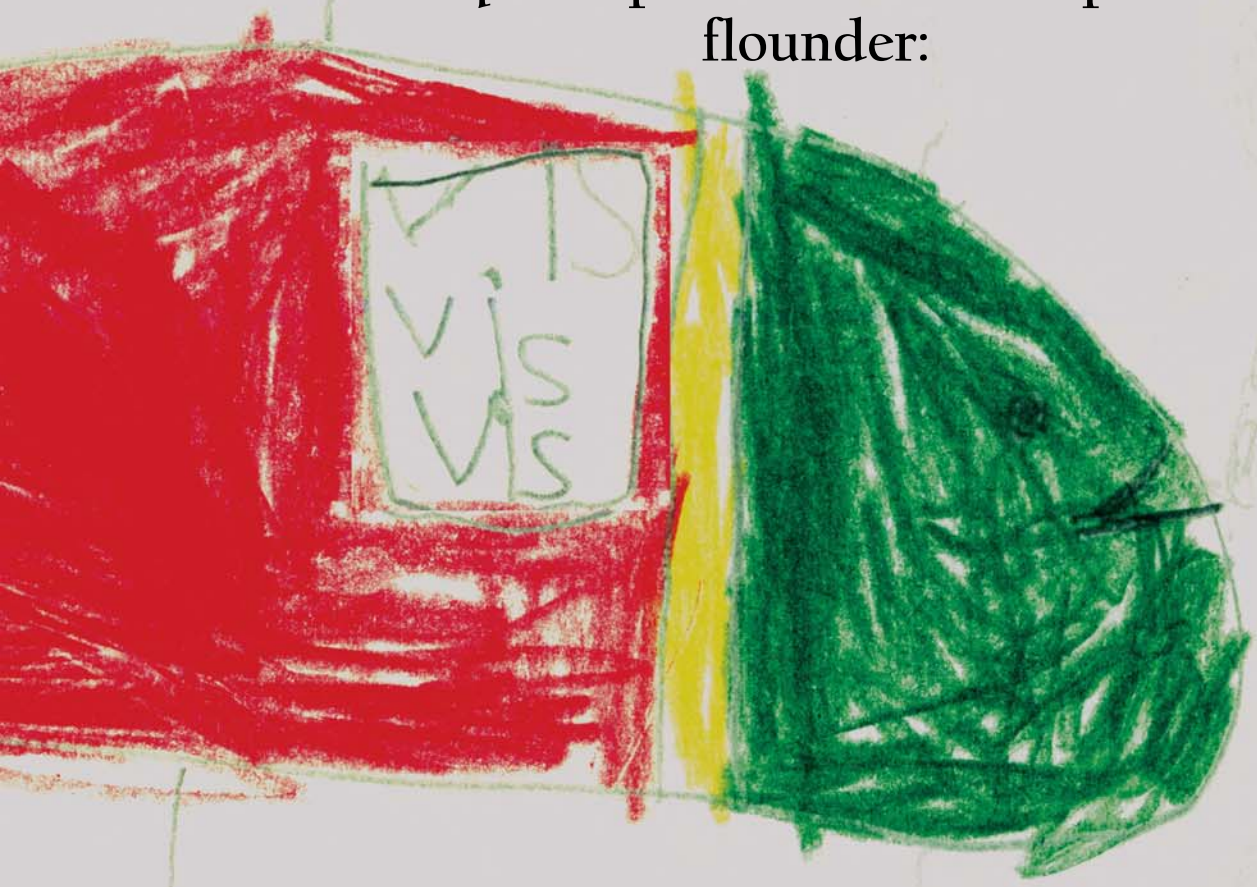
Health effects of some major aquatic pollutants in European flounder:



Laboratory experiments with emphasis on histopathological and immunological aspects

Guy Grinwis

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Health effects of some major aquatic pollutants in European flounder:

Laboratory experiments with emphasis on histopathological and immunological aspects

Gezondheidseffecten van enkele belangrijke verontreinigende stoffen in het aquatische milieu in de bot:

Laboratorium experimenten met de nadruk op histopathologische en immunologische aspecten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 29 mei 2006 des middags te 12.45 uur

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geboren op 26 mei 1962 te Utrecht, Nederland.

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Co-promotoren:	Dr. A.D. Vethaak Dr. P.W. Wester

Contents

Chapter 1

Introduction

Based on:

Toxicology of environmental chemicals in the flounder (*Platichthys flesus*) with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies

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1. General remarks

This thesis is part of a large-scale research project in which several research institutes have participated. The primary driving force for this project was provided by reports of epizootics of grossly visible disease signs in fish in the North Sea. Because of concern with the possible contribution of pollution-mediated diseases to fish mortality and population declines, these diseases and abnormalities of marine and estuarine fish populations have received much attention. Moreover, they could be suitable indicators for monitoring anthropogenic environmental stress (Vethaak and ap Rheinallt, 1992). Studies of fish diseases in relation to pollution were designed to answer the following questions:

1. in general terms, are changes in disease patterns associated with changes in environmental quality (field surveys) ?
2. can we identify cause-and-effect relationships between disease patterns and exposure to the complex mixture of chemical pollutants present in the environment (semi-field experiments) ?
3. can we identify cause-and-effect relationships for specific chemical contaminants (laboratory experiments) ?

The answers to these questions are highly relevant to the potential use of disease as a biomarker of contaminant effects. The questions mentioned above were addressed in an integrated study strategy (Fig. 1) in which field, semi-field and laboratory studies with the euryhaline fish species European flounder (*Platichthys flesus*) were carried out in The Netherlands. Each type of study has its strengths and disadvantages regarding the relevance of its results to natural populations and to relationships between pollution and disease (correlative or causal), but combined they are complementary.

Both field and semi-field studies have been performed in the past. This thesis reports the results of laboratory experiments, the missing link between correlative and causal evidence.

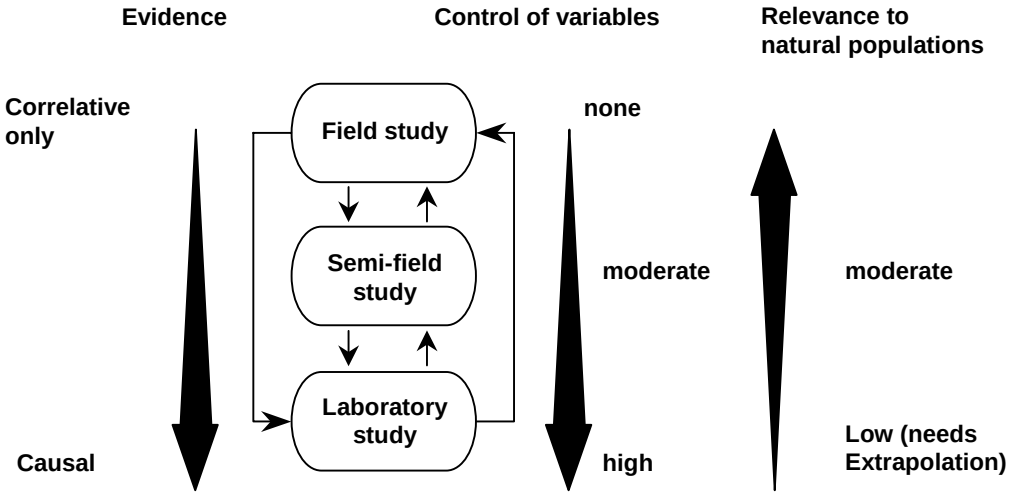

Fig. 1. Integrated approach to study the causes of wildlife diseases (According to Vethaak, 1993).

2. Field experiments

Systematic and routine epizootiological studies in Dutch coastal fish populations were initiated in the early 1980s (Van Banning, 1987; Vethaak, 1987, 1992, 1996; Vethaak and Jol, 1996; Vethaak and Wester, 1996). In these studies attempts were made to correlate the presence of fish disease with chemical pollution and other anthropogenic stressors. European flounder, a coastal and estuarine fish which is widespread in Europe, was chosen as indicator species primarily because of their high disease prevalence (Vethaak and ap Rheinallt, 1992; Bucke et al., 1996). As a result of these studies, a number of pollution-associated diseases/abnormalities were incorporated within (inter)national fish disease monitoring programs. In 1991, these studies became part of the ongoing OSPAR Joint Assessment and Monitoring Program (JAMP). In the guidelines for contaminant-specific biological effects monitoring, European flounder is included as one of the sentinel fish species (Wosniok et al., 1999). The field surveys have shown relatively high prevalences of three grossly visible diseases in European flounder:

- skin ulcers
- lymphocystis disease
- liver tumors.

Various authors have attributed the development of **skin ulceration** in fish to a variety of agents such as viruses, bacteria of a range of genera, and parasitic infections. In the case of flounder, bacteriological examination has resulted in the isolation of bacteria of the genera *Vibrio*, *Pseudomonas*, and *Aeromonas* from the lesions and other tissues of affected fish (reviewed by Wiklund, 1994). Experiments carried out with Baltic flounder have shown that bacteria cultured from diseased fish can infect mechanically abraded skin and contribute to the development of skin ulcers (Wiklund, 1994). Vethaak (1992) carried out bacterial analyses at two sites adjacent to drainage sluices in the Dutch Wadden Sea, one with a much higher prevalence of skin ulcers than the other. Results showed no clear association between bacterial levels in the sediment and bacterial infections of the fish. Nevertheless, the sites with the highest bacterial loads in the water also showed the highest prevalences of ulcers. These findings indicate that in addition to high bacterial loads in the environment, other factors might be of influence such as abrupt and extreme salinity fluctuations (causing osmotic stress) and nutritional deficiencies. It was proposed that a combination of these factors operating near drainage sluices might have had an adverse effect on the immune system of the fish, making them vulnerable to opportunistic bacterial infection (Vethaak, 1993). Recent studies in the vicinity of fresh water drainage sluices have indicated an association of flounder ulcers and certain liver diseases with liver contaminant levels, including PCBs and organotin compounds (Vethaak et al., 2004), supporting an additional role of chemical stress in the etiology of this disease.

Lymphocystis disease is known to be of viral origin. Although its pathology (Wolf, 1962, 1988) and epizootiology are well documented, relatively little is known about the immunological aspects of the disease (Russel, 1974). Lymphocystis disease was found with an average prevalence of 14.3% in the field in 1983-1989 (Vethaak and Jol, 1996), and some evidence for a role of contaminants in the pathogenesis for this disease was found. However, the hypothesis that contaminants may contribute to the development of lymphocystis disease through immunosuppression needs to be

addressed.

Liver tumors and associated lesions were reported in flounder in the early 1980s (Vethaak, 1987). These tumors were present in 1.0% of the fish sampled during the study, prevalences rising steeply with age and locally attaining values of up to 30% in the 6-year group and older fish (Vethaak and Jol, 1996). The majority of these tumors were diagnosed as hepatocellular adenomas after histopathological examination, 13.1% were diagnosed as hepatocellular carcinomas (Vethaak and Wester, 1996). Routine histopathological examination of livers showing no gross pathology resulted in the identification of a range of lesions including hepatocellular adenomas, foci of cellular alteration, regenerative foci, inflammatory lesions, focal necrosis, hydropic vacuolated hepatocytes and bile duct epithelium, and fibrillar hepatocytes (Vethaak and Wester, 1996).

From these retrospective field studies a possible contribution of pollutants to multifactorial disease causation could not be adequately assessed due to interfering factors such as fish migration, salinity, and fishing impact. Disease prevalences showed a strong correlation with fishing activity (possibly indicating an effect of damage by fishing gear) and appeared to be positively related to salinity, but there appeared to be little correlation with concentrations of contaminants in sediments or tissues. However, when only strictly marine sites were considered, a relation with pollution could not be ruled out (Vethaak and Jol, 1996).

In an attempt to overcome the shortcomings of epizootiological field studies in investigating the role of environmental contamination, a large-scale mesocosm study of disease in flounder was initiated in 1990 on the island of Texel in the Netherlands.

3. Semi-field (mesocosm) experiments

This three-year (1990–1993) prospective semi-field study involved the exposure of single cohorts of European flounder, obtained from a relatively clean site, to contaminated dredge spoil (representative for the general pollution load of the river Rhine) in three large-scale mesocosms (40 x 40 x 3m) (Vethaak et al. 1996). The advantage of using mesocosms is that they act as an intermediate between controlled laboratory studies and field studies (Fig. 1). The mesocosm approach allows us to study the long-term development of diseases within a single cohort of fish under ‘natural’ conditions with reduced or eliminated interference from other factors including migratory behavior of the fish, fishing activity and salinity changes (Vethaak et al. 1996). Two of the mesocosms used in the experiment contained clean sand whilst the third, sharing a common water circulation with one of the clean sand mesocosms, was stocked with contaminated dredged spoil from Rotterdam harbor. In this way, one of the clean sand mesocosms was indirectly polluted via the water phase, and analysis of contaminant concentrations showed it to have a status intermediate between the other two, with polychlorinated biphenyls (PCBs) and selected polycyclic aromatic hydrocarbons (PAHs) showing a clear concentration gradient across the three mesocosms (Vethaak et al., 1996). Analysis of the PAH metabolite 1-hydroxy (1-OH) pyrene in the bile of the flounder, using synchronous fluorescence spectrometry, showed a similar gradient (Ariese et al., 1993). Random samples of flounder from the indirectly polluted and reference mesocosms were examined every 2 months for epidermal diseases (lymphocystis, skin ulcers and fin rot) and then released back into the mesocosms. In addition, some fish from all three

mesocosms were sacrificed every 6 months for histological and other investigations. The prevalences of skin-related diseases (lymphocystis disease and skin ulcers) in the reference and indirectly polluted mesocosms showed a similar pattern of temporal variation, and were rather high compared with values observed in the field. Little difference in the prevalence of skin ulcers was found between the reference and the indirectly polluted mesocosm. In contrast, prevalences of lymphocystis disease were consistently higher in the indirectly polluted mesocosm during the latter half of the 3-year period. Additionally, a significant reduction in the number of antibody-positive fish in the mesocosm containing polluted harbor sludge compared to fish in the indirectly polluted mesocosm or reference mesocosm was detected (Dixon et al., 1995). In flounder from the directly and indirectly polluted mesocosm, a significant reduction of vitamin A levels was found accompanied by high PCB tissue-levels and increased cytochrome P4501A (CYP1A) protein levels in these fish, indicating a possible involvement of aryl-hydrocarbon receptor (AhR) mediated biotransformation enzymes in causing the observed Vit A reductions (Besselink et al., 1997). CYP1A activity was measured in flounders from the mesocosm study, using CYP1A-mRNA levels as well as ethoxyresorufin-O-deethylase (EROD) activity as indices (Eggens et al., 1996). Despite the elevated levels of PCB-153 in the liver and 1-OH pyrene in the bile of fish from the polluted mesocosm, the only biomarker that was significantly induced in these mesocosms (by comparison with the reference mesocosm) was CYP1A-mRNA in female fish, not in male animals. This provided a contrast with plaice (*Pleuronectes platessa*) sampled at the same time, where both EROD and CYP1A protein level showed significant induction.

