure 14a-d. Imposex stages of *Buccinum undatum*. — = 40 μm.

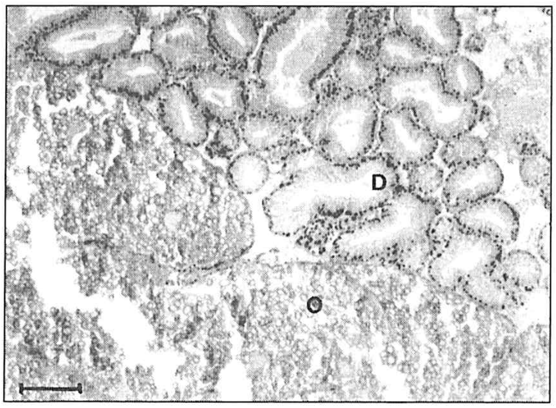


Figure 15a. Ovary (O) and digestive gland (D) of a female from the North Sea. — = 40 μm.

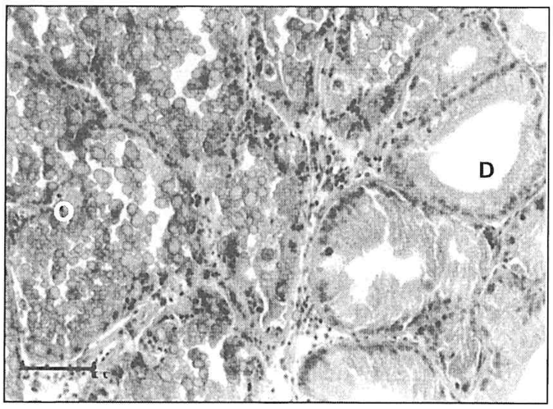


Figure 15b. Ovary (O) and digestive gland (D) of a female with imposex stage 3+ from the Eastern Scheldt. — = 40 μm.

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. 16).

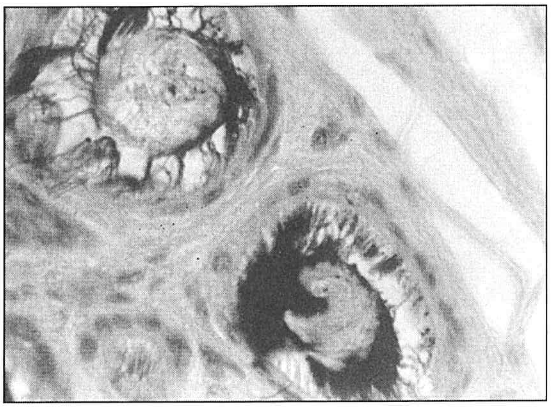


Figure 16. Fertilisation of eggs by spermatoza in the seminal receptacle of a female *B. undatum* suffering from imposex stage 3+.

Clearly, these females were not sterile although they suffered from the most advanced stages of imposex known so far for *Buccinum undatum*. Whether imposex caused a *reduced* fertility could not be studied in these sections. Following the sperm duct from penis to capsule gland in females with imposex stage 3+, the end of the duct in the capsule gland could not be observed. It seemed to stop at the ventral mid section of the egg capsule gland without invading the glandular tissue. The sperm duct in imposex females consists of the same tissues and functional cell structures as in normal males (Fig. 17). The duct is covered with normal skin tissue on the outside; on the inside there is a thick layer of muscle tissue. Within this tissue there is a lumen covered with glandular cells. The sperm duct develops either unidirectional from the penis site towards the egg capsule gland, or synchronously from the egg capsule gland towards the penis homologue and from the penis towards the egg capsule gland, leaving a gap between the two approaching parts. The penis of females is always smaller than that of males but consists of the same elements and tissues. A penis duct with ciliated epithelium and subterminal papilla are normally present.

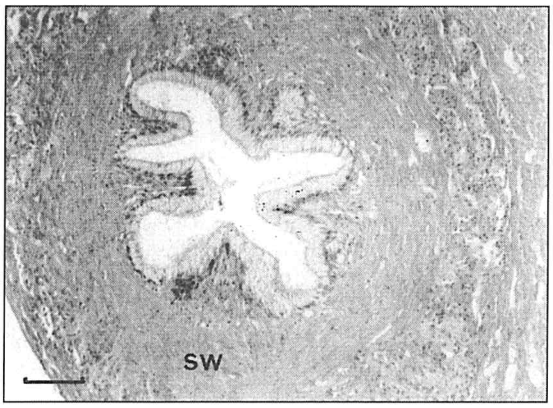


Figure 17. Section of the sperm duct of a normal male.
— = 40 µm. SW = muscle tissue

