

**NATIONAL INSTITUTE OF PUBLIC
HEALTH AND THE ENVIRONMENT
BILTHOVEN**

Report no. 732501 002

**Short term toxicity of bis(tri-*n*-butyltin)oxide in flounder
(*Platichthys flesus*): Pathology and immune function**

G.C.M. Grinwis^{1,2}, E.J. van den Brandhof,
J.A.M.A. Dormans, M. Engelsma, R. Kuiper², R. Leewis,
H. van Loveren, P.W. Wester, M.A. Vaal, A.D. Vethaak³,
J.G. Vos^{1,2}

March 1997

- ¹ Laboratory for Pathology and Immunobiology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands
- ² Department of Pathology, Faculty of Veterinary Science, University of Utrecht, Utrecht, The Netherlands
- ³ Ecotoxicology Section, National Institute of Coastal and Marine Management/RIKZ, Middelburg, The Netherlands

Dit onderzoek werd verricht in opdracht en ten laste van het Directoraat-Generaal Milieubeheer/DWL in het kader van project nummer 732501 "Zoute oppervlakte wateren". This investigation has been performed in order and for the account of the Directorate-General for Environmental Protection/DWL within the framework of project number 732501.

Rijksinstituut voor Volksgezondheid en Milieu
National Institute of Public Health and the Environment
P.O. Box 1, 3720 BA, Bilthoven, The Netherlands
tel. ..(31)30-2740111, fax ..(31)30-2744437

MAILING LIST

1. Drs. G.J.A. Al, DGM, Directie DWL
2. Ir. J. van Vliet, DGM, Directie DWL
3. Drs. D. Jonkers, DGM, Directie DWL
4. Dr.Ir. B.C.J. Zoeteman, Plv. DG. Milieubeheer
5. Prof.Dr. W.J. Wolff, RU Groningen
6. Dr.Ir. P. Reijnders, IBN-DLO Den Burg
7. Prof.Dr. W. van Muiswinkel, Dr. J.H.W.M. Rombout, LUW Wageningen.
8. Prof.Dr. J.H. Koeman, Dr. A. Brouwer, Drs. H. Besselink, LUW Wageningen
9. Drs. M. Scholten, MT-TNO Den Helder
10. Bibliotheek NIOZ Den Burg
11. Dr. J. Boon, Dr. J. Everaarts, Dr. M. Fonds, NIOZ Den Burg
12. Bibliotheek RIKZ Den Haag
13. Prof.Dr. J. Donkers, RIKZ Den Haag
14. Drs. E. Evers, RIKZ Den Haag
15. Dr. R. Laane, RIKZ Den Haag
16. Drs. J. Visser, RIKZ Den Haag
17. Dr. A. van Wezel, RIKZ Den Haag
18. Bibliotheek RIKZ Haren
19. Bibliotheek RIKZ Middelburg
20. Drs. W. Vonck, RIKZ Middelburg
21. Dr. P. Hagel, RIVO-DLO IJmuiden
22. Drs. P. van Banning, RIVO-DLO IJmuiden
23. Dr. M.G. Zeeman, Environmental Protection Agency, Washington, USA
24. Drs. C. vd Guchte, RIZA Lelystad
25. Bibliotheek Faculteit Diergeneeskunde, Universiteit Utrecht
26. Prof. Dr. J.E. van Dijk, Universiteit Utrecht
27. Prof.Dr. J.E. van Dijk, Universiteit Utrecht
28. Prof.Dr. R. Kroes, Prof.Dr. W. Seinen, Dr. M. van den Berg, RITOX Utrecht
29. Dr. J.G.D. Lambert, Universiteit Utrecht
30. Dr. P. de Voogt, Universiteit Amsterdam
31. Dr. T. Bowner, TNO Delft
32. Ir. D. Tromp, Dir. Noordzee der RWS Rijswijk
33. Depot Nederlandse Publikaties en Nederlandse Bibliografie
34. Directie RIVM
35. Dr.Ir. G. de Mik, directeur Sector IV
36. Ir. F. Langeweg, directeur Sector V
37. Prof.Dr. H. Eijsackers, Drs. J.H. Canton, ECO
38. Mw.Ir. C.J. Roghair, ECO
39. Dr. Ir. J. Noteboom, ECO
40. Ir. A.H.M. Bresser, LWD
- 41-51. Auteurs
52. Hoofd Bureau Voorlichting en Public Relations
53. Bureau Rapportenregistratie
54. Bibliotheek RIVM
- 55-75. Bureau Rapportenbeheer

CONTENTS

MAILING LIST	2
SUMMARY	4
SAMENVATTING	5
1. INTRODUCTION	6
2. MATERIALS AND METHODS	9
2.1 Fish species and maintenance	9
2.2 Chemicals and chemical analysis	9
2.3 Experimental design	10
2.4 Calculation of the LC50.	11
2.5 Histological techniques.	11
2.6 Scanning electron microscopy	12
2.7 Immune function tests	12
3. RESULTS	14
3.1 Chemical analysis	14
3.2 Clinical findings	14
3.3 Histopathology	14
3.4 Scanning electron microscopy	15
3.5 Immune function tests	15
4. DISCUSSION	16
5. CONCLUSIONS	18
ACKNOWLEDGEMENTS	18
REFERENCES	19
TABLES	24
FIGURES	28

SUMMARY

The present study is part of a project which focuses on the relation between environmental pollution and fish diseases. Field studies in various polluted coastal areas in Europe and the United States of America clearly indicate a relation between pollution and the increase in prevalence of tumours and infectious diseases in fish. Research under controlled laboratory conditions is necessary in order to prove causal links between pollution with specific xenobiotics and disease prevalence. One of the chemicals of interest in the myriad of xenobiotics found in polluted waters and sediments is the organotin compound tributyltin (TBT), originating mainly from antifouling paints used on the hulls of ships.

This report describes a study in which flounders (*Platichthys flesus*) were exposed to bis(tri-*n*-butyltin)oxide (TBTO) in the water under controlled laboratory conditions. The possible histopathological effects on several organs (gill, skin, eye, liver, mesonephros, ovary/testis, spleen, and gastrointestinal tract) were examined and morphometric analysis of the thymus was performed to assess the target organ(s) for TBTO in this fish species. Also the function of the non specific and specific resistance was studied using *ex vivo/ in vitro* immune function tests.

Exposure of flounder to TBTO, in concentrations which are in the same order of magnitude as maximum TBT levels measured in the field (experiment: 17.3 µg TBT; field: 7.2 µg TBT), caused mortality after 7-12 days, decreased the condition factor, resulted in gill lesions, and induced significant reduction of the non specific resistance. No marked effects on the relative thymus volume, or the specific immune system were noted after exposure to TBTO.

SAMENVATTING

De beschreven experimenten zijn een onderdeel van een project dat zich richt op de relatie tussen milieuverontreiniging en visziekten. Veld onderzoeken uitgevoerd in diverse verontreinigde kustwateren in zowel Europa als de Verenigde Staten laten een duidelijke relatie tussen vervuiling en een toegenomen prevalentie van tumoren en infectieziekten bij vissen zien. Onderzoek onder gecontroleerde laboratoriumomstandigheden is echter noodzakelijk om een eventueel causaal verband tussen vervuiling met specifieke xenobiotische stoffen en het frequenter voorkomen van ziektes aan te tonen. Eén van de chemische verbindingen in de veelheid van xenobiotica die gevonden kunnen worden in vervuilde wateren en sedimenten is de organotin verbinding tributyltin (TBT), hoofdzakelijk afkomstig van verf waarmee scheepshuiden worden behandeld om ongewenste aangroei van onder andere algen en schelpdieren tegen te gaan.

Dit rapport beschrijft een onderzoek waarin botten (*Platichthys flesus*) via het water werden blootgesteld aan bis(tri-*n*-butyltin)oxide (TBTO) onder gecontroleerde laboratoriumomstandigheden. De mogelijke histopathologische effecten aan diverse organen (kieuw, huid, ogen, lever, buiknier, ovarium/testikel, milt en maagdarkanaal) werden onderzocht en tevens werd er een morfometrisch onderzoek van de thymus (zwezerik) uitgevoerd om de doelorganen van TBTO bij de bot vast te stellen. Daarnaast werd het functioneren van zowel de specifieke- als aspecifieke afweer onderzocht door gebruik te maken van *ex vivo* / *in vitro* immuun functietesten.

Blootstelling van botten aan TBTO gehaltes in dezelfde orde van grootte als de maximaal gemeten TBT gehaltes in de veldsituatie (experiment: 17.3 µg TBT; veld: 7.2 µg TBT) veroorzaakte sterfte na 7-12 dagen, een vermindering van de conditiefactor, kieuwlaesies en een significante onderdrukking van de aspecifieke weerstand. Er werden geen duidelijke effecten waargenomen op het relatieve volume van de thymus en op het specifieke immuunsysteem na blootstelling aan TBTO.