Strongest evidence for induction by contaminants was found for liver tumors and preneoplastic foci. Evidence for a chemical carcinogenesis in the mesocosm experiment was further substantiated by a correlation between biliary hydroxy-metabolites of carcinogenic PAHs, such as benzo[a]pyrene and the occurrence of PAH-DNA adducts (Vethaak et al., 1996).

4. Laboratory experiments

From both field and mesocosm experiments the following **hypothesis** was formulated:

Exposure to major environmental chemical contaminants cause adverse health effects in European flounder including:

- *immunodeficiency, making individuals more susceptible to infectious diseases*
- *induction of (pre)neoplastic liver lesions.*

In order to provide evidence for causal links between specific environmental chemicals and fish disease, laboratory experiments were incorporated in the integrated study. This type of experiments was considered to be an essential addition to field and semi-field studies (Fig. 1). Therefore European flounder had to be kept and raised in captivity in order to perform experiments under laboratory conditions.

4.1. *Experimental animals*

Several authors have obtained fertilized eggs of flounder by artificial fertilization of eggs stripped from mature specimen caught on spawning grounds (Solemdal, 1967;

Von Westernhagen, 1970), but to our knowledge at the time our laboratory studies started no attempts had been made to reproduce and rear flounder on a large scale in captivity (Grinwis et al., 1995).

For the first series of laboratory experiments we therefore relied on collection of fingerlings from feral populations. Animals used in these experiments were caught with a dip-net in a relatively clean estuary near Southampton (England) during a short period in spring, and belonged to the 0+ group (animals younger than 1 year) and measured ~ 1 cm upon arrival at the laboratory.

For the second set of laboratory experiments captive-bred European flounder were obtained as fingerlings (1- group) from the Port Erin Marine laboratory, School of Biological Sciences, University of Liverpool (UK).

4.2. *Water quality and holding facilities*

European flounder is an euryhaline species that normally spends a large part of its life-cycle in fresh or brackish water, but requires marine conditions for spawning (Vethaak and Jol, 1996). As a result flounder can be kept in fresh as well as seawater. A bottom-substrate is essential in a tank with flounders. Fish kept in tanks without a bottom-substrate showed continuous bacterial and parasitic problems that could interfere with the experiments and were difficult to treat. Moving fish to a sand-substrate removed these disease problems almost immediately.

At the Dutch National Institute of Public Health and the Environment (RIVM), where the first series of laboratory experiments was performed, the animals were kept in glass aquariums filled with fresh water (Dutch Standard Water (DSW) at 19 ± 2°C under a 16 h light and 8 h dark regimen. The water was circulated and aerated continuously, and passed over a biological filter. Temperature was checked daily, pH, oxygen and NO_2^- contents of the water were checked weekly. The population density depended on the size of the animals, and ranged from maximum of 30 animals per 25 l for animals between 2 and 5 cm, to 10 animals per 100 l for the larger category. The bottoms of the aquaria were covered with a 2 cm layer of silver sand in order to decrease the above-mentioned problems. During a quarantine period of several weeks the animals were monitored in particular for signs of illness. Clinically sick animals were treated once in a water basin containing 324 µl formalin (35% formaldehyde solution) and 0.5 mg malachite-green per liter DSW for 30 min, against bacterial-, mycotic-and parasitic infections. The animals were checked daily for gross abnormalities, condition and behavior.

The second series of laboratory experiments was performed at the Dutch National Institute for Coastal and Marine Management (RIKZ) field station at Jacobahaven, Netherlands, where the test animals were raised for one to two years in glass aquaria in a flow-through system containing 10kg of relatively clean sandy sediment (obtained from the Eastern Scheldt) on a 70x100 cm bottom, and 160 l seawater. The water was renewed by continuous flow-through with salt water of 32 ‰ from the relatively clean Eastern Scheldt at a rate of 175 l per day. Water temperature was 16± 1°C and the animals were subjected to a 12-12 hours dark-light regimen and inspected daily for behavioral changes and signs of disease. Following these procedures flounder was kept and raised in the laboratory without major problems.

4.3. *Nutrition*

Flounder can be fed with a variety of natural food items. Artificial compound feed is

usually not attractive for flounder although flounder has successfully been adapted to commercial sheath-fish feed. During the laboratory experiments at the RIVM facility the animals were fed *Artemia salina* (Rasbora, Veenendaal, The Netherlands), and pelleted feed (Trouvit; Seafarm, Kamperland, the Netherlands) at the Jacobahaven field station.

5. Topographical anatomy, histology and immune system

The normal topographical anatomy and histology of flounder was studied with emphasis on the gills, liver, the hematopoietic system and the gonads at the beginning of the laboratory experiments (Grinwis et al., 1995).

It is known from mammalian studies that the immune system is sensitive for the effects of pollution and we hypothesized a possible effect of environmental pollutants on the immune system of flounder as a predisposing factor for the high disease prevalence encountered in feral flounder populations. Immune parameters could therefore be valuable to evaluate the health condition of fish and to monitor the possible effects of pollutants on the organism. Therefore a more detailed description of the hematopoietic system is given below.

5.1. *Immune system of teleost fish*

The fish immune system shows, to a certain extent, a resemblance with the defense system of mammals. Innate, nonspecific immune reactions are usually the first defense mechanism and can be provoked by invasion by foreign organisms and/or trauma. Phagocytic cells (granulocytes and mononuclear phagocytes) and several mediators play an important role (Zelikoff, 1994). In fish, non-specific cytotoxic cells (NCCs), the counterpart of mammalian natural killer cells, have been identified. These cells belong to a leukocyte subpopulation of large granular lymphocytes and play an important role in immunosurveillance and are able to lyse tumor cells and cells infected by viruses. The NCCs are found mainly in the head kidney, spleen and peripheral blood (Evans et al., 1989; Faisal et al., 1989; Greenly et al., 1991).

The adaptive, specific immune defense mechanisms of fish include cellular and humoral responses and are comparable to the mammalian mechanisms (Van Muiswinkel et al., 1991; Zelikoff, 1994). In fishes, antibodies are produced by B-lymphocytes and can be demonstrated in the serum, bile, and mucus of the skin. The antibodies are of a single class, comparable with IgM of mammals, but are structurally tetrameric instead of pentameric as in mammals (Rombout et al., 1990). During the period that laboratory experiments were performed, antibodies to distinguish lymphocyte subpopulations in European flounder were not available. Thymus, spleen and kidney (the latter containing the bone marrow homologue in fish) are the major organs in the fish immune system, while bone marrow and lymph nodes are absent. Like in mammals the bursa of Fabricius, the central lymphoid organ for B-cells in birds, is lacking.

5.2. *Thymus*

The thymus can be found in all vertebrates, except agnatha, and shows no essential histological variations between vertebrates. In European flounder, the thymus is a paired structure localized in close association with the gill cavity (Grinwis et al., 1995). Due to its irregular shape and small size, reliable weighing of the thymus, a

procedure often used in mammals to detect thymotoxicity is practically not feasible. Also, no clear distinction between cortex and medulla, as seen in mammals, is present in the thymus of European flounder. Therefore, we used morphometric analysis of serial sections of the thymus in our experiments from which the thymus volume was calculated (Grinwis et al., 1998).

5.3. *Spleen*

The spleen is a rounded organ with a dark red to black color. It is situated in the mesentery next to the stomach, guts, liver, and pancreas (Grinwis et al., 1995). The histology of the spleen is comparable with that of mammals: red pulp, white pulp, vessels and ellipsoids can be distinguished (Ellis et al., 1989; Zapata and Cooper, 1990). The red pulp constitutes the major part of the spleen with a cellular reticulum, hematopoietic tissue (which usually shows little activity) and sinusoids. The white pulp is typically not prominent in our experimental animals. Ellipsoids are the terminal capillaries surrounded by a cuff of fibrous tissue with mainly macrophages. In the red pulp larger groups of pigment containing macrophages, the melano-macrophage centers (MMCs) can be found.

5.4. *Kidney*

The kidney of teleosts is a multifunctional organ in which four functional aspects can be distinguished. The kidney has an excretory and a hematopoietic function, plays a role in the immune system and produces hormones (Ellis et al., 1989; Zapata and Cooper, 1990). The kidney can be divided into two parts, the head kidney and the trunk kidney, and is situated retroperitoneally ventral to the vertebral column. The main portion of the head kidney consists of lymphoid, hematopoietic and adrenal (suprarenal) tissue; lymphoid and hematopoietic tissue is also present in the trunk kidney. MMCs can be encountered in both parts of the kidney.

5.5. *Blood*

Teleosts have a small blood volume in relation to the body weight. Usually May–Grünwald Giemsa stained blood smears are used for cytologic evaluation of the blood. The main cellular component of the blood is the erythrocyte. Like in amphibians, reptiles and birds the red blood cells of fish contain nuclei. The cells and nuclei are more or less oval shaped. Several leukocytes can be found in the blood of fish: lymphocytes, granulocytes monocytes, and thrombocytes. An important problem in using blood smears for lymphocyte quantification in fish is the difficulty of distinguishing lymphocytes and thrombocytes as they can have a similar morphology. This might be the main reason for the widely differing data on numbers of circulating lymphocytes within the same species as reported in the literature (Ellis, 1977; Rowley, 1990). Data on lymphocyte counts from May–Grünwald Giemsa stained blood smears are therefore rather unreliable. In order to use blood (smears) for the quantification of lymphocytes, specific immunological reagents are required, but those are not yet available for the flounder.

6. Immune function tests and infection model

To address the putative immunotoxic effects of xenobiotics, attempts were made to adapt ex vivo/in vitro immune function test for the use in flounder, and experiments

were conducted to develop a viral infection model.

6.1. *Immune function tests*

Several laboratory techniques have been described to assess the function of the immune system in fish (Stolen et al., 1990). Our experiments for the development of immune function test focused on the adaptation of existing protocols for mammalian species for the assessment of the mitogen-induced proliferation of lymphocytes and for the assessment of the NCC activity (Boonstra et al., 1996).

Like in mammals, in fish adaptive immunological reactions are mediated by B and T lymphocytes. Flounder lymphocytes were tested for their ability to proliferate following stimulation with the B cell mitogen lipopolysaccharide (LPS), or the T cell mitogen phytohemagglutinin (PHA). The leukocytes were cultured in the presence of LPS or PHA for 4 days; on the third day ^{3}H-thymidine was added. Leukocytes from the spleen, mesonephros and peripheral blood were stimulated with 0.03, 0.08, 0.17 mg/ml LPS (final concentration). As a reference, cells were incubated with medium alone. The highest degree of proliferation was observed in peripheral blood lymphocytes (PBL) compared to the response of spleen and mesonephros leukocytes, with an optimum of 0.08 mg/ml LPS. The mitogen responses of flounder leukocytes to the T cell mitogen PHA showed a completely different pattern than LPS. The spleen showed the highest degree of proliferation following incubation for 4 days with PHA. Its optimum concentration was 0.12 mg/ml PHA. PHA also stimulated the proliferation of the mesonephros leukocytes, but only at the highest concentration (0.30 mg/ml PHA). PBL did not show any response when stimulated with this T cell mitogen.

The activity of NCCs was measured in vitro by determining their ability to lyse a cultured radiolabeled target cell. The amount of radioactivity released is a measure for the number of cells killed by the NCCs. The NCC activity was determined in flounder at the age of 2.5 years (weighing ~ 50 g) at different incubation temperatures (20 vs. 26°C). There appeared to be a trend that the lower incubation temperature augmented the NCC activity as compared to the higher temperature. The effector cells were leukocytes isolated from the spleen, mesonephros and pronephros of flounder. After co-incubation of effector and target cells for 4 h, the mesonephros leukocytes were clearly capable of lysing YAC-1. At an effector to target (E/T) ratio of 25, 19% of the YAC-1 cells were lysed by the mesonephros leukocytes. A ratio of 100 resulted in killing of 37% of the target cells during the 4 h incubation period. The spleen and pronephros leukocytes on the other hand, showed a low NCC activity (< 4%) even at an E/T ratio of 100. Similar experiments performed using P815 or K562 as target cells in stead of YAC-1 showed that these cells were less sensitive targets for determining the cytotoxic activity of NCCs as compared to YAC-1. To ascertain whether the specific release measured could be attributed to the activity of NCCs various inhibition and stimulation experiments were performed. Especially the results obtained from the inhibition studies strongly suggest that the observed specific release following incubation of flounder mesonephros leukocytes with the target cells might indeed be contributed to the activity of NCCs. However, in all immune function tests the standard deviations were very high, which makes incorporation of these tests in experiments difficult.

6.2. *Lymphocystis infection model*

Experiments were performed to develop an infection model with the lymphocystis virus. This virus was chosen because it was one of the disease agents that showed a relatively high disease prevalence in field surveys. The agent belongs to the group of iridoviridae and can cause a chronic, self-limiting infection in fish. Several different virus strains have been recognized, and different strains are infectious to different fish species. The virus induces remarkable hypertrophy of infected fibroblast. Infected fibroblasts can become as large as 1 mm in diameter. The typical lymphocystis lesions are often found in the subcutaneous connective tissue in the fins, but lesions have also been described in internal organs (Nigrelli and Ruggieri, 1965). Although several papers have been published on morphological and virological aspects of the disease, little is known about the involvement of the immune system in the protection against infection with the virus (Russel 1974).

Our infection experiments were largely based on publications reporting the successful propagation of an infection with this virus in the bluegill (*Lepomis macrochirus*; Wolf, 1962) and the plaice (*Pleuronectes platessa*; Roberts, 1975). For exposure purposes, histologically verified lymphocystis virus-induced lesions were used, harvested from freshly caught European flounder and processed into a cell-free suspension. Ten fish were injected in the subcutaneous tissue with 0.1 ml of the virus suspension at a standardized site just dorsal of the vertebrae on a line connecting the dorsal and ventral fin, ten control animals were injected with 0.1 suspension medium only at the same site. Since no grossly visible lesions developed during a 3-month period, a second experiment was initiated. The animals were stressed by alternating the water salinity every 3 days, and animals were fed only once weekly instead of daily. Also, the virus suspension was applied by a multi-needle tattooing device in the tail and both dorsal and ventral fin. Again, no grossly visible lesions were noted during 3 months, rendering the used protocol not suitable as a host-resistance model for our purposes. Histological examination of the injection-site, however, did show typical lymphocystis cells indicating that the virus suspension used did contain infectious viral particles.

7. Chemical exposure experiments

From analyses of sediments and fish, combined with data on the effects in other (mammalian and non-mammalian) species, three (groups) of xenobiotics were identified as being of specific interest:

1. polycyclic aromatic hydrocarbons (PAHs; benzo[a]pyrene (BaP) and dimethyl benz[a]anthracene (DMBA),
2. bis (tri-*n*-butyltin)oxide (TBTO; see chapter 2 and 3)
3. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; see chapter 4) and 3,3',4,4',5 pentachlorobiphenyl (PCB-126; see chapter 5 and 6).

7.1. *PAHs*

BaP and DMBA are for instance formed by combustion of fossil fuels. They have both carcinogenic and immunotoxic properties (Zedeck, 1980; Ward et al., 1985; White et al., 1994) and occur widely, and are therefore important environmental contaminants.