4. DISCUSSION

Histological data showed that it was virtually impossible to discriminate for sex in juvenile common whelks, because after two years no primary sexual characteristics (gonads) had been developed, despite the complete formation of a penis and *vas deferens*.
Adolescent animals from the North Sea (4-6 cm) showed the 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 be reproductively successful.
So far, few histological data on developing young *B. undatum* or other prosobranchs have been published, which makes comparison very difficult. 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 even ceased and even testis tissue could be distinguished (Gibbs *et al.*, 1988).
Although in the Eastern Scheldt a high percentage of female common whelks (>90%) suffers from imposex, the highest stages of imposex do not mechanically sterilise 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 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 in the Eastern Scheldt area.
Stroben *et al.*(1992) have given 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.
However, it should be kept in mind that a possible gradual decrease of the fertility of common whelks could not be established by histological methods.

5. CONCLUSIONS

- Common whelks could not be sexed on histological grounds when they were 2 years old, since besides a penis and vas deferens, no other reproductive organs had been developed. Development of the penis and *vas deferens* can therefore not be controlled by the gonads.
- 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.
- 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. Only at this stage it is possible to say to which sex the animal belongs.

CHAPTER 5

OCCURRENCE OF A CYTOCHROME P450 CONTAINING MIXED FUNCTION OXIDASE SYSTEM IN THE COMMON WHELK

1. INTRODUCTION

Cytochrome P450 (CYP450) is an important component of the mixed-function oxidase system. This system mediates the oxidative metabolism of a variety of endogenous as well as exogenous compounds. It comprises a superfamily of more than 150 individual forms of CYP450, each belonging to one of the 27 gene families identified so far (Nebert *et al.*, 1991).

In mammals and fish, the liver appears to be the primary organ where xenobiotics are metabolised, whereas in crustaceans and molluscs, the main organ appears to be the digestive gland (Livingstone, 1990). In *N. lapillus* the two organs involved in the breakdown of tributyltin are the digestive gland and the kidney (Bryan *et al.*, 1993).

In fish, TBT interacts with the CYP450 enzyme system resulting in an inhibition of its activity by the degradation to cytochrome P420 (CYP420), which is an inactive form of the enzyme. The remaining percentage of intact CYP450 after dosing TBT (up to 1 mM) decreased with increasing concentrations of TBT. The inactive (degraded) CYP420 was formed *in vitro* as well as *in vivo* (Fent, 1996).

Several CYP450 families are involved in steroidgenesis (fig.18). 11β-, 17α-, and 21 hydroxylation and aromatization of androgens all depend on CYP450.

Because hormonal disorders have been found in gastropods suffering from imposex caused by TBT (Oehlmann *et al.*, 1993), it was the aim of this study to investigate whether TBT caused a similar effect on CYP450 of the common whelk as it does in fish.

First had to be elucidated what would be the best method to determine the CYP450 enzyme system of this gastropod species. Liver microsomes of dab, *Limanda limanda*, were used to validate the methods used.

2. MATERIALS AND METHOD

Common whelks were collected at the Dogger Bank and then held under laboratory conditions (temperature 12°C, 12 hL-12hD light-regime, fed ad libitum once a week with mussels, Wadden Sea water). When microsomal samples were prepared, whelk shells were cracked with a vice and 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.