1. INTRODUCTION

The present study is part of a fish disease project which focuses on the relation between environmental pollution and fish diseases. Field studies in various polluted coastal areas in Europe and the United States of America clearly indicate a relation between pollution and the increase in prevalence of tumours and infectious diseases in fish (Malins et al, 1984, 1985; Couch and Harshbarger, 1985; Murchelano and Wolke, 1991; Vethaak and Reinhalt, 1992; Myers et al, 1994; Vethaak and Jol, 1996). Field studies can be useful in providing data supporting the hypothesis of a causal relation between marine pollution and fish diseases. Although these studies have a high pertinence to natural populations, it is not possible to establish a direct link between pollution and disease in a field study mainly due to the variety of potential causal factors involved. In a large scale mesocosm experiment flounders (*Platichthys flesus*), a bottom dwelling fish species that is relevant for the Dutch field situation, were exposed to contaminated harbour sediment. Exposed animals showed an increased prevalence of liver tumours and lymphocystis virus infections compared to the control group (Vethaak et al. 1996). In mesocosm experiments one can confine the number of intervening variables and simulate a natural habitat at the same time, combining a moderate control of variables with a moderate relevance to the natural population. Research under controlled laboratory conditions remains necessary in order to prove causal links between pollution with specific xenobiotics and disease induction (Vethaak 1993; Wester and Vos, 1994). One of the chemicals of interest, in the myriad of xenobiotics found in polluted waters and sediments is, the organotin compound tributyltin (TBT).

Organotins are widely utilised organometallic compounds. Most of these chemicals are man made, except for methyltins, which can be produced under natural conditions (Guard et al., 1981). Organotins are used in a variety of products: as stabilisers in polyvinyl chloride (PVC), catalysts in polyurethane and silicone elastomers and as pesticides. Although the use of bis(tri-*n*-butyltin)oxide (TBTO) in antifouling paints for small ships (length < 25 m) has been banned in the Netherlands (since 1990) and in several other countries, it is still the major source of tributyltin (TBT) in water and sediments (Fent, 1996). This has resulted in TBT concentrations up to 5.76 µg/l in Canadian freshwater (Maguire et al., 1986), 1.5 µg/l in marine waters in France (Alzieu et al., 1989, and 1.66 µg/l in various harbours in the Netherlands (Ritsema and Laane, 1991). Because the degradation of TBT in sediments can be very slow (Stewart and De Mora, 1990; De Mora et al., 1995; Evans et al., 1995), the large reservoirs of TBT polluted sediments may also pose a threat especially to bottom dwelling fish species such as flounder.

Experiments with organotin compounds have shown various toxic effects in experimental animals including effects on the liver, the endocrine system, and especially the immune system. Exposure of rats to organotin compounds, including TBTO, induced a reduction in weight and cellularity of the thymus. Also depletion of T-cell areas in the spleen and lymphnodes was observed (Seinen et al., 1977a,b; Krajnc et al., 1984; Snoeij et al., 1985). Functional impairment of immune system has been found in rats resulting in a

decreased thymus dependent antibody synthesis against sheep red blood cells and *Trichinella spiralis* after short term and long term exposure to TBTO (Vos et al, 1984, 1990; Van Loveren et al., 1990). Also a decreased natural killer cell (NK-cell) activity, and a decreased splenic clearance of *Listeria monocytogenes* (both reactions of the non specific resistance) were found in these studies.

Several effects of TBT have been reported in fish as well. Dose dependent teratogenic effects and delayed hatching after short term exposure of minnow (*Phoxinus phoxinus*) eggs, and a dose dependent decreased survival of short term exposed larvae of minnow have been reported (Fent, 1996). Also histological alterations in a variety of organs were described in these experiments. In experiments studying the effects of TBTO in medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) showed histopathological lesions in liver, kidney, eye and gill epithelium (Wester and Canton, 1987; Wester et al., 1990). Several effects of TBT on the immune system of fish have been reported. It is known from mammalian as well as fish studies that the immune system is sensitive to the effects of pollution (Anderson and Zeeman, 1995) and immune parameters can therefore be valuable as biomarkers to evaluate the health status of fish and to monitor the possible effects of pollutants on the exposed organism. In rainbow trout, a concentration dependent lymphodepletion in the spleen has been reported (Schwaiger et al., 1994), and exposure of rainbow trout yolk sac fry to both TBT and dibutyltin (DBT) significantly reduced trout resistance to *Aeromonas hydrophila* challenge although no effects on the thymus or other lymphoid organs were noted (De Vries et al., 1991). In a comparative study using medaka and guppy, thymus atrophy was induced by aqueous TBTO exposure in the guppy only (Wester and Canton, 1987; Wester et al., 1990). A concentration dependent decrease in phagocytic activity of phagocytes exposed in vitro to TBT in levels ranging from 0.04 to 400 µg/ml was found in Atlantic croaker (*Micropogonias undulatus*), hogchoker (*Trinectes maculatus*) and in oyster toadfish (*Opsanus tau*) (Wishkovsky et al., 1989). Intraperitoneal injection of TBT in channel catfish (*Ictalurus punctatus*) resulted in peripheral blood neutrophilia and suppression of the humoral immune response against heat killed *Edwardsiella ictaluri*. Also reduction of the non-specific cytotoxic cell (NCC) activity and a decreased phagocyte oxidative burst were found in the same study (Rice et al., 1995).

In the present study, flounders were exposed to TBTO under controlled laboratory conditions (Grinwis et al., 1995). Histopathological investigation of the gill, skin, eyes, liver, mesonephros, spleen, ovary/testis and gastrointestinal tract was performed to assess the target organ(s) for TBTO pathology in this fish species. Also scanning electron microscopy of the gills was carried out. A sensitive parameter for thymotoxic effects in mammals is the weight of the thymus. However, it is not possible to excise and weigh the thymus in flounder due to the small size of the organ, its topographical orientation and its similar colour and structure compared to the surrounding muscle tissue. In addition quantitative histological interpretation of the thymus using routine histological techniques is difficult due to the irregular shape and lack of clear cortex-medulla distinction in the flounder thymus (Grinwis et al., 1995). Therefore in the present study morphometric techniques were used on histological serial sections to determine possible changes in thymus volume. *Ex vivo/ in vitro*

assays were also carried out in this study in order to assess effects on immune function. An assay to quantify the activity of an important first line defence mechanism in fish, comparable to mammalian natural killer (NK) cell activity, has been developed for flounder (Boonstra et al., 1996). The activity of non-specific cytotoxic (NCC) cells can be measured *in vitro* by determining their ability to lyse a ⁵¹Cr-radiolabeled target cell. The amount of radioactivity released is a measure for the number of cells killed by the NCC cells. The target cell used in the cytotoxicity assay was a virus-transformed murine lymphoma cell line, YAC-1. This cell line is commonly used in mammalian as well as piscine assays and has proven to be a sensitive target for the action of NK and NCC cells (Faisal et al., 1989; Van Loveren et al., 1990; Greenly et al., 1992). Specific immunological reactions are mediated by T and B lymphocytes. It was shown that flounder lymphocytes can be stimulated to proliferate *in vitro* with a specific T and B cell mitogen (Boonstra et al., 1996). The degree of stimulation is a parameter for the function of the specific defence mechanisms.

2. MATERIALS AND METHODS

2.1 Fish species and maintenance

The test organisms, flounder, with a length of 4-7 cm and 2-3 years of age were obtained from the National Institute for Fisheries Research (RIVO-DLO, IJmuiden, The Netherlands). These fish were caught with a dip-net during a short period in spring from an estuary near Southampton (England). They belonged to the 0+ age-group and measured approximately 1 cm upon arrival at the RIVO-DLO, where the animals were raised. The animals were acclimatised for at least 2 weeks under controlled conditions as described previously (Grinwis et al., 1995) at the test laboratory before they were used in the experiments.

The fish were kept in groups of 5 (experiment 1) or 10 (experiment 2 and 3) animals in 100 litre glass aquaria filled with 25 litre freshwater (Dutch Standard Water (DSW)). The water was aerated continuously, O₂ and pH levels were checked every other day. A water temperature of 19 ± 2 °C and a 16-8 hours light-dark regimen were maintained during the experiments. The animals were fed frozen *Artemia Salina* (SELCO, Artemia Systems N.V., Baas-Rode, Belgium) daily except in weekends. In experiments 2 and 3, 2.7 litres of silversand (M32, van Roon-Vreeswijk, Nieuwegein, The Netherlands) was added in order to improve husbandry conditions in these bottom dwelling fish species (Grinwis et al., 1995).

2.2 Chemicals and chemical analysis

Bis(tri-*n*-butyltin)oxide was obtained from Fluka Chemika a.g. (Buchs, Switzerland). The purity of the lot used (15210) was 96%. A stock solution with a concentration of 20 mg TBTO dissolved in 50 ml dimethylsulfoxide (DMSO; purity >99.5%, Merck, Amsterdam, The Netherlands) was used. In experiments 2 and 3 water samples were taken for TBTO analysis (Table 2). In order to ascertain the effects of the silversand on the actual TBTO water concentration, an additional aquarium without silversand with two flounders was added in experiment 2.

For the organotin analysis, an excess of potassiumtetrahydroborate solution was added to the acidified water samples (1 ml 37% HCl/l, Merck, Amsterdam, The Netherlands) in an oxygen free environment. The produced volatile hydrides were expelled by nitrogen gas and subsequently condensed in a chromosorb GNAW 60/80 column coated with a layer of 3% SP-2100 (Supelco, Sigma-Aldrich, Zwijndrecht, The Netherlands) cooled in liquid nitrogen. Detection of butyltin components was performed with an atom absorption spectrometer (AAS type 2380; wave length 22.4 nm, slit 0.2 nm; Perkin Elmer, Nieuwerkerk, The Netherlands).