Five short-term pilot exposure studies were performed in which European flounders

were exposed to BaP and DMBA. The fish were exposed through the water-phase with 28 µg BaP/l or 190 µg DMBA/l. These levels were much higher than the solubility in water, but the real bioavailability of the PAHs was not determined.

Flounder were also exposed by intraperitoneal injection (25 mg BaP/kg body weight (bw) or 12 mg DMBA/kg bw). Effects on behavior and appearance were monitored, and the effects on gills, thymus, hepatopancreas, digestive tract, pronephros and mesonephros, spleen, and ovary or testicles were examined histopathologically. Induction of the enzyme CYP1A as a measure for the biotransformation of PAHs and the formation of biologically active metabolites was determined immunohistochemically.

Exposure of flounder to the PAHs did not result in striking effects in spite of the high concentrations. Only a slight increase in immunoreactivity for CYP1A in hepatocytes of the injected animals was noted. Therefore we concluded that flounder appears to be not very sensitive to PAHs. However, to use this conclusion in hazard identification and risk assessment, the limitations of the exposure methods used in the present experiments and the fact that bioavailability and body burdens were not measured have to be taken into account. Because of the design of the experiments, possible effects of PAHs on tumor induction could not be adequately assessed.

Studies with TBTO, TCDD and PCB-126 are reported and discussed in the following chapters of this thesis.

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Chapter 2

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

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Abstract

The present study is part of a project that focuses on the relationship between environmental pollution and fish diseases. Field studies in various polluted coastal areas in Europe and the United States of America clearly indicate a relationship between pollution and the increase in prevalence of tumors and infectious diseases in fish. Research under controlled laboratory conditions is necessary to prove causal links between 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 effects on several organs (gills, skin, eye, liver, mesonephros, ovary/testis, spleen, and gastrointestinal tract) were examined using histopathology. 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 were in the same order of magnitude as maximum TBT levels measured in the field (experiment: 17.3 µg TBT/l; field: 7.2 µg TBT/l), caused mortality after 7–12 days, resulted in gill lesions, and induced significant reduction of the non-specific resistance. A significant decrease of the relative thymus volume, but no marked effects on the specific immune system were noted after exposure to TBTO.

1. Introduction

The present study is part of a fish disease project that focuses on the relationship between environmental pollution and fish diseases. Field studies in various polluted coastal areas in Europe and the United States of America clearly indicate a relationship between pollution and the increase in prevalence of tumors and infectious diseases in fish (Malins et al., 1984, 1985; Couch and Harshbarger, 1985; Murchelano and Wolke, 1991; Vethaak and ap Rheinallt, 1992; Myers et al., 1994; Vethaak and Jol, 1996). Field studies can be useful in providing data supporting the hypothesis of a causal relationship 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 harbor sediment. Exposed animals showed an increased prevalence of liver tumors and lymphocystis virus infection 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 potentially at risk. 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 utilized 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 stabilizers 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 7.2 µg/l in harbors 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 lymph nodes was observed (Seinen et al., 1977a,b; Krajnc et al., 1984; Snoeij et al., 1985). Functional impairment of the 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*), exposed animals 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 their 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 at levels ranging from 0.04

to 400 µg/l was found in Atlantic croaker (*Micropogonias undulatus*), hogchocker (*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*. Reduction of the non-specific cytotoxic cell (NCC) activity and a decreased phagocyte oxidative burst were also 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-induced pathology in this fish species. Also, scanning electron microscopy of the gills was carried out. Furthermore, effects of TBTO on the immune system of flounder were investigated to find a possible link between the increased prevalence of infectious disease in flounder under field conditions and exposure to TBTO. 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 color 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 to assess effects on immune function. An assay to quantify the activity of an important primary defense 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 ^{51}Cr-radiolabeled target cell. The amount of radioactivity released is a measure of the number of cells killed by the NCC cells. The target cell used in the cytotoxicity assay was a virus-trans-formed 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 or B cell mitogen (Boonstra et al., 1996). The degree of stimulation is a parameter for the function of the specific immune response.

The goal of the present study was to establish the target organs for TBTO-induced pathology, and examine its effects on the immune system (especially the thymus) of flounder to interpret the role of aquatic TBTO-pollution in disease problems in flounder in the field.

2. Materials and methods

2.1. *Collection and maintenance of experimental fish*

European flounders were initially caught with a dip-net during a short period in spring from an estuary near Southampton (UK). They belonged to the 0 + age-group and

measured approximately 1 cm upon arrival at the National Institute for Fisheries Research (RIVO-DLO, IJmuiden, The Netherlands) where the animals were raised. For the present experiments, animals (4–7 cm total length, 2–3 years of age) were acclimatized for at least 2 weeks under controlled conditions at the test laboratory as described previously (Grinwis et al., 1995) before they were used in the experiments. No signs of disease were found, clinically or histologically, in the batch of fish used for the experiments.

The fish were kept in groups of five (experiment 1) or 10 (experiments 2 and 3) animals in 100-l glass aquariums filled with 25 l of freshwater (Dutch Standard Water (DSW)). The water was aerated continuously. O_2 levels ranged from 5.8 to 9.2 mg/l, and pH levels ranged from 7.23 to 8.25. A water temperature of 19 ± 2°C and a 16-h light, 8-h dark regimen were maintained during the experiments. The animals were fed frozen *Artemia salina* (SELCO, Artemia Systems, Baas-Rode, Belgium) daily except on weekends. In experiments 2 and 3, 2.7 l of silversand (M32, van Roon-Vreeswijk, Nieuwegein, The Netherlands) were added to the aquariums 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 (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. 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 potassium tetrahydroborate solution was added to the acidified water samples (1 ml 37% HCl/l, Merck, Amsterdam, The Netherlands) in an oxygen-free environment. The volatile hydrides produced 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 atomic absorption spectrometer (AAS type 2380; wavelength 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.

2.3.1. *Experiment* 1

This experiment was used for dose range-finding. Four groups of 10 flounders each, five animals per aquarium, were exposed semi-statically 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 water, and two carrier-control groups were included in the experiment. The exposure medium was renewed every Monday, Wednesday and Friday. Clinical parameters like behavior, 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 methanosulfonate (MS222®, Sandoz, Basel, Switzerland), fixed in Bouin's fixative for 24 h and then transferred into 70% alcohol for histological investigation.

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

2.3.2. *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 index (100 × [body weight]/[length]^3) was calculated. The animals were fixed in 4% buffered formaldehyde.

2.3.3. *Experiment* 3

Based on the results of experiment 2 (the mortality rates in the highest dose group and the absence of histological lesions in the lower dose groups), this experiment was performed to determine acute target organ toxicity of TBTO at a concentration of 32 µg/l. The experiment was ended before mass mortality could 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 the spleen and mesonephros were collected for immune function tests as described below. The remaining parts were fixed in 4% buffered formaldehyde.

2.4. *Calculation of the LC_{50}*

The LC_{50} 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*

The selection of organs and tissues for histological examination was based on target organs of TBTO in other fish species (Wester and Canton, 1987; Wester et al., 1990; Wester and Vos, 1994). In experiment 1, the animals were fixed in Bouin's for 24 h after killing, and then transferred into 70% ethanol. Several transverse slices were made at standardized levels (Grinwis et al., 1995) and paraffin-embedded. The first level just caudal from the eyes, revealed the gill cavity, gills and the major part of the thyroid gland. The second level, halfway from the dorsal commissure and the caudal ridge of the operculum showed the gills, gill cavity, thymus, heart and head kidney. The third section was made through the pectoral fin where the hepato-pancreas, stomach, trunk kidney, spleen, and intestinal tract were situated. The fourth section was made just cranial of the caudal part of the body cavity where gonads, trunk kidney and intestinal tract were 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 hematoxylin and eosin.

The histological procedures in experiments 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 in experiment 3. 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 hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) according to routine procedures. Histological samples were examined using a 'blind' system in which the examiner was unaware of the exposure group each specimen came from.

For morphometric analysis of the thymus in experiment 3, a transverse block of each fish 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. Serial sections of 5 µm were made at intervals of 30 µm until the whole thymus was sectioned. 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 also related to the body-height (measured from side to side just caudal to the pectoral fin in a line perpendicular to the body axis), body length and body weight.

2.6. *Scanning electron microscopy*

After fixation in 4% buffered formaldehyde, the gills were postfixed in 2% glutaraldehyde, and in 2% osmium tetroxide, 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 aluminum 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 experiment 3, ex vivo/in vitro immune function tests were performed on mesonephric and splenic 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 900 × *g* at 20°C, cells at the interphase were collected and washed twice with fcRPMI for 10 min at 300 × *g* and 4°C. Finally, the pellet was resuspended in 250 µl fcRPMI. 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 10^7 lymphoid cells/ml were used for measurement of the NCC activity, and 5×10^6 lymphoid cells/ml for the assessment of the mitogen-induced proliferation (lymphocyte transformation test, 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 (YAC-1) at effector-to-target cell ratios ranging from 5 to 20. The plates were centrifuged in order to enhance cell to cell contact (200 × *g*, 5 min). Following a 4-h incubation at room temperature (20°C) in an atmosphere of 5% CO2–95% air, the plates were centrifuged (200 × *g*, 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].

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

3. Results

3.1. *Chemical analyses*

Actual aqueous concentrations were measured in experiments 2 and 3 in control and exposed groups at 0, 6 and 48 h after the start of exposure (Table 2). The recovery in experiment 2 at $t = 0$ was 77% in the 10 µg/l group with silversand and 99% in the 10 µg/l group without silversand. In experiment 3 only 55% of the TBTO was recovered

at *t* = 0 in the 32 µg/l group (with silversand). The actual TBTO concentration in the 32 µg/l group at *t* = 0 was 17.75 µg/kg water in experiment 3. In the same experiment the TBTO level dropped to 7.99 µg/kg water at *t* = 6 h and to 1.89 µg/kg water, which is approximately 10% of the *t* = 0 level, at *t* = 48 h. Levels of mono-and dibutyltin components were very low or even below the detection level.

3.2. *Clinical findings*

Behavioral changes were seen in the highest dose groups (32 µg/l) in experiments 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 signs did not clearly increase over 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 used in our study, was 17.9 µg/l using the trimmed Spearman-Kaber method (Hamilton et al., 1977). In experiment 2, no significant changes in body length, and body weight were found between control and exposed groups after 28 days (Table 3).

The condition index in experiment 2 showed an increase of 0.05 ± 0.26, and of 0.03 ± 0.28, and a decrease of 0.25 ± 0.31 (mean ± standard deviation) in the control, 3.2 and 10 µg group, respectively. The liver weight and the hepatosomatic index, calculated in experiment 3 (Table 4), showed a significant increase in TBTO-exposed animals.

Table 2
Analysis of organotin compounds in the water[a]

	MBT	DBT	TBT	TBTO
Experiment 2				
0 µg/l				
t = 0 h	n.d.	n.d.	n.d.	n.d.
t = 48 h	n.d.	n.d.	n.d.	0.03
10 µg/l				
t = 0 h	0.015	0.03	3.09	7.74
t = 6 h	0.02	0.08	2.68	6.73
t = 48 h	0.2	0.125	0.24	0.58
10 µg/l without silversand				
t = 0 h	0.02	n.d.	3.96	9.94
t = 48 h	0.15	0.04	0.1	0.25
Experiment 3				
0 µg/l				
t = 0 h	n.d.	n.d.	n.d.	n.d.
t = 6 h	n.d.	n.d.	n.d.	n.d.
t = 48 h	n.d.	n.d.	n.d.	n.d.
32 µg/l				
t = 0 h	n.d.	n.d.	7.07	17.75
t = 6 h	n.d.	n.d.	3.18	7.99
t = 48 h	n.d.	0.317	0.75	1.89

[a] MBT (monobutyltin), DBT (dibutyltin) and TBT (tributyltin) were measured by atomic absorption spectrometry and expressed as µgSn/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 (experiment 2) or 0.005 µg/kg (experiment 3).

3.3. *Histopathology*

Animals that died during the experiment were lost for histological investigation since dead fish quickly develop autolytic changes. Gill epithelium is especially sensitive, it desquamates rapidly following death of the fish, and cannot be evaluated properly even a few hours after death. All animals in the 32 µg/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, histopathology of the thyroid gland and the skin was included, and in experiment 2 the eyes were also examined. In experiments 1 and 2, no exposure-related histopathological changes were found in the above-mentioned organs.

Table 3
General toxicity parameters (length, weight, and condition index) of flounder exposed to TBTO for 28 days (experiment 2)

TBTO conc	Length (cm)		Weight (g)		Condition index[a]	
	D0	D28	D0	D28	D0	D28
0 µg/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*

Values are mean ± standard deviation of 10 animals per group.
[a] Condition index = 100[body weight (g)]/[length (cm)]^3.
*P = 0.071 (repeated measures, 2-tailed) compared to condition index at D0.

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

TBTO conc.	Length (cm)	Weight (g)	Liver weight (g)	Condition index[a]	Hepatosomatic index (%)[b]
0 µg/l	6.36 ± 0.54	4.88 ± 1.33	0.053 ± 0.014	1.69 ± 0.14	1.1 ± 0.4
32 µg/l	6.74 ± 0.47	5.11 ± 0.95	0.073 ± 0.023*	1.66 ± 0.12	1.5 ± 0.5**

Values are mean ± standard deviation of 30 animals per group.
[a] Condition index = 100×[body weight (g)]/[length (cm)]^3.
[b] [Liver weight (g)]/[body weight (g)]×100.
* P<0.001 (Student's t-test, 1-tailed), compared to controls.
**P<0.005 (Student's t-test, 1-tailed), compared to controls.

In experiment 3, all animals of the 32 µg/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 93% and 7% resp. of the control animals but frequent in 40% of the animals in the 32 µg/l group. Respiratory epithelial proliferation (Fig. 3) and fusion of secondary lamellae (synechia) (Fig. 4) were present in control animals but not as frequent, prominent and extensive as in animals exposed to 32 µg/l (marked cases 20% and 70% resp.). Respiratory epithelial proliferation was called mild when the epithelial layer was 2–3 cells thick, while marked cases showed epithelial layers thicker than 3 cells as indicated in Fig. 3.

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 ± 0.11 mm^3) was not significantly decreased when compared to control animals (0.394 ± 0.10 mm^3). Also no significant effects on thymus volume in relation to body weight or body length were noted after exposure to TBTO (Table 6).

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

* *P*<0.0001 (Chi-square-test, 2-tailed); absent, rare and frequent classes separately in exposed animals compared to controls.
***P*<0.0001 (Chi-square-test, 2-tailed); absent, mild and marked classes separately in exposed animals compared to controls.

However, when related to the [body height]^3, a statistically signifcant ($P < 0.05$; student's *t*-test, 1-tailed) 20% decrease in relative thymus volume (control 0.024 ± 0.005; exposed 0.019 ± 0.007) was calculated for the exposed group.