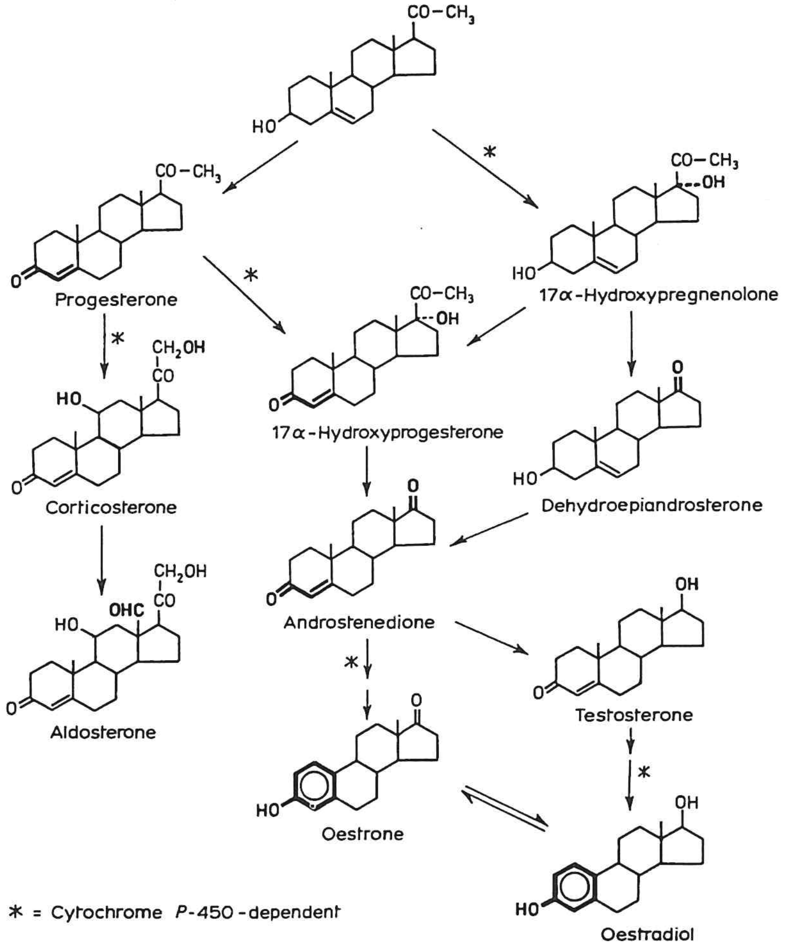


Figure 18. Possible biosynthesis of steroid hormones from pregnenolone.

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) and PMSF (phenyl methyl sulphonyl fluoride, Sigma diagnostics) were also used trying to improve the CYP450/CYP420 ratio in favour of CYP450.
Dab (*Limanda limanda*) liver microsomes were also used to check whether the method showed the same results as 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 doublebeam spectrophotometer (Perkin-Elmer, lambda 6):
I. CO-difference spectrum of sodium dithionite reduced sample
II. Sodium dithionite difference spectrum of CO treated (oxidised) sample.

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%.

Substrate induced difference spectrum
When a substrate binds to CYP450, the 'Soret peak' (characteristic absorbance peak of the haem group of the molecule at 420 nm) can shift. Two types of binding might occur :
Type I substrates bind to the protein part of the molecule, which will change the conformation and hence the ligation of the haem group with the protein of the molecule. This results in a high spin configuration and this shift from low to high spin configuration results in a characteristic spectral change (type I spectral change), with an absorption maximum at around 390 nm and minimum around 420 nm in the difference spectrum (microsomal fraction measured against buffer solution).
Type II substrates are thought to ligate to the haem iron of CYP450, resulting in a low spin haemoprotein, with a 420 nm peak. This CYP420 form represents the denaturated or inactivated form but might also be reactivated to CYP450.
These spectra are measured between 350-450 nm.

Substrate induced cytochrome P450 spectrum
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 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.

3. RESULTS

Cytochrome P450 spectra
The cytochrome P450 and cytochrome P420 spectra were at their maximum 3-4 minutes after dithionite addition. In the digestive gland microsomes of the common whelk, the CYP420 peak was always far greater than the CYP450 peak (Fig.19). To investigate whether this was caused by degradation of P450, BSA (as a substrate for proteases) and PMSF (protease inhibitor) were added to reduce the CYP420 peak. This was, however, unsuccesful. Other attemps to optimize the concentrations of dithionite, CO bubbling time or incubation temperature were not succesful either.
Microsomal fractions of the kidney gave a CYP450 spectrum where the CYP420 and CYP450 peaks were much more equal. Because of this, it was decided to use microsomal fractions from the kidney for the incubation studies with TBT.

To be able to make a good comparison between several spectra the absorbances at 490 nm were mathematically standardised at 1.

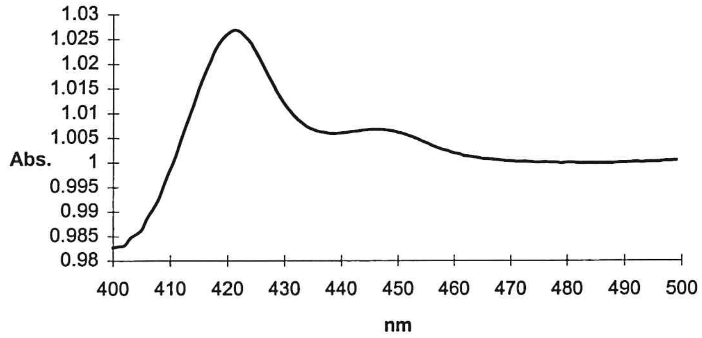


Figure 19. Cytochrome P450 spectrum of the digestive gland of *Buccinum undatum*.