2.3 Experimental design

Table 1 gives an overview of the experimental design, a more detailed description is presented below.

Experiment 1

This experiment was used for dose range finding. Four groups of 10 flounders each, 5 animals per aquarium, were exposed semi-static to 0, 3.2, 10 or 32 µg TBTO/l. DMSO was used as a carrier with a maximum concentration of 20 µl DMSO/l DSW, and two carrier-control groups were included in the experiment. The exposure medium was renewed every Monday, Wednesday and Friday. Clinical parameters like behaviour, food uptake and external features were monitored during feeding.

Five animals per group were killed after 7 and 14 days with an overdose of tricaine methanesulfonate (MS222[®], Sandoz Ltd., Basel, Switzerland), fixed in Bouin's fixative for 24 hours and then transferred into 70% alcohol for histological investigation.

Experiment 2

This experiment was carried out because all animals in the highest dose group died in experiment 1 and were not suitable for histological examination, and animals in the lower dose groups did not show exposure related effects. Four groups of 10 flounders each were exposed to 0, 3.2, 10 or 32 µg TBTO/l. The exposure medium was renewed every Monday Wednesday and Friday for 4 weeks. Clinical parameters were monitored during feeding.

The animals were killed after 28 days with an overdose of MS222[®]. The weight and length were measured. From these data the condition factor ($100 * \text{body weight}/\text{length}^3$) was calculated. Blood samples, and samples of spleen and mesonephros were taken for immune function tests as described below. The remaining parts of the mesonephros and the rest of the fish were fixed in 4% buffered formaldehyde.

Experiment 3

Based on the results of experiment 2 (the mortality in the highest dose group and the absence of histological lesions in the lower dose groups) this experiment was used to determine acute target organ toxicity of TBTO at a concentration of 32 µg/l. The experiment was ended before mass mortality would take place so animals could be used for both histological examination and immune function tests. Two groups of 30 animals each were exposed to 0 or 32 µg TBTO/l for 6 days. The exposure medium was renewed on Wednesday and Friday. Clinical parameters were monitored during feeding.

The animals were killed after 6 days with an overdose of MS222[®]. Their body weight, liver weight and length were measured. Blood samples, and samples of spleen and mesonephros were taken for immune function tests as described below. The remaining parts were fixed in 4% buffered formaldehyde.

2.4 Calculation of the LC 50

The lethal concentration of TBTO for flounder under the experimental conditions was calculated in experiments 1 and 2 using the trimmed Spearman-Kaber method for estimating the median lethal concentration (Hamilton et al., 1977).

2.5 Histological techniques

Selection of organs and tissues for histological examination was based on target organs of TBTO in other fish species (Wester and Vos, 1994; Wester and Canton., 1987; Wester et al., 1990). In experiment 1, the animals were fixed in Bouin's for 24 hours after killing and then transferred into 70 % ethanol. Several transverse slices were made at standardised levels (Grinwis et al., 1995) and paraffin embedded. The first level just caudal from the eyes, revealed gill cavity, gills and the major part of the thyroid gland. The second level halfway the dorsal commissure of the operculum and the caudal ridge of the operculum showed gills, gill cavity, thymus, heart and head kidney. The third section is made through the pectoral fin where the hepatopancreas, stomach, trunk kidney and guts are found. In this third section also the spleen can be encountered but the localisation of the spleen is somewhat variable and this organ may also be found in the more caudal part of the body cavity. The fourth level is made just cranial of the caudal part of the body cavity where gonads, trunk kidney and guts are displayed. The exact position of the organs may differ due to natural variation, which can cause a problem in finding small organs like the thymus and spleen. Sections were cut at 5 μm and routinely stained by haematoxylin and eosin.

The histological procedures in experiment 2 and 3 were different from the procedures in experiment 1, because the spleen and (part of the) mesonephros were removed for immune function tests. The remaining part of the mesonephros, the gastro-intestinal tract, ovary/testis, liver, skin, and the head (including the caudal edge of the opercula) were fixed in 4% buffered formaldehyde. These tissues were subsequently paraffin embedded and cut into 5 μm sections which were stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) according to routine procedures. Histological samples were examined and scored under cover.

For morphometric analysis of the thymus in experiment 2 and 3, a transverse block of each fish of about 0.5 cm thick was taken cranially from the caudal edge of the opercula. This block, which contained the thymus was decalcified for 5 days in formic acid after fixation in 4% buffered formaldehyde before it was paraffin embedded and was used for morphometric analysis of the thymus. For this purpose serial sections of 5 μm were made with an interval of 30 μm until the whole thymus was cut. The surface area of the slides of the thymus was measured using an IBAS 2000 (Kontron, Munich, Germany) image analysis system. In this way an indirect measurement of the thymus volume was made. If possible, the thymus at both sides of the body was measured, and the mean values were used in our results. The thymus volume was related to the body-height ([body height just caudal of

pectoral fin]³), because a better correlation between the thymus volume and the body height was found in the control group than between thymus volume and body length or body weight.

2.6 Scanning electron microscopy

After fixation in 4% buffered formaldehyde, the gills were postfixed in 2% glutaraldehyde, and in 2% osmiumtetroxide, both in a 0.1 M phosphate buffer. The specimens were dried in a critical point drying device (Baltzer CPD 030) after dehydration in a graded acetone series. The dried specimens were mounted on aluminium stubs, sputter coated with a thin layer of gold in a Polaron coating unit E5000, and examined in a Philips PSEM 501 B scanning electron microscope.

2.7 Immune function tests

In experiments 3 *ex vivo/ in vitro* immune function tests were performed on mesonephros and spleen leukocytes.

Cell suspensions of spleen, and mesonephros were prepared in culture medium which consisted of RPMI-1640 (GIBCO, Grand Island, USA) supplemented with 18% distilled water, 10% heat inactivated fetal calf serum (FCS; PAA, Linz, Austria), 100 IU/ml penicillin, 100 mg/ml streptomycin (hereafter called fcRPMI, flounder complete RPMI) by squeezing the tissues through a 70 µm nylon cell strainer (Becton Dickinson, Rutherford, USA). Blood was diluted with 2 volumes fcRPMI. The cell suspensions and diluted blood were purified by density gradient centrifugation using Ficoll Paque (1.077 g/ml, Pharmacia LKB Biotechnology, Uppsala, Sweden). Following centrifugation for 20 min at 900xg at 20°C, cells at the interphase were collected and washed twice with fcRPMI for 10 min at 300xg and 4°C. Finally, the pellet was resuspended in 250 µl fcRPMI. (hereafter called fcRPMI, flounder complete RPMI). The viability of the cells was determined using trypan blue. In all experiments the viability exceeded 95%. Total cell numbers were counted manually in a hemocytometer. Cytospin samples were prepared, and stained with May-Grünwald Giemsa. The percentage of lymphoid cells was determined, and the total lymphoid cell number was calculated. A total of 10⁷ lymphoid cells/ml was used for measurement of the NCC activity, and 5x10⁶ lymphoid cells/ml for the LTT.

Cell suspensions from the mesonephros were incubated overnight (26°C, 5% CO₂-95% air) since preincubation is known to enhance the NCC activity (Graves et al., 1984) and because macrophages are removed simultaneously making use of their tendency to adhere to plastic. Following the preincubation, 100 µl effector cell suspension was added to the labeled target cells at effector to target cell ratios ranging from 5 to 20. The plates were centrifuged in order to enhance cell to cell contact (200xg, 5 min). Following a 4 hours incubation at room temperature (20°C) in an atmosphere of 5% CO₂-95% air, the plates were centrifuged (200xg, 5 min) and supernatant from each well was collected. Radioactivity was determined using a gamma counter (Packard, Tilburg, the Netherlands). The percentage specific release

was calculated as: [radioactive counts in the supernatant minus the spontaneous release by the target cells] divided by [the maximum release by the target cells minus their spontaneous release].

Lymphocytes obtained from the spleen were stimulated by adding the T-cell mitogen phytohaemagglutinin (PHA, HA 15, Murex Diagnostics Ltd, Dartford, UK) and peripheral blood lymphocytes with the B-cell mitogen lipopolysaccharide (LPS, LPS W, E. coli 0127:B8, Difco Laboratories, Detroit, USA). A more detailed description of these function tests is reported elsewhere (Boonstra et al., 1996).

.

3. RESULTS

3.1 Chemical analysis

Actual water concentrations were measured in experiments 2 and 3 in control and exposed groups at 0, 6, and 48 hours after the start of exposure. Data are shown in Table 2. The recovery in experiment 2 at $t=0$ was 77 % in the 10 $\mu\text{g/l}$ group with silversand and 99% in the 10 $\mu\text{g/l}$ group without silversand. In experiment 3 only 55% of the TBTO was recovered at $t=0$ in the 32 $\mu\text{g/l}$ group (with silversand). The actual TBTO concentration in the 32 $\mu\text{g/l}$ group at $t=0$ was 17.75 $\mu\text{g/kg}$ water in experiment 3. In the same experiment the TBTO level dropped to 7.99 $\mu\text{g/kg}$ water at $t=6$ hours and to 1.89 $\mu\text{g/kg}$ water, which is approximately 10% of the $t=0$ level, at $t=48$ hours. Levels of mono- and dibutyltin components were very low or even below the detection level. The exposure medium was renewed 3 times a week in the present study.