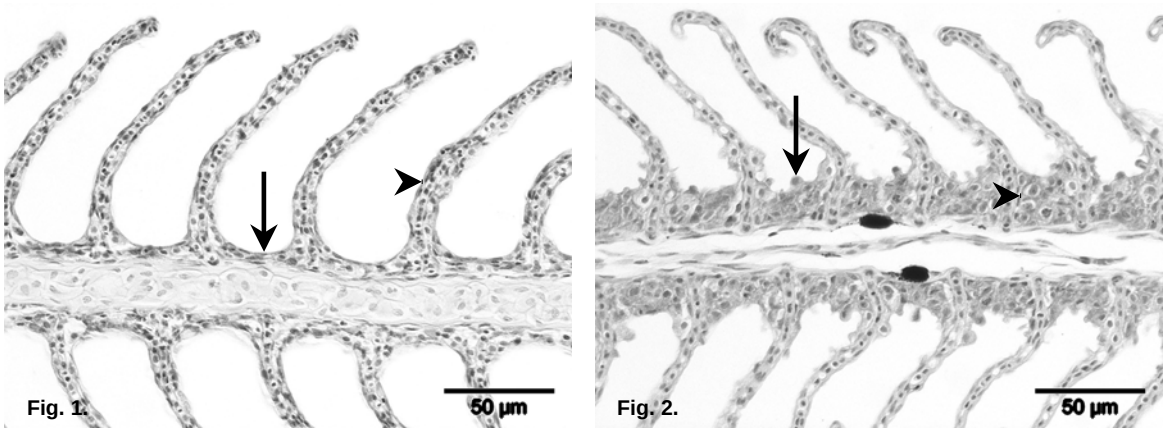


Fig. 1. Gill of a control animal with normal primary (arrow) and secondary (arrowhead) lamellae. *H&E*.
Fig. 2. Gill of an animal exposed to 32 µg TBTO/l for 6 days showing budding (arrow) of epithelial cells; some minor epithelial hyperplasia is also present (arrowhead). *H&E*.

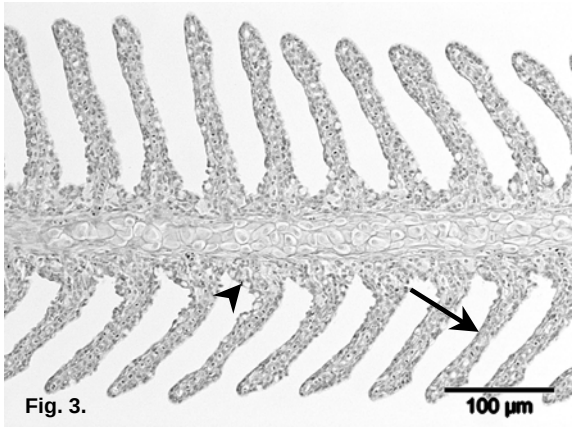
3.4. *Scanning electron microscopy*

Scanning electron microscopy of the affected gills (Figs. 5–8) of animals exposed to 32 µg TBTO/l for 6 days in experiment 3, showed the localized 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 in percentage of lymphocytes and total lymphocyte number in the spleen was found in TBTO-exposed flounder in experiment 3 (Table 7). The proliferative response to stimulation with PHA (in a concentration of 0.12 mg/ml) in experiment 3 showed no statistically significant changes between TBTO-exposed and control animals (Table 7).



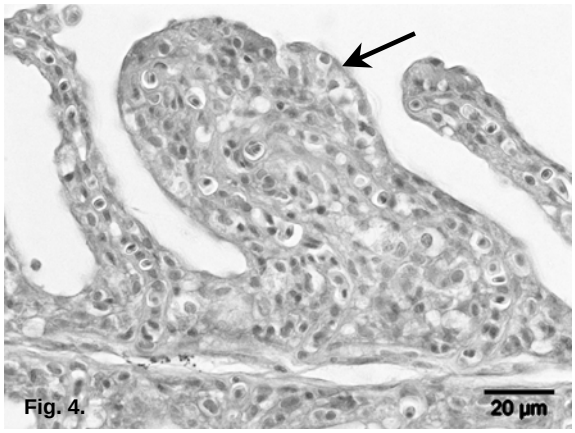


Fig. 3. Gill of an animal exposed to 32 µg TBTO/l for 6 days with mild (arrow), and marked (arrowhead) respiratory epithelial hyperplasia. Also budding of epithelial cells is visible. *H&E*.
Fig. 4. Gill of an animal exposed to 32 µg TBTO/l for 6 days with respiratory epithelial proliferation and fusion of secondary lamellae (synechia; arrow). *H&E*.

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

TBTO conc.	Absolute thymus volume (mm^3)	Thymus volume/ body weight	Thymus volume/ body length	Thymus volume/[body height]^{3a}
0 µg/l	0.394 ± 0.10	0.069 ± 0.022	0.055 ± 0.016	0.024 ± 0.005
32 µg/l	0.339 ± 0.11	0.059 ± 0.018	0.046 ± 0.013	0.019 ± 0.007*

Values are mean ± standard deviation of 13 (0 µg TBTO/l) or 10 (32 µg TBTO/l) animals.
[a] [Thymus volume]/[body height just caudal of pectoral fin]^3.
* $P<0.05$ (Student's *t*-test, 1-tailed) compared to controls.

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 TBTO-exposed animals, to approximately 30% of the control values, at all E/T ratios (5, 10 and 20) in experiment 3 (Table 8). In this experiment, 2 of 14 TBTO-exposed flounder (14%) exhibited no or only insignificant NCC activity.

4. Discussion

In the present study, flounders kept in fresh water under controlled conditions were exposed to three concentrations (3.2, 10 and 32 µg/l) of TBTO. A nominal

concentration of 32 µg/l (maximum actual water concentration: 17.75 µg TBTO/l) induced 100% mortality after 7–12 days (14-day LC50 = 17.9 µg/l; 9.9 µg/l when related to the actual water concentration), 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).

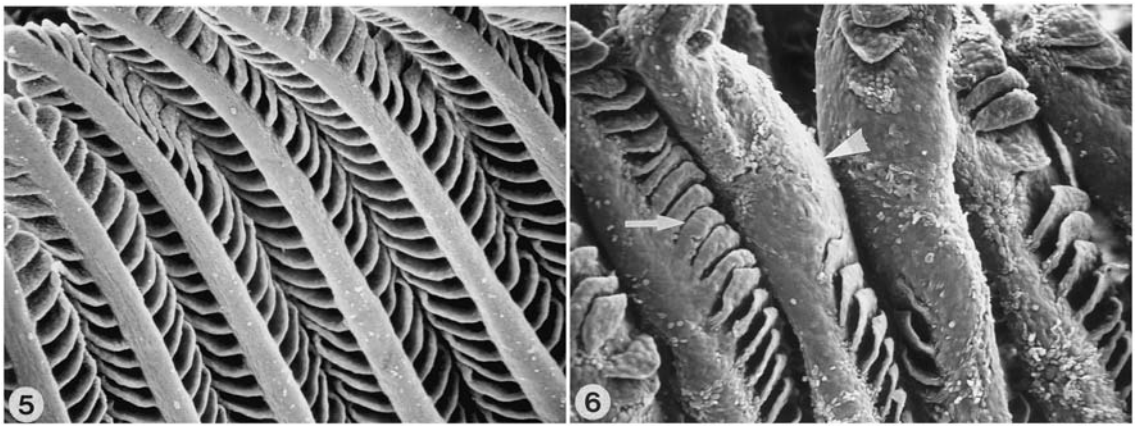


Fig. 5. Gill of a control flounder showing six primary lamellae. Each primary lamella has many secondary lamellae on both sides. The surface, covered with epithelium, is smooth. *SEM*, 160×.
Fig. 6. Gill of a flounder exposed to 32 µg TBTO/l for 6 days. Six primary lamellae are visible showing marked thickening (arrow) and fusion of many secondary lamellae (arrowhead). Note also rougher surface of the thickened parts. *SEM*, 160×.

After 28 days, a trend towards a decrease of the condition index was seen in the group exposed to 10 µg TBTO/l in experiment 2. This decrease was not statistically significant (P = 0.071, repeated measures, 2-tailed). 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 to the occurrence of histopathological lesions at non-lethal concentrations in other fish species.

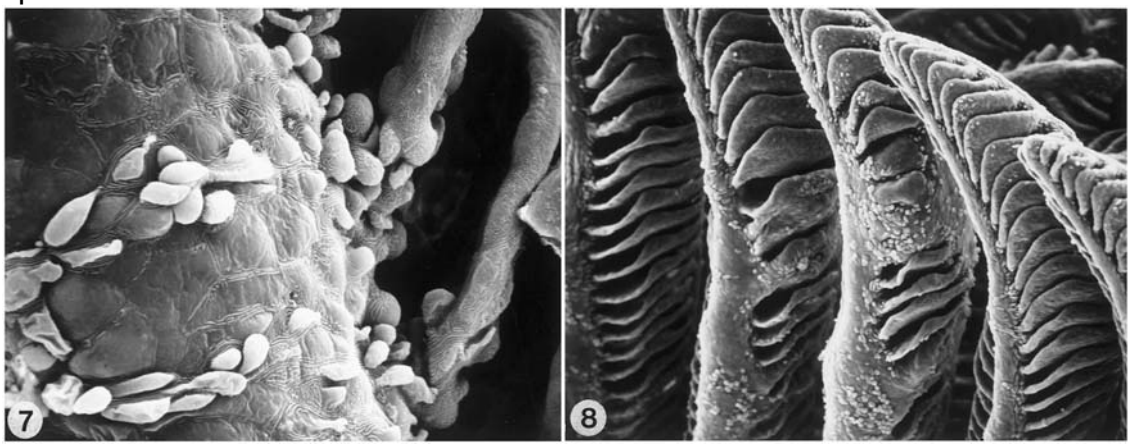


Fig. 7. Gill of a flounder exposed to 32 µg TBTO/l for 6 days. Detail of primary and secondary lamellae with frequent budding of epithelial cells resulting in an irregular surface. *SEM*, 1250×.
Fig. 8. Gill of a flounder exposed to 32 µg TBTO/l for 6 days. Five primary lamellae are visible, showing varying degrees of fusion of secondary lamellae. Also swelling and budding of epithelial cells are seen on the surface of primary lamellae. *SEM*, 160×.

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 histological alterations in a variety of organs (skin, muscle, kidney, cornea, lens and retina) were described in experiments with minnow (*Phoxinus phoxinus*) eggs after short-term exposure to TBTO. In these experiments a dose-dependent decreased survival of minnow larvae was also 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).

Table 7
Cell counts and mitogen responsiveness to PHA of leukocytes from the spleen of flounders exposed to TBTO in experiment 3

TBTO conc.	Total cell number ($\times 10^6$)	Lymphocyte number ($\times 10^6$)	% Lymphocytes	PHA Δcpm[a]
0 µg/l	5.9 ± 2.1	1.9 ± 0.7	35.9 ± 6.1	307 ± 329 (59 – 774)
32 µg/l	5.0 ± 4.7	0.8 ± 0.3*	25.6 ± 3.2**	321 ± 202 (148 – 645)

Values are mean ± standard deviation of seven animals per group.
[a] The mitogen responsiveness is expressed as counts per min (Δcpm; the proliferation of unstimulated cultures has been subtracted). The range is given in parentheses.
* $P<0.005$ (Student's *t*-test, 1-tailed) compared to controls.
** $P<0.01$ (Student's *t*-test, 1-tailed) compared to controls.

Table 8
NCC activity of mesonephros leukocytes at different effector to target (E/T) ratios of flounders exposed to TBTO in experiment 3

TBTO conc.	E/T5	E/T10	E/T20
0 µg/l	8.7 ± 10.5 (1 – 31)	19.6 ± 15.7 (5 – 50)	28.7 ± 18.3 (11 – 56)
32 µg/l	2.2 ± 1.8 (0 – 4)*	4.4 ± 3.4 (0 – 8)*	8.7 ± 7.0 (1 – 18)*

Mean values are expressed as percentage specific release ± standard deviation of seven animals per group, the range is given in parentheses.
$P<0.02$ (Student's *t*-test, 1-tailed) compared to controls.

These lesions are also seen in animals caught in the wild due to infections with parasites (e.g. Gyrodactylus and trichodinid ciliates), but none of the animals used in the present study showed signs of parasitic infections. The budding of epithelial cells as seen in animals exposed to 32 µg TBTO/l in the present study, is a remarkable lesion. This lesion has not been described in the review article on gill lesions induced by toxicants (Mallatt, 1985). Immunohistochemical staining for apoptosis (using the TUNEL assay) did not reveal an increased number of apoptotic cells in the affected gill epithelium (data not shown). Although the gill lesions are the only significant histopathological changes 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 major part of the respiratory surface, even in severely

affected gills with fusion of secondary lamellae and epithelial proliferation, appeared not dramatically reduced.

Experimental data in rainbow trout have shown neurotoxicity after exposure to 0.5 or 2.0 µg TBTO/l, resulting in histopathological lesions in the tectum opticum and optical nerves (Triebskorn 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 localized 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 signs were clinically 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 TBTO exposure on the non-specific immune response 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 in percentage lymphocytes and total lymphocyte numbers. These findings agree with the effects of TBT exposure reported in rainbow trout by Schwaiger et al. (1994), but contrast with data obtained by De Vries et al. (1991) in the same species. There was, however, no statistically significant change in the proliferative response after 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 the 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 [thymus volume]/[body length] or body height. The ratio [thymus volume]/ [body height]^3 showed a 20% reduction ($P < 0.05$, *t*-test, 1-tailed) in TBTO-exposed animals compared to controls. The functional significance of this finding needs further investigation. In guppy exposed to TBTO concentrations ranging from 0.032 to 10 µg/l for 1 month, thymus atrophy was much more obvious (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).

In experiment 3, chemical analysis data showed that TBTO levels dropped to approximately 45% and 10% of initial values at $t = 6$ and $t = 48$ h, respectively. This indicates a pulse exposure of flounder to TBTO in the present study, and shows that the animals are exposed to the maximum TBTO levels for a relatively short period.

Also, the analysis data show that renewal of the exposure medium three times a week did not result in accumulation of TBTO in the water. The decrease of actual TBTO-levels in time could be explained by absorption of TBTO to the aquarium (glass or kit) or the fish. Also, some effect of silversand on the actual TBTO concentrations in the water was shown. At t = 0 in the 10 µg TBTO/l group, the presence of silversand 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 h, 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. In order to investigate the actual bioavailability of TBTO and the effects of silversand on it, tissue analysis should be performed. Because the TBTO used in the experiments contained 4% unknown contaminants one cannot completely rule out some effects of these contaminants.

5. Conclusions

Organotin levels obtained from field measurements show TBT levels in water up to 5.76 µg/l (Maguire et al., 1986), and levels up to 7.2 µg TBT/l were found in Dutch harbors. 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 harbors. The maximum TBT level at t = 48 h 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 at 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, as shown by a decreased NCC activity, may have detrimental effects on health status, such as increased susceptibility to viral infections. Mortality data for 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, and indicate a steeper concentration-effect curve in flounder. Further studies on long-term TBTO exposure will be performed in the near future, including infection experiments, in order to elucidate the effects on (target) organs and the immune system. These studies should clarify a possible causal role of TBT exposure in the increased prevalence of diseases observed in field-and mesocosm studies.