Substrate induced difference spectra

The substrate induced difference spectra gave different results for fish and whelk microsomal samples. After incubation with TBT (conc. in cuvet 0.5 mM) dab liver microsomes showed a typical type I binding spectra with a peak at 380-390 nm and a through at 420 nm, although the base line increased (350→450 nm). With increasing TBT concentrations this binding spectrum became less clear (Fig.20).

After incubation of kidney microsomes of the whelk with TBT only minor changes in the base-line occurred. No binding spectrum could be observed (Fig.21).

Substrate induced cytochrome P450 spectra

In dab a clear decrease in CYP450 coinciding with an increase in CYP420 could be observed at increasing concentrations of TBT. In the absence of TBT, 77% was present as CYP450 and 23% as CYP420. The addition of TBT to a concentration of 1.5 mM in the cuvette reduced the percentage of CYP450 to 64% in favour of CYP420 (36%). Addition of more TBT further reduced CYP450, until only CYP420 was present at 3 mM TBT (Fig. 22).

For the common whelk a similar pattern could be observed. 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.23).

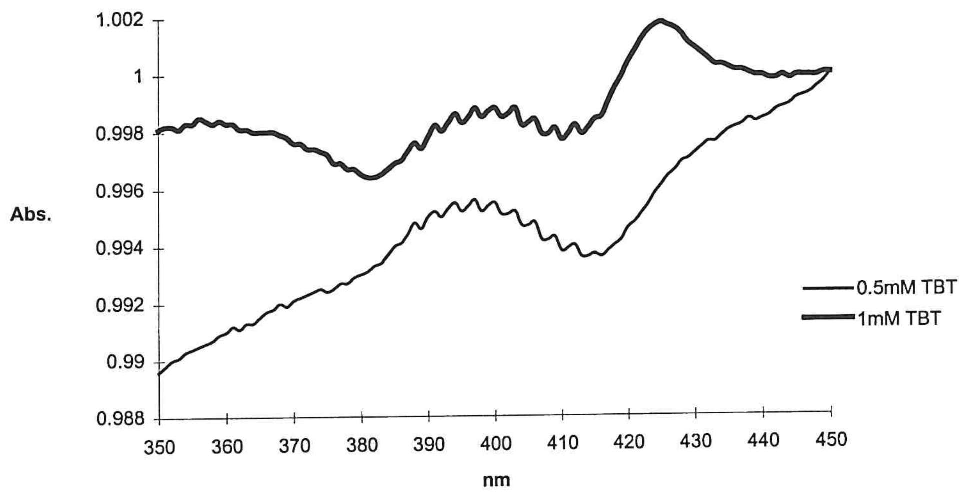


Figure 20. TBT incubation spectrum of dab, *Limanda limanda*, liver microsomes

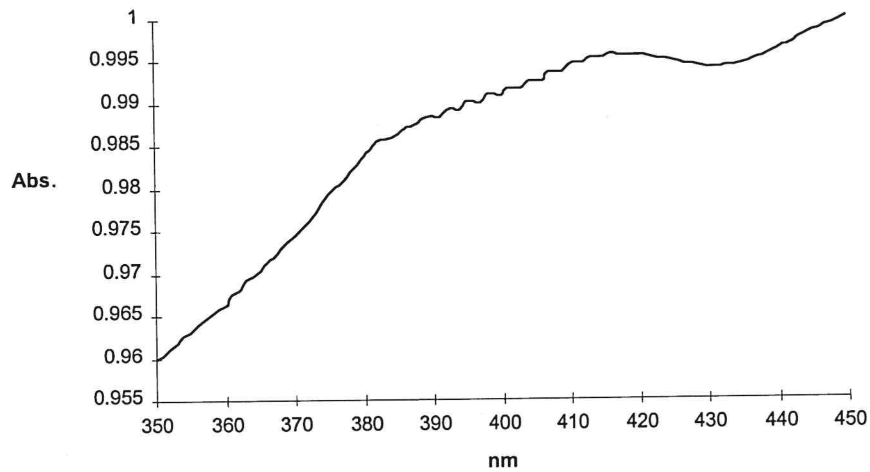


Figure 21. TBT (1 mM) incubation spectrum of common whelk, *Buccinum undatum*, kidney microsomes.