3.2 Clinical findings

Behavioural changes were seen in the highest dose groups (32 $\mu\text{g/l}$) in experiment 2 and 3, but were not obvious in experiment 1. The animals showed a decreased food uptake and a decreased activity. The severity of these symptoms did not clearly increase in time. In experiment 1 the first animal in the 32 μg TBTO/l group died after 2 days, the mortality was 100% after 7 days. In experiment 2 mortality in the 32 μg TBTO/l group started after 3 days, and was 100% after 12 days. The LC50 for TBTO after 14 days, in flounder kept under the conditions as used in our study, was 17.9 $\mu\text{g/l}$ calculated using the trimmed Spearman-Kaber method (Hamilton et al., 1977). In experiment 2 no significant changes in body weight were found between control and exposed groups after 28 days (Table 3). Animals exposed to 10 μg TBTO/l showed a significant decrease of the condition factor after 28 days of exposure in experiment 2 (Table 3). The liver weight and the hepatosomatic index, calculated in experiment 3 (Table 4), showed a significant increase in exposed animals.

3.3 Histopathology

Animals that died during the experiment were lost for histological investigation since dead fish rapidly develop autolytic changes. Especially gill epithelium is very sensitive, desquamates rapidly and can not be evaluated properly even a few hours after death. All animals in the 32 $\mu\text{g/l}$ groups in experiments 1 and 2 died before the end of the experiment, so no histopathological data from these groups were available. In all experiments gills, hepatopancreas, mesonephros, gastro-intestinal tract and ovary/testis of the surviving animals were examined. In experiment 1 also histopathology of the thyroid gland and the

skin was performed, and in experiment 2 also the eyes were examined. In experiments 1 and 2 no exposure related histopathological changes were found in the above mentioned organs.

In experiment 3 all animals of the 32µg/l group showed gill lesions (Table 5; Figs. 1-4). Budding of epithelial cells (Fig.2) of the primary and secondary lamellae was absent or rare in control animals but frequent in animals in the 32µg/l group. Epithelial proliferation (Fig.3) and fusion of secondary lamellae (synechia) (Fig.4) was present in control animals but not as frequent, prominent and extensive as in animals exposed to 32µg/l.

The volume of the thymus was calculated by measuring the surface areas of serial sections (about 45 sections per thymus) with the use of an image analysis system. In exposed animals, the absolute thymus volume ($0.339 \pm 0.11 \text{ mm}^3$) was not markedly changed when compared to control animals ($0.394 \pm 0.10 \text{ mm}^3$). Also no effects on thymus volume in relation to body weight or body length were noted after exposure to TBTO (data not shown). However, when related to the (body height)³ a 20% decrease in relative thymus volume (control : 0.024 ± 0.005 ; exposed: 0.019 ± 0.007) was calculated in animals in the exposed group (Table 8).

3.4 Scanning electron microscopy

Scanning electron microscopy of the affected gills (Figs. 5-8), of animals exposed to 32 µg TBTO/l for 6 days in experiment 3, shows the localised character and three dimensional extension of these lesions. The roughened surface of primary and secondary lamellae in the exposed animals caused by budding of the epithelial cells is clearly visible (Fig. 7). As compared to control animals (Fig. 5) the functional surface area of the gills in exposed animals is markedly decreased due to fusion of secondary lamellae (Fig. 6), but often normal gill tissue is present adjacent to these fused lamellae (Fig.8).

3.5 Immune function tests

A significant decrease of the lymphocyte percentage and total lymphocyte number in the spleen was found in experiment 3 (Table 6). The proliferative response to stimulation with PHA in experiment 3 showed no statistically significant changes between exposed and control animals (Table 6). The lymphocyte transformation test (LTT) using LPS as the mitogen was not performed due to insufficient numbers of peripheral blood lymphocytes.

A strong and significant decrease of the NCC activity was observed in exposed animals, to approximately 30% of the control values ,at all E/T ratios (5,10 and 20) in experiment 3 (Table 7). In this experiment 2 of 14 animals (14 %) had no or only insignificant NCC activity.

4. DISCUSSION

In the present study flounders, kept in fresh water under controlled conditions, were exposed to 3 concentrations (3.2, 10 and 32 µg/l) of TBTO. A concentration of 32 µg/l induced 100% mortality after 7-12 days (14 days LC_{50} =17.9 µg/l), gill lesions, an increase in liver weight, an increase of the hepatosomatic index, a decrease in relative thymus volume and a reduction of the NCC activity. The mortality data are comparable to data obtained from experiments using medaka and guppy where exposure to 32 µg TBTO/l also resulted in 100% mortality after 2 weeks (Wester and Canton, 1987; Wester et al., 1990). At a TBTO concentration of 10 µg/l a significant decrease in condition factor was observed in experiment 2. Since no exposure related histopathological lesions were detected at non lethal concentrations in our experiments, a steep concentration-effect curve is indicated. This is in contrast with the occurrence of histopathological lesions at non lethal concentrations in other fish species. In the guppy and medaka lesions in liver, kidney, eye and gill epithelium were found (Wester and Canton, 1987; Wester et al., 1990). Teratogenic effects, delayed hatching and histologic alterations in a variety of organs (skin, muscles, kidney, cornea, lens and retina) were described in experiments of minnow (*Phoxinus phoxinus*) eggs after short term exposure to TBTO. In these experiments also a dose dependent decreased survival of minnow larvae was found (Fent, 1996). In 4 month old rainbow trout a concentration related depletion of lymphocytes in the spleen was seen, accompanied by degeneration and necrosis of epithelial cells and chloride cells in the gills after exposure to TBTO in concentrations ranging from 0.6 to 4.0 µg/l for 28 days (Schwaiger et al., 1994).

Exposure to 32 µg TBTO/l for 6 days resulted in gill lesions in all animals. Some of these lesions found in the present study, like the epithelial hyperplasia and fusion of secondary lamellae are non specific and can also be seen after exposure to several other irritating substances (Mallatt, 1985) and in animals caught in the wild. The budding of epithelial cells as seen in animals exposed to 32µg TBTO/l in the present study, is likely a specific reaction to the exposure to TBTO. This lesion has not been described in the review article on gill lesions induced by toxicants (Mallatt, 1985). Immunohistochemical staining for apoptosis (using the tunnel assay) did not reveal an increased number of apoptotic cells in the affected gill epithelium (data not shown). Although it is the only significant histopathological lesion in the highest dose group, it is unclear if these gill lesions are responsible for the clinical symptoms (anorexia, decreased activity) and death due to hypoxia, because the rate of gas exchange across the respiratory epithelium depends on the dimensions of the epithelium, the concentration gradient, and the diffusion coefficient of the gas (Randall, 1970) and the major part of the respiratory surface, even in severely affected gills with fusion of secondary lamellae and epithelial proliferation, appeared not clearly reduced .

Experimental data in rainbow trout have shown neurotoxicity after exposure to 0.5 or 2.0 µg TBTO/l resulting in ultrastructural lesions in the tectum opticum and optical nerve (Tribskorn et al., 1994). On the other hand, in rats (Krajnc et al., 1984), guppy and medaka (Wester and Canton, 1987; Wester et al., 1990) no histopathological effects were seen in the

central nervous system. The brain tissue was not incorporated in the histopathological evaluation of the present study (because it is localised in the part of the head that was used for morphometric analysis of the thymus) so an effect of TBTO on the central nervous system in flounder can therefore not be excluded although no neurological symptoms were observed.

Exposure to 32 µg TBTO/l for 6 days resulted in a strong suppression of the NCC activity at all E/T ratios. This effect of exposure to TBTO on the non specific resistance shows similarities to data in other species. The statistically significant decrease of the NCC activity is comparable to the decreased NK cell activity found in rats after oral exposure to TBTO (Vos et al., 1990, van Loveren et al., 1990). The high inter-individual variability in the results obtained from the NCC activity assay and other immune function tests is a problem often reported in piscine immunological studies (Zeeman, 1986; Dunier et al., 1995; Pulsford et al., 1995). The spleen showed a decrease of lymphocyte percentage and total lymphocyte numbers. This is in line with the effects of TBT exposure reported in rainbow trout by Schwaiger et al. (1994), but in contrast with data obtained by De Vries et al. (1991) in the same fish species. There was however no statistically significant change in lymphocyte stimulation with PHA (a T-cell mitogen) even if the reduced spleen cell number was taken into account. This could be due to the high variability in the results. No marked effect on thymus morphology could be demonstrated by light microscopy. Using morphometry, an indirect measurement of the thymus volume was made. Results indicated that there was no difference between the groups with respect to the absolute thymus volume and ratio thymus volume/body weight. However, since body weight could equally be affected by treatment as thymus volume (or thymus weight), a more stable parameter was also taken as reference, such as the ratio thymic volume/body length or body height. Only the ratio thymic volume/body height³ showed a 20% reduction (p. 0.049, t-test, 1 tailed) but this is considered of unclear biological significance. In guppy exposed to TBTO concentrations ranging from 0.032 to 10 µg/l for 1 month, thymus atrophy was much clearer (Wester and Canton, 1987). In rainbow trout exposed to TBTO concentrations ranging from 0.6 to 4.0 µg/l for 28 days, a concentration related lymphocyte depletion and increased phagocytic activity was found in the spleen (Schwaiger et al., 1994). Exposure of rainbow trout yolk sac fry to both TBT and DBT significantly reduced trout resistance to *Aeromonas hydrophila* challenge but no effects on the thymus or other lymphoid organs were noted (De Vries et al., 1991).