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Chapter 3

Chronic aqueous exposure to bis(tri-*n*-butyltin)oxide (TBTO) causes thymotoxicity in European flounder (*Platichthys flesus*)

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Abstract

Although the use of tributyltin in antifouling paints has been restricted, this compound is still a serious pollutant of the aquatic environment. This paper describes a unique study in which European flounder (*Platichthys flesus*) were chronically (8 months) exposed to bis(tri-*n*-butyltin)oxide (TBTO) in the water under controlled laboratory conditions. Chemical residue levels in selected tissues (liver, muscle) and general health-state indices were measured and the effects on several organs (gills, liver, mesonephros, ovary/testis, spleen, and gastrointestinal tract) were examined histo-pathologically. Additionally, morphometric analysis of the thymus was performed.

The major finding of our chronic study, a type of study that is pre-eminently suitable for risk assessment purposes, is the observation that exposure of flounder to 5 µg TBTO/l over a period of 8 months, resulting in body burdens comparable to high field levels, induced significant reduction of thymus volume, possibly affecting immuno-competence of the animals. Also a dose-related marked shift from a glycogen-rich to a fatty liver, with a no-effect level of 1.0 µg TBTO/l, was noted accompanied by a trend in reduction in body weight gain.

1. Introduction

The present long-term study is a follow-up on previously reported short-term experiments with bis(tri-*n*-butyltin)oxide (TBTO) and flounder (Grinwis et al., 1998). These studies are part of a fish disease project investigating the impact of environmental contamination on fish health.

Organotins are widely utilized organometallic compounds, most of which are man-made, except for methyltins that can also be produced under natural conditions (Guard et al., 1981). Organotins are used in a variety of products: as stabilizers in polyvinyl chloride (PVC), catalysts in polyurethane and silicone elastomers, and as pesticides (WHO, 1999).

TBTO has been used worldwide in marine antifouling paints as a biocide. Although the application of TBTO in antifouling paints has been partially banned for some time (in the Netherlands since 1989), it is still the major source of tributyltin (TBT) in marine water and sediments (Fent, 1996). TBT levels in marinas in the Netherlands in 2000-2003 are still in the range of 10-100 ng/l (RIKZ, unpublished data). Due to the slow degradation of TBT in sediments (Stewart and De Mora, 1990; De Mora et al., 1995; Evans et al., 1995; Viglino et al., 2004), 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 immune system, the endocrine system and the liver. It is well known that gastropods, such as the dogwhelk (*Nucella lapillus*), bioaccumulate TBT and its endocrine disruptive effects in female snails result in the development of structures typical of the male reproductive system like a vas deferens and a penis-homologue (“imposex”) (Matthiessen and Gibbs, 1998).

Immune parameters can be valuable biomarkers of effects of xenobiotic substances in fish since the immune system has been shown to be sensitive to the effects of pollution (Anderson and Zeeman, 1995; Vos et al., 1996), and organotin compounds affect the immune system in particular. Thymus atrophy was induced by

TBTO in flounder (Grinwis et al., 1998) and in the guppy (Wester and Canton, 1987; Wester et al., 1990). Also a reduction of the non-specific cytotoxic cell (NCC) activity in European flounder (Grinwis et al., 1998) and channel catfish (*Ictalurus punctatus*) (Rice et al., 1995) as well as a concentration-dependent decrease in phagocytic activity of phagocytes in Atlantic croaker (*Micropogonias undulatus*), hogchocker (*Trinectes maculatus*) and in oyster toadfish (*Opsanus tau*) (Wishkovsky et al., 1989) were noted after exposure to TBT. Exposure to TBT also caused histopathological lesions in liver, kidney, eye and gill epithelium in medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) (Wester and Canton, 1987; Wester et al., 1990), and masculinization in Japanese flounder *Paralichthys olivaceus* (Shimasaki et al., 2003). Dose-dependent teratogenic effects, delayed hatching, and decreased survival have been reported after short-term exposure in minnow (*Phoxinus phoxinus*) (Fent, 1996). Both morphological and functional effects on lymphoid organs have also been recorded in the rat, including a reduction in weight and cellularity of the thymus, depletion of T-cell areas in the spleen and lymph nodes (Seinen et al., 1977a,b; Krajnc et al., 1984; Snoeij et al., 1985) and a decreased thymus-dependent antibody synthesis against sheep red blood cells and *Trichinella spiralis* (Vos et al., 1984, 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.

For hazard identification and dose-response assessment in humans, immunotoxicity is adopted as the critical effect for TBTO by the Environmental Protection Agency (EPA, 1997), the World Health Organization (WHO, 1999) and the European Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE, 1998). These organizations base their perspective on the effects of TBTO reported in rats (Vos et al., 1990).

The present study was conducted to investigate the effects of chronic exposure to TBTO in the bottom-dwelling flatfish species European flounder (*Platichthys flesus*) under controlled laboratory conditions. Since our previous acute toxicity studies resulted in mortality in the highest concentrations, lower exposure levels were chosen in the chronic studies. Special attention was paid to the effects on the thymus that could possibly have implications for host resistance in flounder.

2. Materials and methods

2.1. *Experimental fish*

Captive-bred European flounder, obtained as fingerlings (1- group) from the Port Erin Marine laboratory, the School of Biological Sciences, University of Liverpool (UK), and grown for 1 year at the National Institute for Coastal and Marine Management (RIKZ) field station at Jacobahaven, the Netherlands, were used as experimental animals. The 2-year old mature animals were ranging from 13-18 cm in length and 25-68 gram body weight at the start of the experiment (D0). The fish were kept in glass aquaria in a flow-through system containing 10 kg of clean sediment on a 70x100 cm bottom, and 160 l seawater. The water was renewed by continuous flow-through with salt water of ca. 32 promille from the relatively clean Eastern Scheldt (background TBT levels <1 ng/l on basis of Sn) at a rate of 160 l per day. Water temperature was 16 $\pm$ 1°C and the animals were subjected to a 12-12 hours dark-light regimen.

Table 1
Analysis of organotin burdens in muscle and liver tissue of flounder exposed to TBTO for 8 months

Exposure group		Tributyltin[a]	Dibutyltin[a]	Monobutyltin[a]	Σ Butyltin[a]	Total extractable lipid[b]
0	Muscle	3 ± 1	b.d.	b.d.	3 ± 1	130 ± 70
	Liver	2 ± 1	6 ± 3	2	9 ± 6	400
0.2 µg/l	Muscle	83 ± 40	11 ± 6	b.d.	95 ± 46	90 ± 50
	Liver	45 ± 22	274 ± 180	59 ± 42	377 ± 244	470
1.0 µg/l	Muscle	511 ± 59	115 ± 49	14 ± 6	640 ± 114	110 ± 90
	Liver	351 ± 24	1103 ± 23	278 ± 103	1732 ± 149	720
5.0 µg/l	Muscle	644 ± 89	170 ± 72	18 ± 8	833 ± 168	96 ± 50
	Liver	433	1233	373	2039	650

[a] Values are mean ± standard deviation expressed as µg Sn/kg wet weight.
[b] Values are mean ± standard deviation in g/kg.
If only 1 value is given, sample-size required pooling of tissues of all animals.
b.d.= below detection limit of 3 µg/kg.

Table 2
General toxicity parameters (length, weight, liver weight, condition index and hepatosomatic index) of flounder exposed to TBTO for 8 months

TBTO conc. (µg/l)	n[a]	Length (cm)	Weight (g)	Liver weight (g)	Condition index[b]	Hepatosomatic index[c]
0	19	15.5 ± 2.6	72.0 ± 9.2	1.1 ± 0.9	1.8 ± 0.2	1.2 ± 0.6
0.2	17	14.9 ± 2.5	63.7 ± 37.9	0.7 ± 0.8	1.8 ± 0.2	1.1 ± 0.6
1.0	18	14.5 ± 1.9	54.8 ± 24.5	0.7 ± 0.5	1.7 ± 0.3	1.2 ± 0.6
5.0	20	14.4 ± 2.0	53.3 ± 28.2	0.8 ± 0.7	1.6 ± 0.2	1.4 ± 0.7

Values are mean ± standard deviation.
[a] *n*= number of animals per group.
[b] Condition index = 100 x [bodyweight (g)]/[length (cm)]^3.
[c] Hepatosomatic index = [liver weight (g)]/[bodyweight (g)] x 100.

Fish were fed pelleted feed (Trouvit; Seafarm, Kamperland, the Netherlands) at 1% of the estimated total body weight three times a week, and inspected daily for behavioral changes and signs of disease. Seawater quality was checked twice a week for dissolved oxygen and pH, and measured values remained within a normal range for the duration of the experiment. Accumulating organic debris was removed from the tank bottoms regularly.

Table 3
Bodyweight gain and increase of condition index of flounder exposed to TBTO for 8 months

TBTO conc. (µg/l)	*n*[a]	Weight gain (g)	Condition index[b]
0	19	33.7 ± 36.3	0.47 ± 0.30
0.2	17	21.0 ± 44.7	0.55 ± 0.37
1.0	18	12.6 ± 28.7	0.43 ± 0.28
5.0	20	13.5 ± 31.9	0.42 ± 0.27

Values are mean ± standard deviation.
[a] *n*= number of animals per group
[b] Condition index = 100 x [bodyweight (g)]/[length (cm)]^3

2.2. *Chemicals and chemical analysis*

Bis(tri-*n*-butyltin)oxide was obtained from Fluka Chemika (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.

Tissue samples of hepatopancreas and muscle from all animals were taken for determination of the internal mono, di, and tributytlin-levels. These tissue samples were wrapped in aluminum foil (cleaned with acetone) and frozen until analysis. Due to the limited size of the samples, muscle tissue was analyzed in 3 mixed samples and liver tissue in two mixed samples in all exposure groups, except for the 5.0 µg group where only one mixed liver sample was available. Detection of butyltin components was performed with an atomic absorption spectrometer (AAS type 2380; wavelength 22.4 nm, slit 0.2 nm; Perkin Elmer, Nieuwerkerk, The Netherlands).

2.3. *Experimental design*

Glass aquaria, including sediment, were charged with TBTO before the experiment and TBTO-water levels were monitored until a steady state was reached. Exposure was continuous by adding TBTO dissolved in DMSO to the inflow water using a stock of 1.57 mg TBTO per ml DMSO.

To each dose-group (0, 0.2, 1.0 and 5.0 µg TBTO/l) 20 European flounder were randomly assigned. Animals were exposed for 8 months after which they were killed with an overdose of MS222® (ethyl 3-amino benzoate methanesulfonate, Sigma-Aldrich, Steinheim, Germany). Feeding of the animals was discontinued 3 days before the end of the experiment to avoid substantial effects of the gastrointestinal contents on body weight. Animals were weighed and length was measured after killing. From these data the condition index (100 x [bodyweight (g)]/[length (cm)]^3) was calculated. During subsequent necropsy, the fishes were checked for gross external abnormalities and organ lesions.

The present study was approved by the ethical committee for animal welfare in experiments of the RIKZ, and complies with Dutch laws.

Table 4
Prevalence of histopathological gill lesions of flounder exposed to TBTO for 8 months

	0 µg/l	0.2 µg/l	1.0 µg/l	5.0 µg/l
Budding of epithelial cells[a]				
Absent	7	10	7	8
Rare	8	7	10	8
Moderate	4	0	1	4
Epithelial proliferation/fusion of lamellae[a]				
Absent	11	4	4	14
Mild	3	9	1	2
Moderate	5	4	13	4

[a] number of cases of 19 (control), 17 (0.2), 18 (1.0) or 20 (5.0) animals per group.

2.4. *Histological techniques*

Organs and tissues used for histological examination were selected based on previous experiments and target organs of TBTO in other fish species (Grinwis et al., 1998; Wester and Canton, 1987; Wester et al., 1990; Wester and Vos, 1994).

After severing the spinal cord, the abdominal cavity was opened and hepatopancreas, gastrointestinal tract, spleen, gonads and trunk kidney were removed for histological examination. The hepatopancreas was weighed, the whole head up to the caudal edge of the opercula was removed and together with all organs mentioned above, fixed in a neutral-buffered 4 % formaldehyde solution. All organs and tissues were inspected during necropsy for gross lesions. After fixation, two gill arches of the upper side of the gill cavity were removed for histological evaluation. All fixed organs were routinely processed and embedded in paraffin. Sections were cut at 3-5 µm and routinely stained with hematoxylin and eosin.

Observers scoring the histological sections were not aware of which exposure-group the samples originated.

2.4 *Morphometry of the thymus*

The weight of the thymus is a sensitive parameter for thymotoxic effects in mammals. However, due to the small size of the organ, its localization and its similar color and structure compared to the surrounding muscle tissue, it is not possible to reliably excise and weigh the thymus in flounder. 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., 1998). Therefore histological morphometric analysis of the thymus was performed following a similar procedure as previously described (Grinwis et al., 1998). In short, a transverse block of about 0.5-1 cm thick of each fish was taken just cranial 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. Serial sections of 3-5 µm of 6 control animals and 6 animals of the highest dose group were made at intervals of 32 µm until the whole thymus was sectioned. Digital pictures of all sections (ranging from 53-121 sections per animal) containing the thymus were made, and surface-areas of the thymus were measured using a Leica QWin® image analysis software. Surface areas (expressed as μm^2) of all thymus sections were added and multiplied by 36 resulting in the actual thymus volume. Because previous research revealed no difference in the volume of

the left or right side of the thymus (dependent of the orientation of the fish) only one side was measured. The thymus volume was also related to the body length and body weight.

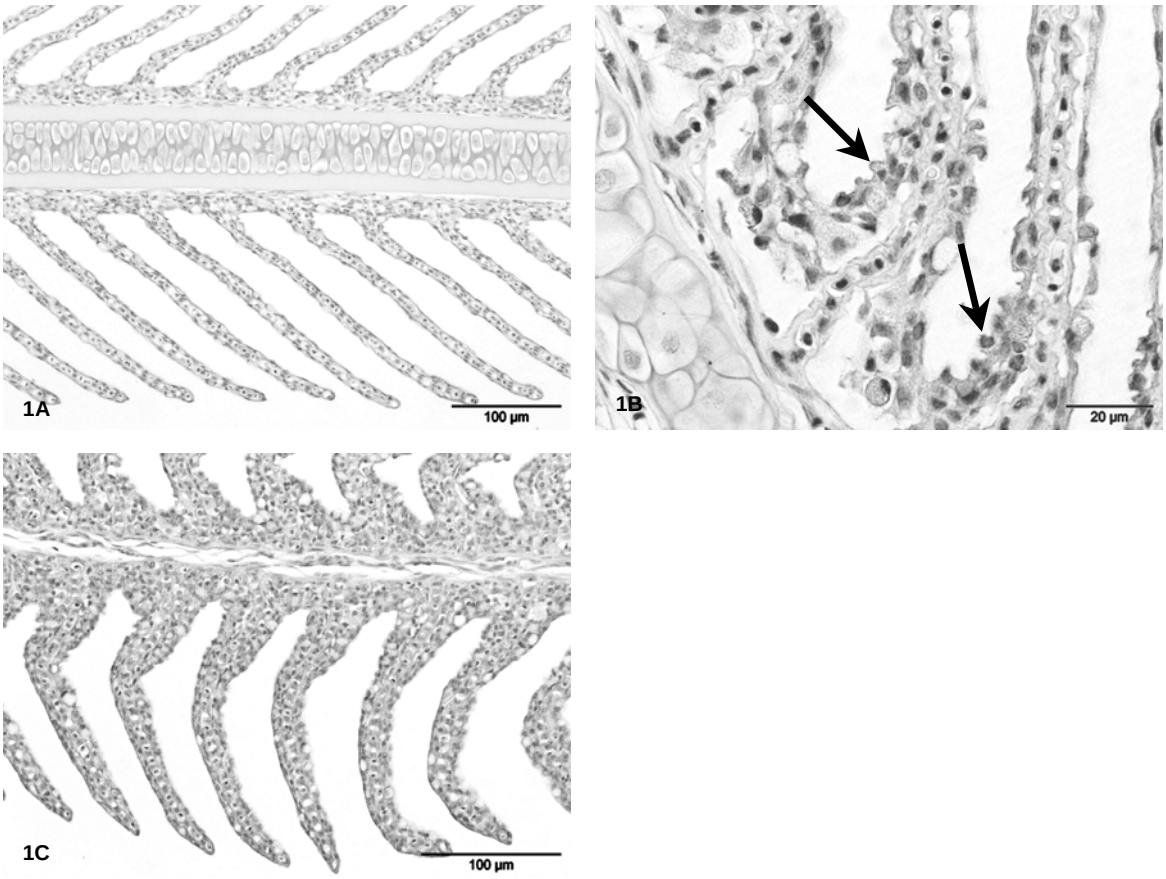

Fig. 1. Gill lesions in a control animal (1A) and animals exposed to TBTO for 8 months (1B and 1C).
Fig. 1A shows slender secondary lamellae and the central cartilaginous core of a primary lamella. Fig. 1B shows budding of epithelial cells lining the secondary lamellae (arrows). The epithelium of the secondary lamellae in Fig. 1C shows moderate hyperplasia. None of these changes appeared to be dose related. *H&E*.