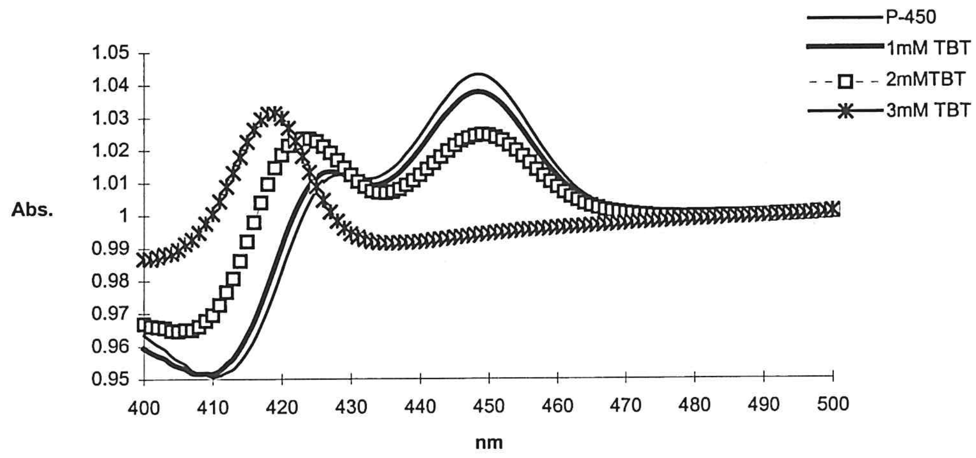


Figure 22. Cytochrome P450-TBT incubation spectrum of dab, *L. limanda*, liver microsomes.

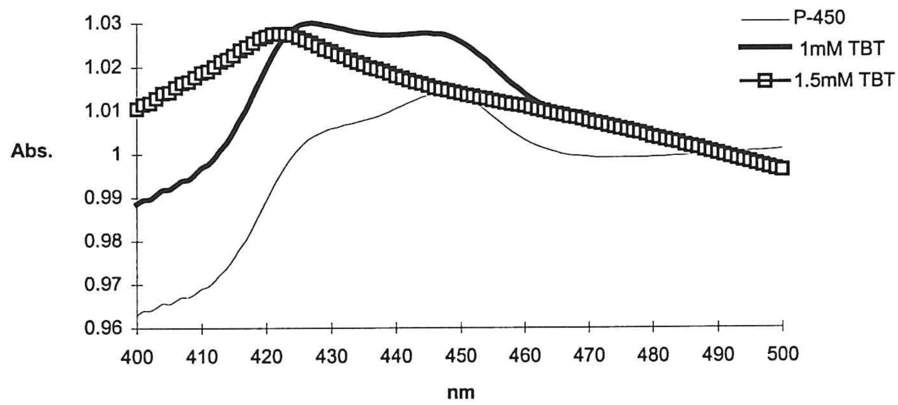


Figure 23. Cytochrome P450-TBT incubation spectrum of *B. undatum* kidney microsomes.

4. DISCUSSION

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, *B. undatum*. 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. By using the Potter teflon homogenizer these lysosomes could be destroyed and the enzymes released could break down a part of the CYP450.
It was difficult to quantify the exact amounts of cytochrome P450 or CYP420 in whelks 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. In this study the objective was a qualitative study of the cytochrome P450 enzyme system. Therefore relative values have been used to compare and describe the processes after addition of TBT.
In this study 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. Whelk microsomes did not, however, show such a binding spectrum.
In contrast, 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 to the inactive form CYP420 was observed after addition of TBT. This effect has already been observed in fish (Fent, 1996).
Although in several gastropods the presence of a CYP450 enzyme system has been found, no effects of TBT on this enzyme system in gastropods have been investigated. However, a possible effect was thought to play a role in the induction of imposex in gastropod species, leading to an imbalance in steroid hormone titres (Stroben, 1994 and Spooner *et al.*, 1991). In the biosynthesis pathway of steroid hormones in mammals and fish, many steps are regulated by isoenzymes of the CYP450 family (fig.18). Thus, an effect on these enzymes could, if relevant for invertebrates because biosynthesis of steroid hormones has never been properly investigated in gastropods, indeed cause hormonal disturbances.
In this study we present some evidence to support that 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 concentrations. 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 is probably not the organ where hormonal metabolism occurs.

5. CONCLUSIONS

- Common whelks have a cytochrome P450 enzyme system. The microsomal fraction of the digestive gland as well as the kidney contain this enzyme system, which is held responsible for the degradation of xenobiotic compounds like TBT.
- Cytochrome P450 is transformed by TBT in dab as well as in common whelks. A dose dependent decrease in the amount of CYP450 was observed whilst the inactive CYP420 form increased with increasing TBT concentrations. It is, however, questionable whether these concentrations will actually occur in the animals.
- Substrate (TBT) induced difference spectra did show a type I binding spectrum for dab. However, for the whelk no binding spectrum could be observed.

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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

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