Chemical analysis data showed some effect of silversand on the actual TBTO concentrations in the water. At t=0 in the 10 µg TBTO/l group, the presence of silver sand caused a reduction of the actual water concentration, resulting in a concentration of 7.74 µg TBTO/kg (9.94 µg TBTO/kg without silversand). But at t= 48 hours the actual TBTO water concentration was higher in the presence of silversand (0.58 µg/kg compared to 0.25 µg TBTO/kg). This phenomenon might be explained by a reservoir function of the silversand. TBTO levels dropped to approximately 10% at t=48 hours, which shows that the renewal of the exposure medium 3 times a week was adequate. In order to investigate the real bioavailability, tissue analysis should be performed.

5. CONCLUSIONS

TBTO levels obtained from field measurements show TBT water-levels of up to 5.76 µg/l (Maguire et al., 1986), in Dutch harbours levels up to 7.2 µg TBT/l were found. In the present study, the maximum actual water TBT concentration was 17.3 µg/kg water (expressed as TBT/kg water, not as Sn/kg water) at t=0, which is only 2.4 times higher than the highest levels measured in Dutch harbours. The maximum TBT level at t=48 hours in the present study dropped to 1.83 µg/kg water. This indicates that under field conditions animals can be exposed, over a long period of time, to TBT concentrations that are in the same order of magnitude as the concentration in which mortality, gill lesions, a decrease in relative thymus volume and significant reduction of NCC activity were found in the present study. Suppression of non specific resistance, shown by a decreased NCC activity may have detrimental effects on the health status, such as increased susceptibility to viral infections. Mortality data of flounder exposed to TBTO are comparable to data obtained from guppy and medaka. The absence of histopathological lesions at sub-lethal concentrations however is in contrast with findings in guppy and medaka indicating a steeper concentration effect curve than in flounder. Further studies on long term TBTO exposure will be performed in the near future including lymphocystis infection experiments, in order to elucidate the on (target) organs and the immune system. These studies should clarify a possible causal role of TBT exposure in the increased prevalence of lymphocystis infections as observed in field- and mesocosm studies.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude towards A.S. de Jong of the National Institute for Coastal and Marine Management (RIKZ, Haren, The Netherlands) for performing the chemical analysis of butyltin compounds in the exposure medium.

REFERENCES

- Alzieu, C.I., J. Sanjuan, P. Michel, M. Borel, and J.P. Dreno (1989) Monitoring and assessment of butyltins in atlantic coastal waters. *Mar. Poll. Bull.*, 20, 22-26.
- Anderson, D.P., and M.G. Zeeman (1995) Immunotoxicology in fish. In: *Fundamentals of aquatic toxicology: effect, environmental fate, and risk assessment*, edited by Rand, G. Taylor & Francis, Washington D.C, pp. 371-404.
- Boonstra, A., G.C.M. Grinwis, P.W. Wester, H. Van Loveren, M.A. Vaal, A.D. Vethaak, and J.G. Vos (1996) Development of *ex vivo/ in vitro* immune function tests in flounder, *Platichthys flesus*. Report number 732501001. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.
- Couch, J.A., and J.C. Harshbarger (1985) Effects of carcinogenic agents on aquatic animals: an environmental and experimental overview. *Environ. Carcinogenesis Revs.*, 3(1), 63-105.
- De Mora, S.J., C. Stewart, and D. Phillips (1995) Sources and rate of degradation of tri(*n*-butyl)tin in marine sediments near Auckland, New Zealand. *Mar. Poll. Bull.*, 30, 50-57.
- De Vries, H., A.H. Penninks, N.J. Snoeij, and W. Seinen (1991) Comparative toxicity of organotin compounds to rainbow trout (*Oncorhynchus mykiss*) yolk sac fry. *Sci. Total Environ.*, 103, 229-243.
- Dunier, M., C. Vergnet, A.K. Siwicki, and V. Verlhac (1995) Effect of lindane on rainbow trout (*Oncorhynchus mykiss*) immunity. IV. Prevention of nonspecific and specific immunosuppression by dietary vitamin C (ascorbate-2-polyphosphate). *Ecotoxicol. Environ. Safety*, 30, 259-268.
- Evans, S.M., T. Leksono, and P.D. McKinnel (1995) Tributyltin pollution: A diminishing problem following legislation limiting the use of TBT-based ant-fouling paints. *Mar. Poll. Bull.*, 30, 14-21.
- Faisal, M., Ahmed, I.I., G. Peters, and E.L. Cooper (1989) Natural cytotoxicity of tilapia leucocytes. *Dis Aquatic Org.*, 7, 17-22.
- Fent, K (1996) Ecotoxicology of Organotin Compounds. *CRC Crit. Rev. Toxicol.*, 26 (1), 1-117.
- Graves S.S., D.L. Evans, D. Cobb, and D.L. Dawe (1984) Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). I. Optimum requirements for target cell lysis. *Dev Comp Immunol.*, 8: 293-302.

Greenly, A.R., R.A. Brown, and S.S. Ristow (1992) Nonspecific cytotoxic cells of rainbow trout (*Onchorhynchus mykiss*) kill YAC-1 targets by both necrotic and apoptotic mechanisms. *Dev Comp. Immunol.*, 29, 1181-1187.

Grinwis, G.C.M., P.W. Wester, A. Kamstra, E.J. Van den Brandhof, J.E. Van Dijk, R.J. Leewis, M.A. Vaal, A.D. Vethaak, and J.G. Vos (1995) Laboratory maintenance, topographical anatomy and histology of flounder, *Platichthys flesus*. Report number 732402001. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Guard, H.E., A.B. Cobet, and W. M. Coleman III (1981) Methylation of trimethyltin compounds by estuarine sediments. *Science*, 213, 770.

Hamilton, M.A., R.C. Russo, and R.V. Thurston (1977) Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.*, 11(7), 714-719.

Krajnc, E.I., P.W. Wester, J.G. Loeber, F.X.R. Van Leeuwen, J.G. Vos, C.A. Vaessen, and C.A. van der Heijden (1984) Toxicity of bis(tri-*n*-butyltin)oxide in the rat. I. Short-term effects on general parameters and on the endocrine and lymphoid systems. *Toxicol. Appl. Pharmacol.* 75, 363-386.

Maguire, R.J., R.J. Tkacz, Y.K. Chau, G.A. Bengert and P.T.S. Wong (1986) Occurrence of organotin compounds in water and sediments in Canada. *Chemosphere*, 15, 253-274.

Malins, D.C., B.B. McCain, D.W. Brown, S.L. Chan, M.S. Myers, J.T. Landahl, P.G. Prohaska, A.J. Friedman, L.D. Rhodes, D.G. Burrows, W.D. Gronlund, and H.O. Hodgins (1984) Chemical pollutants in Sediments and diseases of bottom-dwelling fish in Puget Sound, Washington. *Env. Sci. Technol.*, 18, 705-713.

Malins, D.C., M.M. Krahn, D.W. Brown, L.D. Rhodes, M.S. Myers, B.B. McCain, and S-L. Chan (1985) Toxic chemicals in marine sediment and biota from Mukilteo, Washington: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). *J. Natl. Cancer Inst.*, 74, 487-494.

Mallatt, J (1985) Fish Gill Structural Changes Induced by Toxicants and Other Irritants: A Statistical Review. *Can. J. Fish. Aquat. Sci.*, 42, 630-648.

Murchelano, R.A. and R.E. Wolke (1991) Neoplasms and nonneoplastic liver lesions in winter flounder, *pseudopleuronectes americanus*, from Boston harbour, Massachusetts. *Env. Health Perspectives*, 90, 17-26.

Myers, M.S., C.M. Stehr, O.P. Olson, L.L. Johnson, B.B. McCain, S.L. Chan and U. Varanasi (1994) Relationships between toxicopathic hepatic lesions and exposure to chemical contaminants in English sole (*pleuronectes vetulus*), starry flounder (*platichthys stellatus*), and white croaker (*genyonemus lineatus*) from selected marine sites on the pacific coast, USA. *Env. Health Perspectives*, 102, 200-215.

Pulsford, A.L., M. Crampe, A. Langston, and P.J. Glynn (1995) Modulatory effects of disease, stress, copper, TBT and vitamin E on the immune system of flatfish. *Fish Shellfish Immunol.*, 5, 631-643.

Randall, D.J. (1970) Gas Exchange in Fish. In: *Fish Physiology*, Vol. IV, edited. by W.S. Hoar, and D.J. Randall. Academic Press, New York, pp. 253-292.

Rice, C.D., M.M. Banes, and T.C. Ardel (1995) Immunotoxicity in channel catfish, *Ictalurus punctatus*, following acute exposure to tributyltin. *Arch. Environ. Contam. Toxicol.*, 28, 464-470.

Ritsema, R., and R.W.P.M. Laane (1991) Dissolved butyltins in fresh and marine waters of the Netherlands in 1989. *Sci. Total Environ.*, 105, 149-156.