2.5 Statistical analysis

Statistical analysis was performed using SPSS for Windows 9.0 software (SPSS Chicago, Ill., USA). Data were analyzed using an analysis of variance (ANOVA) followed by an independent t-test if a significant difference between groups was observed in the ANOVA. Semi-quantitative data were tested for significance with the Fisher's exact test. Data were considered significantly different if $P < 0.05$.

3. Results

3.1. *Chemical analyses*

Actual butyltin and phenyltin body concentrations were measured in liver and muscle tissue (Table 1). Background levels in the control group were relatively low. Internal levels increased with rise in nominal concentrations. The tributyltin (TBT) concentration was higher in muscle tissue than in the liver, whereas the concentrations of the break-down products dibutyltin (DBT) and monobutyltin (MBT) showed higher levels in the liver, indicating a certain degree of biotransformation

capacity in the flounder. There was no clear relationship with lipid content.

3.2. *Clinical and gross morphological findings*

None of the test fish showed any visible behavioural changes or signs of disease during the course of the experiment. In the 8 months exposure period 6 animals died (1 control, 3 from 0.2 µg/l and 2 from 1.0 µg/l) with no mortality in the highest dose group. Mortality was statistically not exposure related. A trend in exposure related reduction of weight gain over a period of eight months was noted, but due to relatively high standard deviation this retardation is not statistically significant (Table 3). Other allometric data (e.g. liver weight, hepatosomatic index, gonadal weight) revealed no exposure related effect (Table 3). Three animals (one control, two in the 1 µg/l group) showed small (0.5 mm in diameter) pale foci diffusely spread through the liver. No other gross lesions were noted during necropsy.

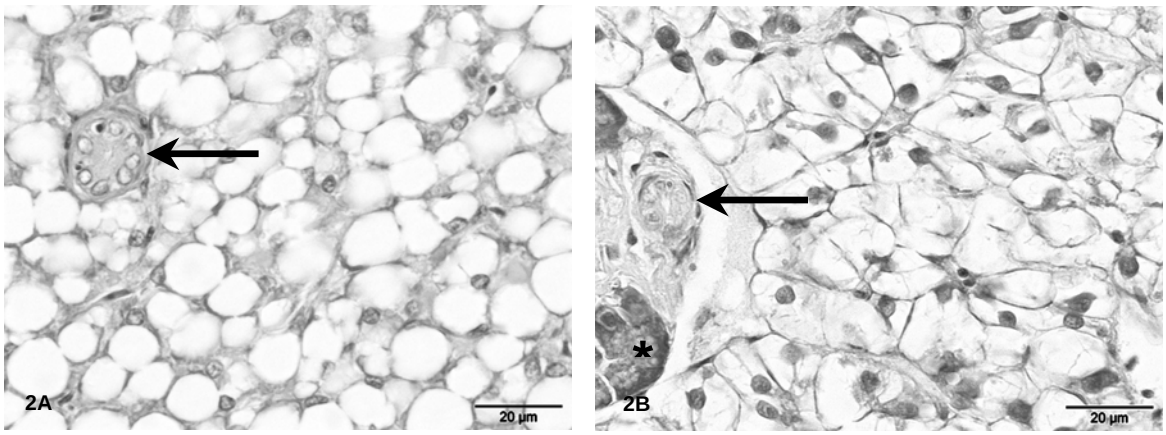

Fig. 2. Hepatopancreas of animals exposed to TBTO for 8 months. Hepatocytes in Fig. 2A contain large clearly demarcated empty vacuoles and peripherally located nuclei characteristic of fat accumulation (steatosis). In contrast, the cytoplasm of the hepatocytes in Fig. 2B show distinctive features of glycogen storage with pale cytoplasm containing reticular structures and centrally located nuclei. Flounder showed a dose related shift from hepatic glycogen storage to fat accumulation. Arrows indicate bile ducts, the asterisk shows a part of the exocrine pancreas. *H&E*.

3.3. *Histopathology*

Because of the rapid development of autolytic changes, the few animals that died during the experiment were lost for histological investigation. The cause of death could therefore not be determined.

Gills of the killed animals showed mild to moderate epithelial hyperplasia and budding of surface epithelium. These lesions showed no significant relation to exposure (Table 4, Fig. 1). No significant fusion of secondary lamellae was noted in any of the exposure groups. The spleen usually was poor in lymphoid cells but showed marked differences in blood contents. Neither the amount of blood nor the number of lymphoid cells in this organ revealed a relation to exposure. The contents of cytoplasm of the hepatocytes showed a significant shift from rich in glycogen in the control group to rich in fat (steatosis, as shown by hepatocytic vacuolation and by biochemical analysis) in the two highest dose-groups (Table 1 and Table 5, Fig. 2). Accumulations of protozoal structures (xenomas) reminiscent of the microsporidian parasite *Glugea stephani* were encountered in the hepatopancreas of all experimental groups (Table 6, Fig. 3). The pale foci noted during necropsy were caused by accumulations of these parasites. Severity varied but no significant

relation with exposure could be detected. Typically, the accumulations did not evoke any reaction of the surrounding tissue. Occasionally however, a more or less extensive granulomatous inflammation with connective tissue related to the parasitic accumulations was present (Fig. 3). Gonads were well differentiated with a varying amount of mature germinal cells (oocytes or spermatozoa) and without indications of hermaphroditism.

Table 5
Semiquantitative analysis of cytoplasm contents of hepatocytes of flounder exposed to TBTO for 8 months

TBTO conc. (µg/l)	-	±	+	++
Glycogen storage[a]				
0	3	0	1	12
0.2	1	0	0	8
1.0	2	0	0	1
5.0[b]	6	0	0	2
Lipid storage[a]				
0	3	0	3	0
0.2	1	0	4	4
1.0	2	2	5	8
5.0[b]	6	3	1	8

[a] number of cases of 19 (control), 17 (0.2), 18 (1.0) or 20 (5.0) animals per group.
[b] Significantly different from controls at $P<0.025$ (Fisher's Exact test, 2-sided)

3.4. *Morphometry of the thymus*

The volume of the unilateral thymus of the animals was calculated by measuring surface areas of serial sections of the control group and the highest dose group. The number of serial sections measured ranged from 53 to 124 per animal. Sectioning artefacts caused by insufficient decalcification of bones and teeth occasionally resulted in fragmentation of the thymus section. In these cases, an interpolation of the thymus section was made with the morphometric equipment. Both the absolute thymus volume as well as the thymus volume related to body length showed a statistically significant reduction in animals of the highest dose group (Table 7).

4. Discussion

The significant reduction in absolute thymus size and thymus size related to body length in the present study was a confirmation of TBTO-induced effects on thymus size reported previously in flounder (Grinwis et al., 1998) and other fish species

Table 6
Prevalence of *Glugea stephani* cysts in the hepatopancreas of flounder exposed to TBTO for 8 months

TBTO conc. (µg/l)	*n*[a]	*Glugea stephani* xenomas
0	19	4
0.2	17	3
1.0	18	5
5.0	20	6

[a] *n*= number of animals per group

(Wester and Canton, 1987; Wester et al., 1990). However, in the short-term experiment (Grinwis et al., 1998) we only recorded a significant effect on the thymus size related to the height of fish, not on absolute thymus size or on thymus size related to body length. In the present study adult animals were used and effects of TBTO on the thymus in juvenile European flounder is to our knowledge not studied. In rats TBTO caused a reduction in thymus weight in both juvenile and adult animals at the same dose (Vos et al., 1990) but whether this holds true for juvenile flounder is not known. Actual effects on immunocompetence of TBT in flounder need to be investigated for instance by using infection models. In any case, no difference in severity of the incidental background hepatic protozoan infection was observed in relation to TBTO exposure. Since the morphometric technique described above is very laborious, also attempts were made to measure the thymus volume by means of computer tomography (CT) and magnetic resonance imaging (MRI). However, both techniques appeared not applicable due to insufficient resolution and difficulties in differentiating thymus from surrounding soft tissue components like muscle and pronephros.

Table 7
Absolute and relative volume of the thymus (one side) of flounder exposed to TBTO for 8 months

TBTO conc. (µg/l)	*n*[a]	Absolute thymus volume (mm^3)	Thymus volume/bodyweight (mm^3/g)	Thymus volume/body length (mm^3/cm)
0	19	3.45 ± 1.39	0.043 ± 0.013	0.211 ± 0.081
5.0	20	2.02 ± 1.14[b]	0.038 ± 0.001	0.162 ± 0.050[c]

[a] *n*= number of animals per group
[b] Significantly different from controls at *P= 0.040*, Student's *t*-test (1-tailed).
[c] Significantly different from controls at *P= 0.048*, Student's *t*-test (1-tailed).

Compared to our previously reported short-term study (Grinwis et al., 1998), in which European flounder was also exposed to TBTO, no exposure related mortality was noted in the present experiment. In contrast, in one of the experiments of the short-term study mortality mounted up to 100% after 12 days. This discrepancy can be explained by the difference in the concentrations used in both studies. The maximum TBTO level in the short-term study (that was not conducted under flow-through conditions) was 32 µg/l with levels dropping rapidly to approximately 50% of the initial concentration after 6 hours. The highest nominal concentration in the present study was 5 µg/l. It is likely that acute effects of the high TBTO dose in the short-term study (Grinwis et al., 1998) were responsible for the mortality, perhaps through effects on the gills (and therefore in oxygen exchange) since the gill lesions were much less severe and lacked a dose-relation in the chronic study whereas marked and exposure related gill lesions were noted in the short-term study. The effects of TBT on the number of chloride cells as has been reported (Hartl et al., 2001) could not be confirmed but in our experiments there was no change from a fresh-water to a salt-water environment as in the experiments of Hartl et al.

Effects of TBT on the genital system have been reported in non-vertebrates as well as in vertebrates. The masculinization of whelks has been attributed to an aromatase inhibiting effect of TBT (Matthiessen and Gibbs, 1998). In Japanese flounder (*Paralichthys olivaceus*) exposure to TBTO has resulted in masculinization

(Shimasaki et al., 2003). Dietary intake of 1 µg TBTO caused testicular differentiation in 31% of genetically female (XX) Japanese flounder. However, no morphological indications for effects of TBT on the morphogenesis and differentiation of the genital tract have been noted in the present experiment. This might be influenced by the fact that the organ systems of the experimental animals were already fully developed at the start of the experiment.

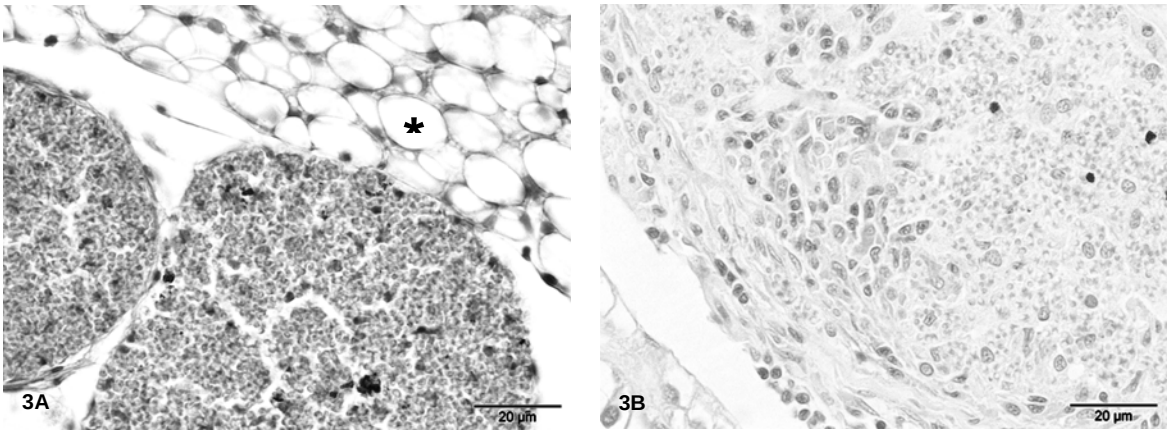

Fig. 3. Accumulations of microsporidian protozoa (xenomas), probably *Glugea stephani*, in the liver of flounder exposed to TBTO for 8 months. Most protozoa appear to be situated inside phagocytes. Note the absence of a marked tissue reaction in Fig. 3A whereas in Fig. 3B clear fibrosis and infiltration of mononuclear inflammatory cells are visible. Asterisk indicates fatty aspect of hepatocytes. *H&E*.

No attempts were made to weigh the spleen because this organ revealed marked differences in blood contents with major effects on both spleen size as well as weight largely superseding any differences in amount of lymphoid cells.

The shift of hepatocellular cytoplasm from rich in glycogen to fat storage was marked and significant with a no-effect level of 1.0 µg TBTO/l. Fatty change as a result of a negative energy balance is a know phenomenon in mammals and was also observed in guppies and medaka exposed to TBTO (Wester and Canton, 1987), and decreased caloric intake in well-fed animals can result in storage of lipids (steatosis) in hepatocytes. Although a dose-related reduction in feed-uptake was not obvious from observations during the experiment, a possible effect of TBT exposure on feeding cannot be ruled out because an adequate measurement of food that was not eaten has not been performed. TBT also has a suppressive effect on oxidative phosphorylation (Aldridge and Cremer, 1955) and may therefore interfere with lipid and carbohydrate metabolism in hepatocytes. Recently, stimulation of adipogenesis in lipocytes has been described (Inadera and Shimomura, 2005). Whether this phenomenon is also applicable to hepatocytes remains to be investigated.