Schwaiger, J., H.F. Falk, F. Bucher, G. Orthuber, R. Hoffmann, and R.D. Negele (1994) Prolonged exposure of rainbow trout (*Oncorhynchus mykiss*) to sublethal concentrations of bis(tri-*n*-butyltin)oxide: effects on leucocytes, lymphatic tissues and phagocytosis activity. In: *Sublethal and Chronic Effects of Pollutants on Freshwater Fish*, edited by R. Müller, and R. Lloyd. Blackwell Scientific, Cambridge M.A., pp 113-123.

Seinen, W., J.G. Vos, R. Van Krieken, A. Penninks, R. Brands, and H. Hooykaas (1977a) Toxicity of organotin compounds . III Suppression of thymus-dependent immunity in rats by di-*n*-butyltin dichloride and di-*n*-octyltin chloride. *Toxicol. Appl. Pharmacol.*, 44, 213.

Seinen, W., J.G. Vos, I. Van Spanje, M. Snoek, R. Brands, and H. Hooykaas (1977b) Toxicity of organotin compounds. II. Comparative in vivo and in vitro studies with various organotin organolead compounds in different animal species and with special emphasis on lymphocyte toxicity. *Toxicol. Appl. Pharmacol.*, 42, 197-212.

Snoeij, N.J., A.J.J. Van Iersel, A. Penninks, and W. Seinen (1985) Toxicity of triorganotin compounds: Comparative in vivo studies with a series of trialkyltin compounds and triphenyltin chloride in male rats. *Toxicol. Appl. Pharmacol.*, 81, 274-286.

Stewart, C., and S.J. De Mora (1990) A review of the degradation of tri(*n*-butyl)tin in the marine environment. *Environ. Technol.*, 11, 565-570.

Triebskorn, R., H.R. Köhler, K.H. Körtje, R.D. Negele, H. Rahmann, and T. Braunbeck (1994) Evaluation of bis(tri-*n*-butyltin)oxide (TBTO) neurotoxicity in rainbow trout (*Oncorhynchus mykiss*). II. Ultrastructural diagnosis and tin localisation by energy filtering transmission electron microscopy (EFTEM). *Aquat. Tox.* 30, 199-213.

Van Loveren, H., E.I. Krajnc, P.J.A. Rombout, F.A. Blommaert, and J.G. Vos (1990) Effects of ozone, hexachlorobenzene, and bis(tri-*n*-butyltin)oxide on natural killer cell activity in the rat lung. *Toxicol Appl Pharmacol.*, 102, 21-33.

Vethaak, A.D., and J.G. Jol (1996) Diseases of flounder (*Platichthys flesus*) in Dutch coastal and estuarine waters, with particular reference to environmental stress factors, Part 1. Epizoötiology of gross lesions. *Dis. Aquat. Org.*, 26, 81-97.

Vethaak, A.D., and T. Reinallt (1992) Fish disease as a monitor for marine pollution: the case of the North Sea. *Rev. Fish Biol. Fish.*, 2, 1-32.

Vethaak, A.D (1993) Fish disease and marine pollution: a case study of the flounder (*Platichthys flesus*) in Dutch coastal and estuarine waters. Ph.D-thesis Amsterdam.

Vethaak, A.D., J.G. Jol, A. Meijboom, M.L. Eggens, T. Rheinallt, P.W. Wester, T. Van der Zande, A. Bergman, N. Danker, F. Ariede, R.A Baan, J.M. Everts, A. Opperhuizen, and J. M. Marquenie (1996) Skin and liver diseases induced in flounder (*Platichthys flesus*) after long-term exposure to contaminated sediments in large-scale mesocosms. *Environ. health pers.*, 104(11), 1218-1229.

Vos, J.G., A. de Klerk, E.I. Krajnc, W. Kruizinga, W. Van Ommen, and J. Rozing (1984) Toxicity of bis(tri-*n*-butyltin)oxide in the rat II. Suppression of thymus-dependent immune responses and parameters of non-specific resistance after short term exposure. *Toxicol. Appl. Pharmacol.*, 75, 387.

Vos, J.G., A. de Klerk, E.I. Krajnc, H. Van Loveren, and J. Rozing (1990) Immunotoxicity of bis(tri-*n*-butyltin)oxide in the rat: effects on thymus-dependent immunity and on non specific resistance following long-term exposure in young versus aged rats. *Toxicol. Appl. Pharmacol.*, 105, 144-155.

Wester, P.W., and J.H. Canton (1987) Histopathological study of *Poecilia reticulata* (guppy) after long-term exposure to bis(tri-*n*-butyltin)oxide (TBTO) and di-*n*-butyltinchloride (DBTC). *Aquat. Toxicol.* 10, 143-165.

Wester, P.W., J.H. Canton, A.A.J. Van Iersel, E.I. Krajnc, and H.A.M.G. Vaessen (1990) The toxicity of bis(tri-*n*-butyltin)oxide (TBTO) in small fish species *Oryzias latipes* (medaka) and *Poecilia reticulata* (guppy). *Aquat. Toxicol.* 16, 53-72.

Wester, P.W., and J.G. Vos (1994) Toxicological pathology in laboratory fish: an evaluation with two species and various environmental contaminants. *Ecotoxicology*, 2, 21-44.

Wishkovsky, A., E.S Mathews, and B.A. Weeks (1989) Effect of tributyltin on the chemiluminescent response of phagocytes from three species of estuarine fish. *Arch. Environ. Contam. Toxicol.*, 18, 826-831.

Zeeman M (1986) Modulation of the immune response in fish. *Vet Immunol Immunopath.*, 12, 235-241.

Table 1 *Toxicity study of TBTO in flounder: experimental design.*

	Experiment		
	1	2	3
TBTO conc.	0, 3.2, 10 and 32 µg/l	0, 3.2, 10 and 32 µg/l	0 and 32 µg/l
No. of animals/group	10	10	30
Duration	7 and 14 days	28 days	6 days
Silversand added	No	Yes	Yes

Table 2 Analysis of organotin compounds in the water^a.

TBTO conc.	Experiment 2						Experiment 3										
	0 µg/l		10 µg/l		10 µg/l ^b		0 µg/l		0 µg/l		32 µg/l						
	MBT	DBT	TBT	TBTO	MBT	DBT	TBT	TBTO	MBT	DBT	TBT	TBTO	MBT	DBT	TBT	TBTO	
t=0 hours	n.d.	n.d.	n.d.	n.d.	0.015	0.03	3.09	7.74	0.02	n.d.	n.d.	9.94	n.d.	n.d.	n.d.	n.d.	17.75
t= 6 hours					0.02	0.08	2.68	6.73					n.d.	n.d.	n.d.	n.d.	7.99
t= 48 hours	n.d.	n.d.	n.d.	0.03	0.2	0.125	0.24	0.58	0.15	0.04	0.1	0.25	n.d.	n.d.	n.d.	n.d.	1.89

^a MBT (monobutyltin), DBT (dibutyltin) and TBT (tributyltin) were measured by atom absorption spectrometry and expressed as µg Sn/kg water. TBTO (bis(tri-*n*-butyltin)oxide) levels were calculated from TBT levels and expressed as µg TBTO/kg water.

n.d.= not detectable; detection limit was 0.01 µg/kg (exp. 2) or 0.005 µg/kg (exp. 3).

^b without silversand

Table 3 *General toxicity parameters (weight, length and condition factor) of flounder exposed to TBTO for 28 days (experiment 2)^a.*

TBTO conc.	Length (cm)		Weight (gram)		Condition factor ^b	
	D0	D28	D0	D28	D0	D28
0µg/l	9.0 ± 0.58	9.10 ± 0.58	12.25 ± 2.45	13.06 ± 2.49	1.67 ± 0.21	1.71 ± 0.11
3.2 µg/l	8.55 ± 0.76	8.79 ± 0.83	10.62 ± 2.83	11.94 ± 3.25	1.70 ± 0.29	1.72 ± 0.09
10 µg/l	8.85 ± 0.91	9.06 ± 0.91	13.19 ± 4.22	12.19 ± 3.31	1.86 ± 0.28	1.61 ± 0.12 [*]

^a Mean values ± standard deviation of 10 animals per group

^b Condition factor = 100(body weight)/length³

^{*} Statistically significant (Student's t-test) p < 0.03

Table 4 *General toxicity parameters (length, weight, liver weight, condition factor and hepatosomatic index) of flounder exposed to TBTO for 6 days (experiment 3)^a.*

TBTO conc.	Length (cm)	Weight (gram)	Liver Weight (gram)	Condition factor ^b	Hepatosomatic index ^c
0µg/l	6.36 ± 0.54	4.88 ± 1.33	0.053 ± 0.014	1.69 ± 0.14	0.011 ± 0.004
32µg/l	6.74 ± 0.47	5.11 ± 0.95	0.073 ± 0.023 [#]	1.66 ± 0.12	0.015 ± 0.005 [*]

^a Mean values ± standard deviation of 30 animals per group

^b Condition factor = 100(body weight)/length³

^c Liver weight/body weight

[#] Statistically significant (Student's t-test) p < 0.001

^{*} Statistically significant (Student's t-test) p < 0.005

Table 5 *Incidence of histopathological gill lesions in flounder exposed to TBTO for 6 days (experiment 3).*

	0 µg TBTO/l (n=30)	32 µg TBTO/l (n=27)
Budding of epithelial cells		
absent	28	5
rare	2	10
frequent	0	12
Epithelial proliferation/ fusion of lamellae		
absent	10	2
mild	14	4
marked	6	21

Table 6 *Cell counts and mitogen responsiveness of leukocytes to PHA in the spleen of flounders exposed to TBTO in experiment 3^a.*

TBTO conc.	Total cell number ^b	Lymphocyte number ^b	% lymphocytes	PHA \odot cpm ^c
0 μ g/l	5.9 \pm 2.1	1.9 \pm 0.7	35.9 \pm 6.1	307 \pm 329 (59- 774)
32 μ g/l	5.0 \pm 4.7	0.8 \pm 0.3 [*]	25.6 \pm 3.2 [#]	321 \pm 202 (148-645)

^a Mean values \pm standard deviation of 7 animals per group

^b $\times 10^6$

^c The mitogen responsiveness is expressed as counts per minute (\odot cpm; the proliferation of unstimulated cultures has been subtracted). The range is given in brackets.

^{*} Statistically significant (Student's t-test) $p < 0.005$

[#] Statistically significant (Student's t-test) $p < 0.01$

Table 7 *NCC activity of mesonephros leukocytes at different effector to target (E/T) ratios of flounders exposed to TBTO in experiment 3.^a*

TBTO conc.	E/T 5	E/T 10	E/T 20
0 μ g/l	8.7 \pm 10.5 (1-31)	19.6 \pm 15.7 (5-50)	28.7 \pm 18.3 (11-56)
32 μ g/l	2.2 \pm 1.8 (0-4) [*]	4.4 \pm 3.4 (0-8) [*]	8.7 \pm 7.0 (1-18) [*]

^a Mean values are expressed as percentage specific release \pm standard deviation of 7 animals per group, the range is given in brackets.

^{*} Statistically significant (Student's t-test) $p < 0.02$

Table 8 *Thymus volume, and relative thymus volume of flounder exposed to TBTO for 6 days calculated from morphometric analysis (experiment 3)^a.*

TBTO conc.	Thymus volume (mm ³)	Body height (cm)	Relative Thymus volume ^b
0 μ g/l	0.394 \pm 0.10	2.5 \pm 0.2	0.024 \pm 0.005
32 μ g/l	0.339 \pm 0.11	2.6 \pm 0.1	0.019 \pm 0.007 [*]

^a mean \pm standard deviation in mm³ of 13 (0 μ g TBTO/l) or 10 (32 μ g TBTO/l) animals.

^b Thymus volume/[body height just caudal of pectoral fin]³

^{*} Statistically significant (Student's t-test) $p < 0.05$

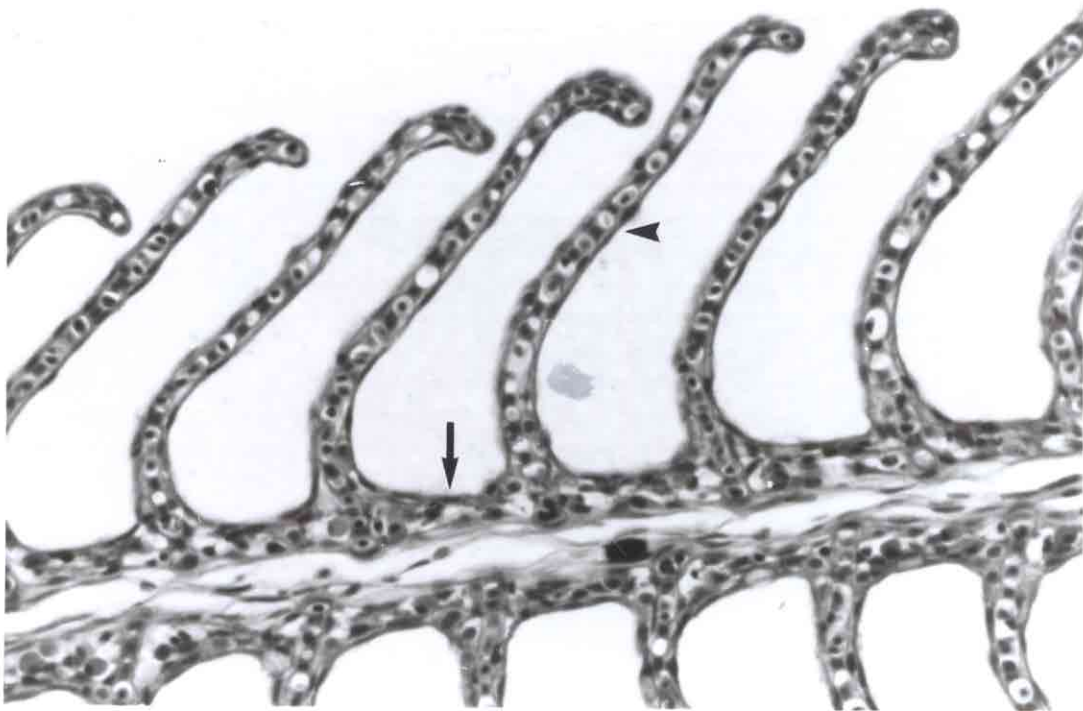


Fig. 1.
Gill of a control animal with normal primary (arrow) and secondary (arrowhead) lamellae. *H&E, obj. 40x.*

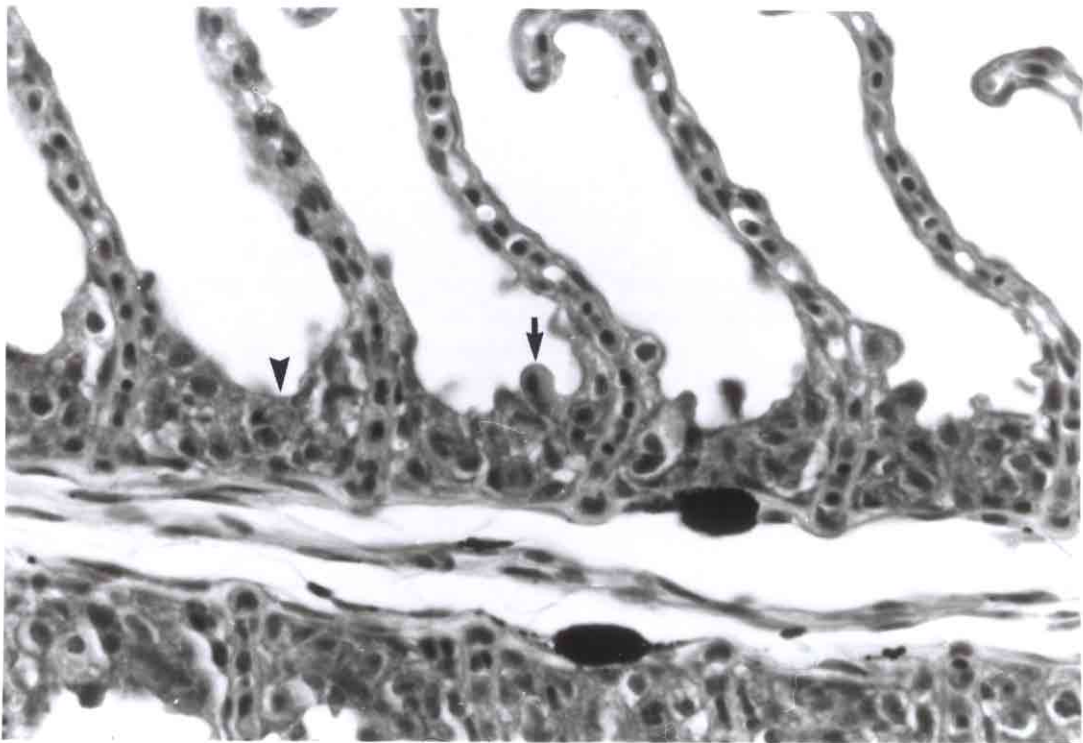


Fig. 2.
Gill of an animal exposed to 32 µg TBTO/l for 6 days showing budding (arrow) of epithelial cells, also some epithelial hyperplasia is present (arrowhead). *H&E, obj. 60x.*