5. Conclusions

Chronic exposure studies, as the present one, are pre-eminently suitable for hazard identification and risk-assessment purposes and the results of our experiment therefore provide important information on the effects of chronic exposure of European flounder to TBTO.

Maximum body burdens in the present study (433 µg TBT/kg and 2039 µg ΣBT/kg w.w. in the liver) are lower than maximum field levels measured in the liver of

lizardfish (*Trachinocephalus myops*) in Taiwanese coastal water (1559 µgTBT/kg and 11473 µg ΣBT/kg w.w.; Dong et al., 2004). Maximum levels measured in the liver of European flounder in the Gdansk Bay, Poland (35 µgTBT/kg and 369 µg ΣBT/kg; Albalat et al., 2002) are one order of magnitude lower than those in the present study. Preliminary measurements in livers from Dutch Wadden Sea flounder are two orders of magnitude lower than those observed in Polish waters (3.3 µg TBT/kg and 39 µg ΣBT/kg w.w.) (Vethaak et al., 2004).

Since chronic exposure of mature European flounder to TBTO resulted in significant reduction of the size of the thymus in the present study, it is likely that thymotoxicity and impairment of immunocompetence due to TBTO exposure will occur at least at highly contaminated field sites. However, to get reliable information on the actual impact of TBT on the efficacy of the immune system, chronic exposure of juveniles accompanied with infection models and immune function test need to be performed. Exposure experiments with juvenile animals or even eggs are also important to assess the effects of TBT on the development of the genital tract in European flounder.

Acknowledgements

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Chapter 4

Toxicity of TCDD in European flounder (*Platichthys flesus*) with emphasis on histopathology and cytochrome P4501A induction in several organ systems

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Abstract

The present study is part of a series of experiments, set up to elucidate the impact of aquatic pollution on fish health in the marine and estuarine environment. In the Dutch coastal and estuarine waters, European flounder (*Platichthys flesus*) showed a relatively high prevalence of (pre)neoplastic liver lesions and lymphocystis virus disease. The hypothesis of a causal relationship between pollution and these diseases was supported by semi-field experiments. Therefore a series of laboratory experiments was performed to further substantiate causality and identify the xenobiotics that may play a major role in the field. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are important environmental pollutants. They are highly persistent, highly lipophilic, and have shown to induce several toxic effects in mammalian and non-mammalian species at relatively low concentrations. This report describes a study in which European flounder were orally exposed to the most toxic PCDD congener, 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD) or to harbor sludge extract under controlled laboratory conditions. The effects on several organs (liver, gills, gastro-intestinal tract, thyroid gland, gonads, spleen and mesonephros) were examined microscopically. Induction and localization of cytochrome P4501A (CYP1A) immunoreactivity, and effects on hepatocyte-proliferation were visualized immunohistochemically. Effects on thymus size were examined by morphometric analysis. Oral exposure of flounder to 0.0125 or 0.3125 µg TCDD/kg bw, or to 0.3125 µg TEQ/kg bw of a harbor sludge extract, weekly for 8 weeks, induced a significant increase in CYP1A immunoreactivity in hepatocytes. Single administration of higher doses (20, 100 and 500 µg/kg bw) of TCDD also induced a significant increase CYP1A immunoreactivity in the endothelium in all organs examined, and in the epithelium of the digestive tract, liver, and mesonephros. Remarkably, strong immunoreactivity was noted in a distinct cell population of the hematopoietic tissue in the mesonephros and spleen, which has not been described in fish previously. Moreover, oral exposure to 20 µgTCDD/kg bw resulted in an increased mitotic activity, and an increased hepatosomatic index was found after exposure to 500 µg TCDD/kg bw. In the thymus only a trend in size reduction was noted, again in the highest dose group. Nevertheless, no marked pathology was detected even in fish exposed to a single dose of 500 µg TCDD/kg body weight. The present experiments show that, under the actual experimental conditions, European flounder is relatively insensitive to the toxic effects of TCDD. However, we assume that exposure to TCDD (and related substances) may promote the development of tumors in the field.

1. Introduction

Strong indications for a relationship between water pollution and fish disease have been found by several researchers (Couch and Harshbarger, 1985; Malins et al., 1984, 1985; Murchelano and Wolke, 1991; Myers et al., 1994; Vethaak and ap Rheinallt, 1992; Vethaak and Jol, 1996; Vethaak and Wester 1996). In European flounder (*Platichthys flesus*) caught in polluted Dutch coastal waters and estuaries, a relatively high prevalence of hepatic tumors, lymphocystis virus infections and ulcerative skin lesions was reported (Vethaak and ap Rheinallt, 1992; Vethaak and Jol, 1996; Vethaak et al., 1996). Vethaak et al. (1996) showed a causal relationship between exposure to polluted sediment and water, and an increased prevalence of (pre)neoplastic liver lesions and lymphocystis infections in European flounder kept in

semi-field conditions. To investigate which individual substance plays an important role in the induction of the effects observed in European flounder in the field, experiments under controlled conditions are imperative. Therefore, a series of laboratory experiments has been performed with the same fish species that showed disease in the wild, the European flounder. Previous experiments in this series showed gill lesions and effects on the immune system after exposure to TBTO (Grinwis et al., 1998).

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are important environmental pollutants (reviewed by Safe, 1990). They form a group of over 200 chemicals that are highly lipophilic and persistent. PCDDs and PCDFs are formed as unintentional by-products of many industrial processes involving chlorine. Incinerators burning chlorinated wastes form the major source of PCDDs and PCDFs in the environment (95%), but pollution with these chemicals is also related to paper mills (which use chlorine bleaching in their production process) and to the production of polyvinyl chloride (PVC) plastics. Levels of PCDDs, PCDFs and related PCBs in Dutch sediments range between 1 and 20557 ng toxic equivalents (TEQ)/kg dry weight. Highest levels have been measured in harbor sediments in Rotterdam. Although TEQ levels in sedimentation areas have decreased by 70–80% since 1975, levels in suspended matter from the rivers Rhine and Scheldt have not declined significantly between 1985 and 1993 (Evers et al., 1996). The most toxic congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Most, if not all, of the effects of TCDD and of other PCDDs, PCDFs and the structurally related planar polychlorinated biphenyls (PCBs) are mediated through the cytosolic aryl-hydrocarbon (Ah) receptor. This receptor has been identified in many mammalian and non-mammalian species (Denison et al., 1986). The relative potency of single PCDDs, PCDFs and structurally related PCBs compared to TCDD is expressed in the toxicity equivalence factor (TEF). The relative toxicity of complex mixtures of these chemicals is expressed as toxic equivalents (TEQs) (Safe 1993; Safe, 1998). The TEF/TEQ approach is a useful utility in the hazard and risk assessment of PCDDs and PCDFs although some problems and limitations with this approach have been described (Safe, 1993, 1994, 1998).

PCDDs and PCDFs induce the same type of toxic reactions in a variety of organ systems. Target organs for PCDD/PCDF toxicity differ between species, and the difference in species sensitivity is remarkable. The LD50 of TCDD after oral exposure ranges from 0.6–2.1 µg/kg body weight (bw) in the guinea pig (Schwetz et al., 1973; McConnell et al., 1978) to 1157–5051 µg/kg bw in the Syrian golden hamster (Henck et al., 1981). Amongst the effects reported is a substantial reduction of fat tissue (Peterson et al., 1984) and muscle tissue (Max and Silbergeld, 1987) resulting in a loss of body-weight in most mammalian species. A dose dependent decrease in body weight gain is noted after administration of sub-lethal doses of TCDD in mice and guinea pigs (McConnell et al., 1978). In addition, a broad array of morphological changes in the liver has been reported mainly in laboratory rodents. Lesions included are fatty change, hypertrophy and hyperplasia of hepatocytes, multinucleated giant hepatocytes, increase of smooth endoplasmic reticulum, necrosis and inflammatory changes (Kociba et al., 1978; McConnell et al., 1978; Turner and Collins, 1983). Inflammation, hepatocellular necrosis and sinusoidal dilatation were noted in rainbow trout (*Oncorhynchus mykiss*) (Van der Weiden et al., 1992). Growth retardation, degenerative effects in liver cells and teratologic effects have been described in early

life stages of rainbow trout (Helder, 1981). In mirror carp (*Cyprinus carpio*), fat depletion and pericholangitis was described after administration of TCDD (Van der Weiden et al., 1994). Chronic studies in rats have also shown an increased incidence of malignant liver cell tumors and squamous cell carcinomas in for instance the lung (Kociba et al., 1978). Cytochrome P4501A (CYP1A) is one of several enzymes induced by TCDD. It plays an essential role in the TCDD metabolism and induction of CYP1A is widely used as an indicator (biomarker) for TCDD exposure (reviewed by Bucheli and Fent, 1995). Atrophy of the thymus and suppression of the thymus-dependent immunity is the most important immunotoxic effect of TCDD and it is seen in all animal species tested (reviewed by Vos and Luster, 1989, and Holsapple et al., 1991). As a result of TCDD-induced immunosuppression, host-resistance to various infectious agents is impaired (reviewed by Vos et al., 1991). The data on the immunotoxicity of TCDD in fish are far less extensive. In rainbow trout (*O*. *mykiss*), ex vivo/in vitro suppression of the pokeweed mitogen (PWM) response of splenic lymphocytes was found, but the concanavalin A (Con A) response of thymic and splenic lymphocytes and phagocytic activity of peritoneal macrophages were not altered (Spitsbergen et al., 1986).

In the present study, the sensitivity of European flounder to TCDD was examined to elucidate the role of TCDD in the increased disease prevalence as was shown in mesocosm experiments in which flounders were exposed to contaminated harbor spoil containing TCDD and related substances. In two of the reported experiments high levels of TCDD were used since European flounder turned out to be relatively insensitive to TCDD exposure in the course of the study.

2. Materials and methods

2.1. *Fish species and maintenance*

In all experiments, 3-year-old European flounders (*Platichtys flesus*) with a length of 16–22 cm. were used. The animals had been caught with a dip-net as young animals (0 + age group) in a relatively unpolluted site in the wild, close to the British coast in an estuary near Southampton, and subsequently raised at the Netherlands Institute for Fisheries Research (RIVO-DLO, IJmuiden) (Grinwis et al., 1995). The present experiments were performed at two different institutes, and prior to all experiments, an acclimatization period of at least 4 weeks was taken into account.

In experiment 1 and 2, the animals were kept in glass aquaria (75 × 60 × 50 cm) filled with salt water (salinity 32%) and clean sand on a 12 h dark/light regimen. The water was aerated continuously, water temperature was kept at 14 ± 1°C, and the animals were fed defrosted *Mysis relicta* (Landman, Zwartebroek, The Netherlands) daily at 16:00 h. Before the start of the experiments, a photograph of the bottom side of the animals was made for individual identification.

In experiment 3a, 3b and 3c, the fish were kept in glass aquaria (80 × 40 × 40 cm) filled with freshwater (Dutch Standard Water (DSW)). The water was aerated continuously. The water temperature was 19 ± 2°C and a 16–8 h light-dark regimen was maintained during these experiments. The animals were fed defrosted *Artemia salina* (SELCO, Artemia Systems N.V., Baas-Rode, Belgium) daily except on weekends. Three liters of silversand (M32, van Roon-Vreeswijk, Nieuwegein, The Netherlands) were added to the aquariums in order to improve husbandry conditions for this bottom dwelling fish species (Grinwis et al., 1995).

2.2. *Experimental design*

European flounders were exposed to low and high doses of TCDD or to a polluted harbor spoil extract in five experiments (Table 1). 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin used in experiment 1 and 2(~ 99% pure) was obtained from Promochem, Wesel, Germany. In experiment 3a, 3b, and 3c, performed in a different laboratory, 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (99.5% pure) was obtained from Smidt, Amsterdam, The Netherlands. Freeze-dried Rotterdam harbor spoil was extracted using the Soxhlet extraction method. The TEQ-level of the extract used in experiment 1 was measured by means of the Calux assay (assay described by Murk et al., 1996).

Table 1
Toxicity study of TCDD in flounder: experimental design

Experiment	1	2	3a	3b	3c
TCDD dose in µg/kg bw	0.0125 or 0.3125	20 or 100	100 or 500	100 or 500	100 or 500
Oral dosing	Weekly	Single	Single	Single	Single
Duration	8 weeks	8 weeks	1 week	2 weeks	4 weeks
No. of animals/group	14	6	6	6	6

In all experiments, animals were exposed by oral administration using a 200 µl gelatin capsule as previously described by Boon et al. (1992). Corn oil was used as solvent in all experiments; hence animals of the control groups were given a capsule with corn oil only. The animals were not sedated prior to administration of the capsule. After administration of the capsule, the animals were individually kept in aquariums for approximately 30 min to check for oil regurgitation.

In experiment 1, six groups of 14 animals each were formed. Two groups served as controls, the other groups were exposed to 0.0125 or 0.3125 µg TCDD/kg bw or 0.0125 or 0.3125 µg TEQ harbor spoil extract/kg bw. Capsules were administered weekly for 8 weeks. At the time of exposure, the length and weight of the animals were measured.

In experiment 2, groups of six animals each were exposed to a single oral dose of 0, 20 or 100 µg TCDD/kg bw, and euthanized after 8 weeks.

In experiments 3a, 3b, and 3c, three groups of six animals each were treated with a single dose of corn oil, 100 µg TCDD/kg bw or 500 µg TCDD/ kg bw. The animals were euthanized after one, two or four weeks. For a schematic presentation of the experiments, see Table 1.

2.3. *Sampling*

In experiment 1, the animals were stunned by a blow on the back of the head. Their length and weight were measured. The gall bladder was carefully removed and the liver was washed in ice-cold 0.9% NaCl and weighed. Part of the liver was stored at − 80°C until further processing for CYP1A (technique described by Omura and Sato, 1964) and EROD assays (technique described by Prough et al., 1978)(data not shown). The remainder of the liver and gill tissue was fixed in a solution of phosphate buffered 4% formaldehyde for histological investigation.

In experiment 2, the animals were stunned as described above. Body length and weight, weight of liver, spleen, and mesonephros were measured. The liver, gills,

gastro-intestinal tract, the head (including the caudal edge of the opercula, and the major part of the thyroid gland), gonads, the spleen and caudal 2/3 of the kidney (containing the mesonephros) were fixed as mentioned above for histology.

In experiments 3a–c, the animals were killed with an overdose of tricaine methanosulfonate (MS222®, Sandoz , Basel, Switzerland). Their body length and weight, and liver weight were measured. The liver, gills, gastro-intestinal tract, the head (including the caudal edge of the opercula, and the major part of the thyroid gland), gonads, the spleen and caudal 2/3 of the kidney (containing the mesonephros) were fixed as mentioned before.

2.4. *Histological techniques*

Histopathology was performed on the above-mentioned organs and tissues. All fixed materials were routinely processed and paraffin embedded. Sections of 3–5 µm were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The latter staining was used for identification of glycogen. Cryostat sections were made of a part of the formalin fixed livers and stained with Oil-red-O for the identification of lipids in hepatocytes.