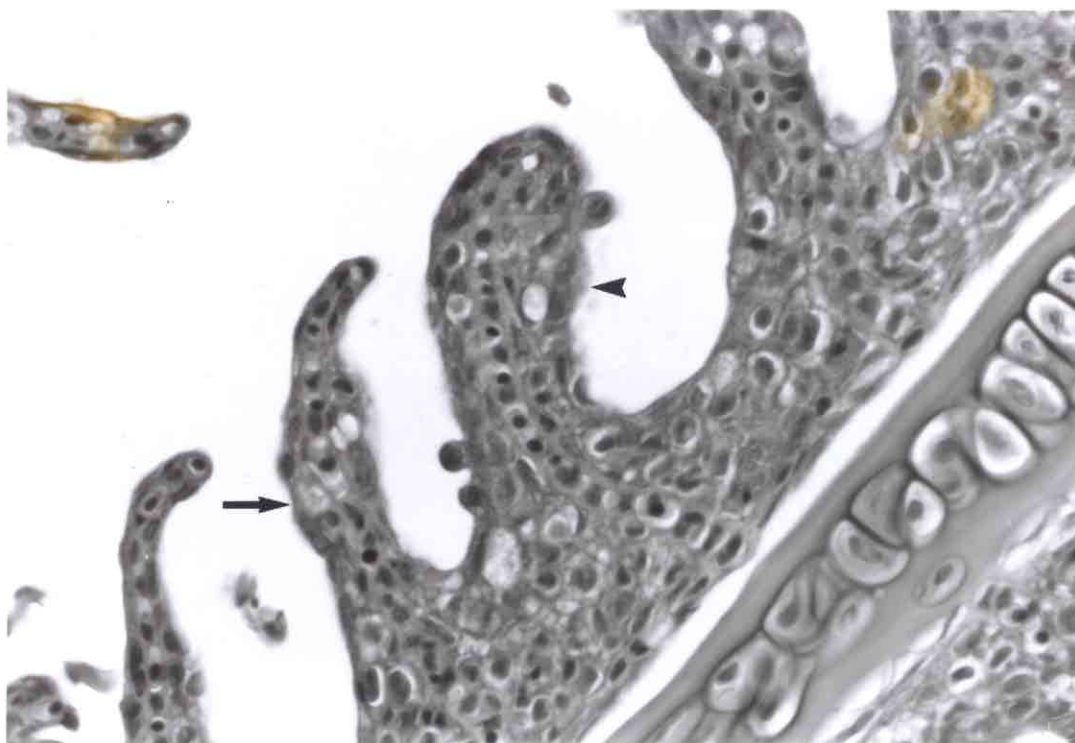


Fig. 3.
Gill of an animal exposed to 32 μ g TBTO/l for 6 days with mild (arrow), and marked (arrowhead) epithelial hyperplasia. Also budding of epithelial cells is visible. *H&E, obj. 60x.*

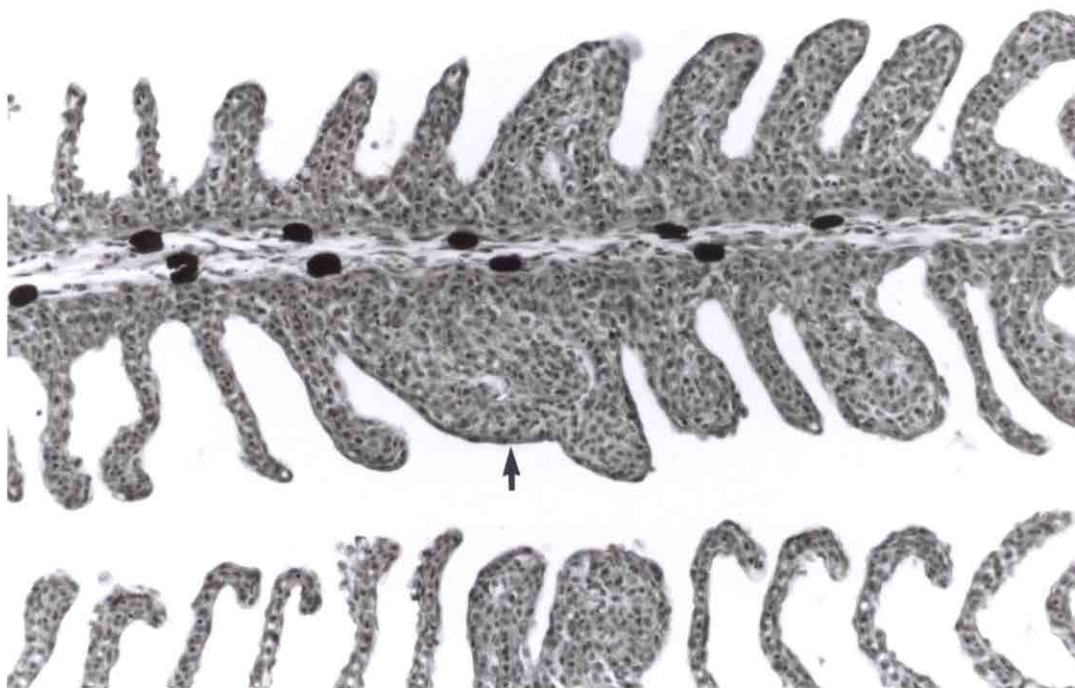


Fig. 4.
Gill of an animal exposed to 32 μ g TBTO/l for 6 days with epithelial proliferation and fusion of secondary lamellae (synechia; arrow). *H&E, obj. 20x.*

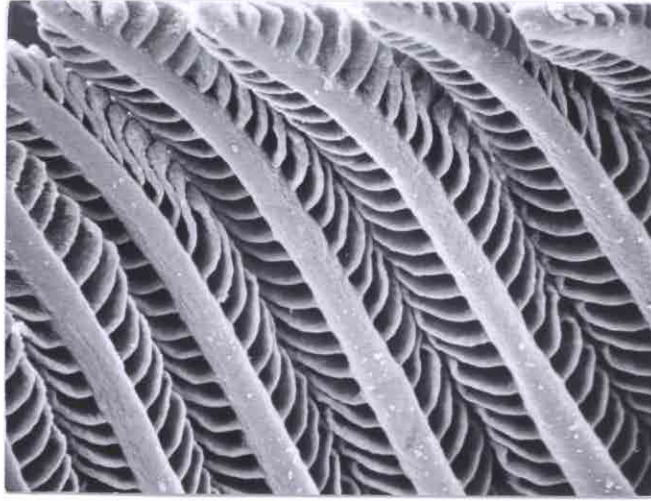


Fig. 5. Gill of a control flounder showing six primary. Each primary lamella has many secondary lamellae on both sides. The surface, covered with epithelium, is smooth. *SEM, 160x.*

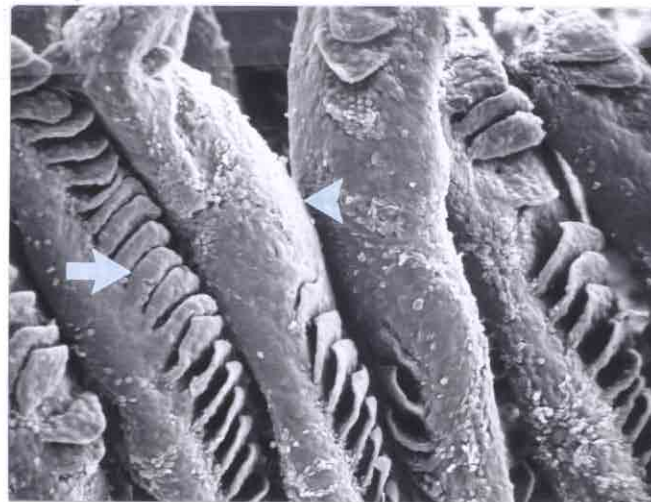


Fig. 6. Gill of a flounder exposed to 32 µg TBTO/l for 6 days. Six primary lamellae are visible showing marked thickening (arrow) and fusion of many secondary lamellae (arrowhead). Note also a rougher surface of the thickened parts. *SEM, 160x.*

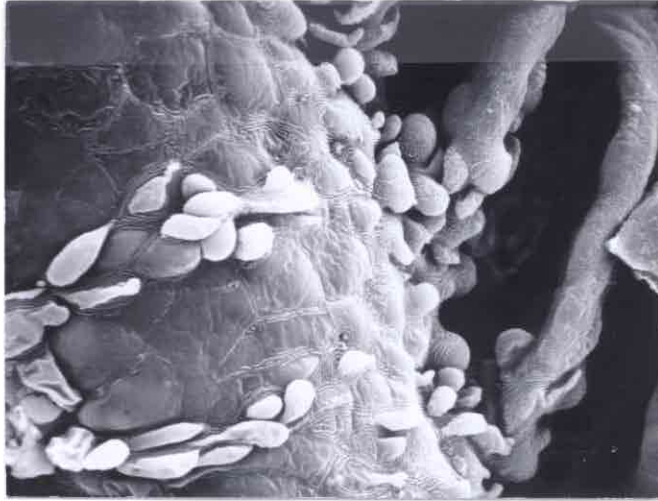


Fig. 7. Gill of a flounder exposed to 32 μ g TBTO/l for 6 days. Detail of primary and secondary lamellae with swelling and frequent budding of epithelial cells resulting in an irregular surface. *SEM. 1250x.*

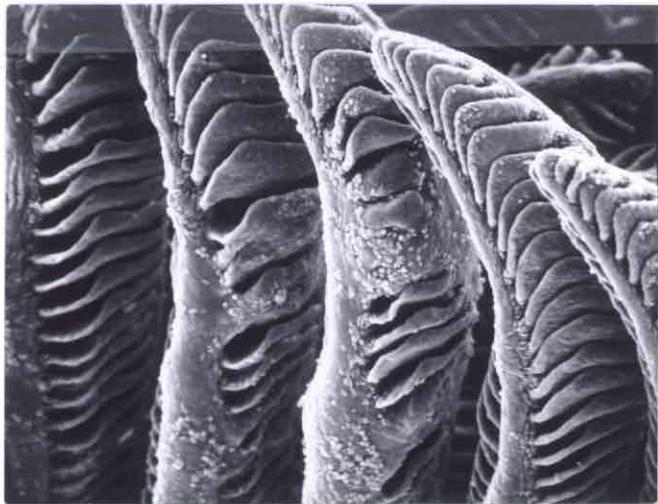


Fig. 8. Gill of a flounder exposed to 32 μ g TBTO/l for 6 days. Five primary lamellae are visible, showing a varying degree of fusion between secondary lamellae. Also swelling and budding of epithelial cells is seen on the surface of primary lamellae. *SEM. 160x.*