Immunohistochemistry was performed on the liver using a monoclonal antibody (moab) against CYP1A (kindly provided by J.J Stegeman, Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA) in an indirect immunoperoxidase staining procedure, using the avidin–biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Tissue sections (3–5µm) were mounted on slides coated with 0.01% poly-l-lysine (Brunswick Chemie, Amsterdam, The Netherlands). After deparaffinization and rehydration, endogenous peroxidase was blocked by incubating the slides with 1% H_2O_2 in methanol for 30 min at room temperature. Then, slides were rehydrated and washed with a phosphate buffered saline (PBS)-Tween solution (PBS/Tween, three times 5 min), and preincubated with normal horse serum in PBS (1:10) for 15 min at room temperature. Subsequently the primary antibody was applied in a 1:800 dilution in 10% normal horse serum, and incubated overnight at 4°C. After incubation, slides were rinsed in PBS/Tween (three times 5 min) and incubated with biotinylated horse anti-mouse IgG (Vetcor Laboratories) diluted 1:125 in PBS and 1% normal horse serum for 30 min at room temperature. Next, the sections were rinsed in PBS/Tween (three times 5 min), and incubated with avidin–biotin complex, freshly prepared according to the manufacturer's instructions for 30 min at room temperature. After rinsing the slides with PBS (three times 5 min), immunoreactive CYP1A was visualized using 0.3% H2O2 and 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, MO) diluted in 0.05 mol/l Tris/HCl buffer during a 30 min incubation step. Then, sections were rinsed in distilled water for 10 min, dehydrated, sealed and covered with coverslips. Staining intensity of the liver tissue with the CYP1A moab was measured using an IBAS 2000 (Kontron, Munich, Germany) image analysis system. For this measurement, the monochrome picture of a liver section at a magnification of 300 X , was translated into a 'grey-scale' (0, black and 220, blank slide). The grey-values of 10 fields per section were measured. The more intense stained edges and less intense stained centers of the sections were avoided as well as sinusoids, bile ducts, and pancreatic tissue. Counterstaining of the slides with hematoxylin was not used to avoid interference with the morphometric analysis.

The relative immunoreactivity as displayed in Figs. 1–3 was calculated by subtraction of the measured grey-value from the grey-value of a blank slide (220).

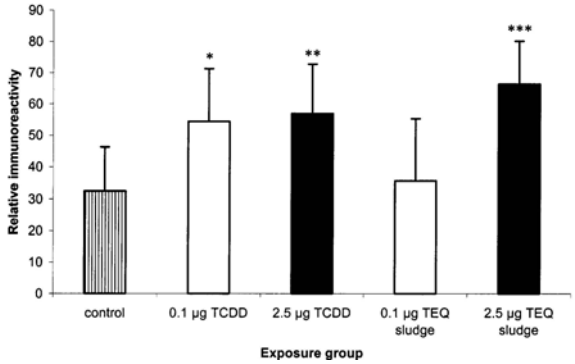

Fig. 1. Morphometric analysis of cytochrome P4501A immunoreactivity in hepatocytes of flounder exposed to TCDD or harbor sludge for 8 weeks (experiment 1).
Relative immunoreactivity = mean of (grey value of a blank slide) − (measured grey value) ± standard deviation of eight animals per group.
* Significantly different from controls at $P< 0.04$ (Student's *t*-test, 2-tailed).
** Significantly different from controls at $P< 0.002$ (Student's *t*-test, 2-tailed).
*** Significantly different from controls at $P< 0.001$ (Student's *t*-test, 2-tailed).

Liver sections were also stained with a monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Glastrup, Denmark) in an indirect immunoperoxidase staining procedure. Tissue sections (3–5µm) were mounted on slides coated with 0.01% poly-l-lysine (Brunswick Chemie). After deparaffinization and rehydration, antigen retrieval was performed by boiling of the slides in distilled water in a microwave (700 W) for 10 min. Slides were cooled-down at room temperature for 20 min. Endogenous peroxidase was blocked by incubating the slides with 1% H_2O_2 in methanol for 30 min at room temperature. Subsequently, slides were rehydrated and washed with PBS-Tween (three times 5 min) slides were incubated overnight at 4°C with the primary antibody in a 1:5000 dilution. After incubation, sections were rinsed in PBS/Tween (three times 5 min) and incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (Zymed Laboratories, San Francisco, CA) in a 1:50 dilution for 30 min at room temperature.

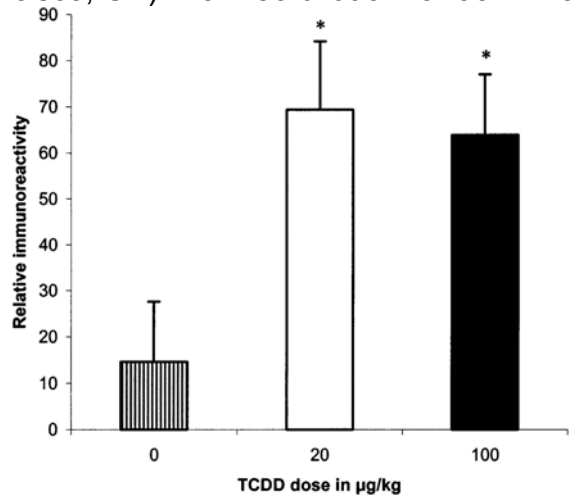

Fig. 2. Morphometric analysis of cytochrome P4501A immunoreactivity in hepatocytes of flounder exposed to TCDD for 8 weeks (experiment 2).
Relative immunoreactivity = mean of (grey value of a blank slide) − (mean measured grey value) ± standard deviation of six animals per group.
*Significantly different from controls at $P< 0.001$ (Student's *t*-test, 2-tailed).

Next, slides were rinsed with PBS/Tween (three times 5 min) and incubated with a HRP-labeled rabbit anti-goat antibody (Dako) for 30 min at room temperature. After rinsing with PBS, immunoreactive PCNA was visualized using 0.3% H_2O_2 and 0.5% 3,3-di-aminobenzidine tetrahydrochloride (Sigma Chemical) diluted in 0.05 mol/l Tris/HCl buffer, during a 10 min incubation step. A rinsing step with distilled water for 2 min followed, and then sections were counterstained with hematoxylin for 1 min. Finally, slides were rinsed in water, dehydrated, sealed and covered with coverslips.

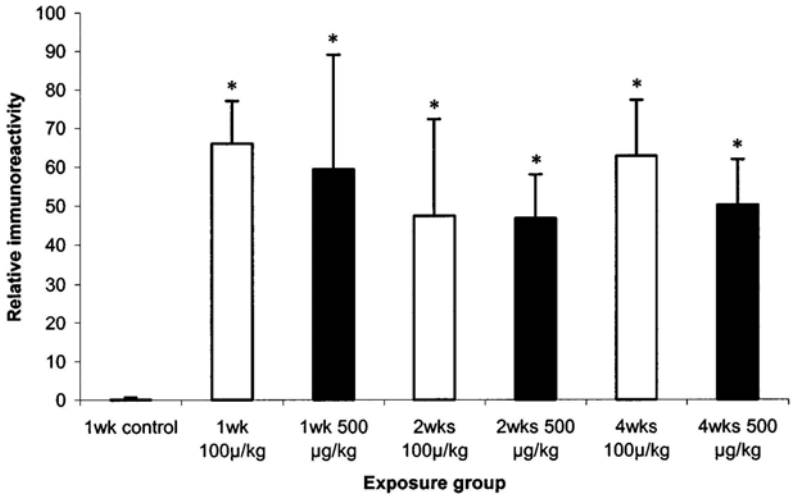

Fig. 3. Morphometric analysis of cytochrome P4501A immunoreactivity in hepatocytes of flounder exposed to TCDD (experiment 3a, 3b,and 3c).
Relative immunoreactivity = mean of (grey value of a blank slide) − (mean measured grey value) ± standard deviation of six animals per group.
*Significantly different from controls at $P < 0.005$ (Mann– Whitney test, 2-tailed).

All immunohistochemically stained sections from the same experiment were stained as one batch to prevent technique-induced staining differences. If slide numbers were too large to handle in one batch, batches of random samples of the slides from one experiment were made and stained. Therefore relative immunoreactivity values cannot be compared between experiments. To test for false positive results, negative controls were included in which the primary antibody was omitted or a primary monoclonal mouse anti chlamydia antibody (Clone C5, Argene Biosoft, Varilhes, France) was used whilst all other procedures were as described above. Observers scoring the histological sections were not aware of which exposure group the samples originated.

Due to its irregular shape and small size, weighing of the thymus, a procedure often used in mammals to detect thymotoxicity is practically not feasible. Besides, a clear distinction between cortex and medulla as seen in mammals, is not present in the thymus of European flounder. Therefore we used morphometric analysis of serial sections of the thymus in our experiments (Grinwis et al., 1998) from which the volume was calculated. To obtain sections of the thymus, a transverse block of each fish of about 0.5 cm thick was taken cranial from the caudal edge of the opercula. This block was decalcified for 5 days in a formic acid solution after fixation in 4% buffered formaldehyde before it was paraffin embedded. Serial sections of 5 µm were made at intervals of 30 µm until the whole thymus was sectioned. The surface areas of the slides of the thymus were 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 either side of the body was measured, and the mean values were used in our results. The thymus volume was also related to the body length and body weight.

2.5. *Data treatment and statistical analysis*

Statistical analysis was performed using SPSS for Windows 9.0 software (SPSS, Chicago, IL). Data were analyzed using an analysis of variance (ANOVA) followed by an independent t-test if a significant difference between groups was observed in the ANOVA. If parametric testing was not appropriate, the Kruskal–Wallis test, followed by the Mann–Whitney test if a significant difference between groups was observed, was used. Semi-quantitative data were tested for significance with the Fisher's exact test. Data were considered significantly different if $P < 0.05$.

3. Results

3.1. *General toxicological parameters*

No regurgitation of corn oil was detected in the experiments. No exposure related mortality, gross lesions or significant changes in body weight, body length, condition index or hepatosomatic index were found in experiment 1, 2, 3a, and 3b (data not shown). However, in experiment 3c, a significant 60% increase of the hepatosomatic index after 4 weeks of exposure was noted in animals exposed to 500 µg TCDD/kg (Table 2).

3.2. *Histopathology*

No exposure-related light microscopical alterations were noticed in the liver, thyroid gland, stomach, gut, spleen, mesonephros, gonads and gills of both treated and control animals in H&E, PAS and Oil-red-O stained sections in any of the experiments.

In all experiments, a highly significant exposure-related increase of immunoreactivity against CYP1A was found in the hepatocytes (Figs. 1–3). This increase was present diffusely throughout the liver without any indication of a zonal distribution (Fig. 4). Induction of CYP1A immunoreactivity was also seen in endothelial cells of portal vein branches and arteries in the liver. However, immunoreactivity of the endothelium of the sinusoids in the liver was not as marked as the immunoreactivity of the endothelium in other organ sites. The induction of CYP1A in the liver tissue was confirmed by biochemical analysis in experiment 1 (data not shown). Also, an exposure-related induction of CYP1A immunoreactivity was found in the endothelium of veins and arteries in the spleen, digestive tract, mesonephros, and gonads in experiment 3a–c (Table 3). Pillar cells and the endothelium of the larger gill-vessels also showed clear exposure-related CYP1A immunoreactivity (Table 3). Induction of CYP1A immunoreactivity, although less impressive than in endothelial cells, was recorded in the epithelium of the digestive tract (Fig. 5), the mesonephros (Table 3) and the pancreas. In the stomach, induction of CYP1A immunoreactivity was mainly visible in the glandular epithelium and less intense in the surface (mucous) epithelium (Fig. 5B). The epithelium of the proximal renal tubules showed stronger CYP1A immunoreactivity than the distal parts and

collecting ducts. Strong CYP1A immunoreactivity was also noted in cartilage cells of the skull in sections containing the thymus of one animal. This finding could not be confirmed since sections of other animals did not contain these cartilage cells.

Table 2
General toxicity parameters (length, body weight, liver weight, condition index, and hepatosomatic index) of flounder exposed to TCDD (experiment 3c)[a]

TCDDdose in µg/kg bw	Length (cm)	Body weight (g)	Liver weight (g)	Condition index[b]	Hepatosomatic index[c]
0	19.5 ± 2.0	85.9 ± 15.3	0.59 ± 0.22	1.15 ± 0.16	0.68 ± 0.2
100	20.7 ± 1.2	94.1 ± 11.5	0.97 ± 0.36	1.07 ± 0.12	1.0 ± 0.32
500	19.4 ± 1.1	89.9 ± 12.6	1.01 ± 0.39	1.23 ± 0.16	1.11 ± 0.37*

[a]Mean values ± standard deviation of six animals per group.
[b]Condition index = 100(body weight (g)/length^3 (cm)).
[c]Hepatosomatic index = 100(liver weight (g)/body weight (g)).
* Significantly different from controls at $P<0.04$ (Student's *t*-test, 2-tailed).

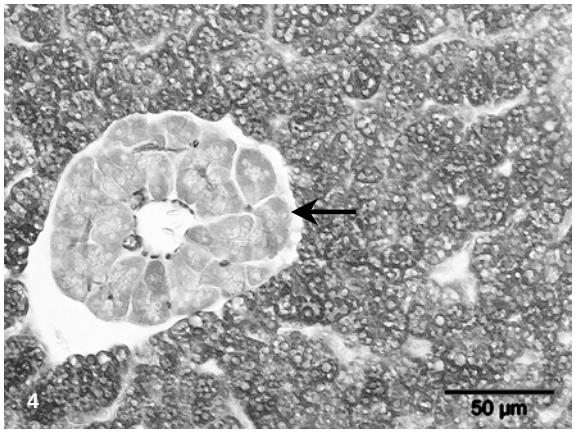

Fig. 4. Immunohistochemical localization of cytochrome P4501A in the liver of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks. Strong immunoreactivity is visible in hepatocytes and in the exocrine pancreas tissue (arrow).

No (experiments 3a–c) or little (experiment 2) immunoreactivity against CYP1A was seen in all tissues examined in the control animals.
In experiment 2, and 3a–c, a distinct population of mononuclear cells in the hematopoietic tissue of the mesonephros with strong CYP1A immunoreactivity was seen (Table 3, Fig. 6). These cells were also visible in the spleen but in smaller numbers, and only incidentally in the thymus. Indications for the presence of im-immunoreactive cells in circulating blood were found in one animal. Macrophages, as seen in for instance melanomacrophage-centers, did not show CYP1A immunoreactivity.
Exposure-related elevation of the mitotic activity in hepatocytes was shown by an increase in immunoreactivity against PCNA at the 2.5 µg/kg dose level (Fig.7). In experiment 2, increased immunoreactivity against PCNA was found at the 20 µg/kg dose level only, and exposure to 100 µg TCDD/kg bw resulted in reduction of the percentage of PCNA-positive cells (Fig. 8). However, this reduction could not be confirmed in hepatocytes of animals exposed to 100 or 500 µg TCDD/kg bw in experiment 3.

No significant effect of TCDD exposure on the absolute or relative thymus volume was found in experiment 3c (Table 4) although a trend in reduction of both absolute and relative thymus size was present.

No significant effect of gender on any of the results was noted.

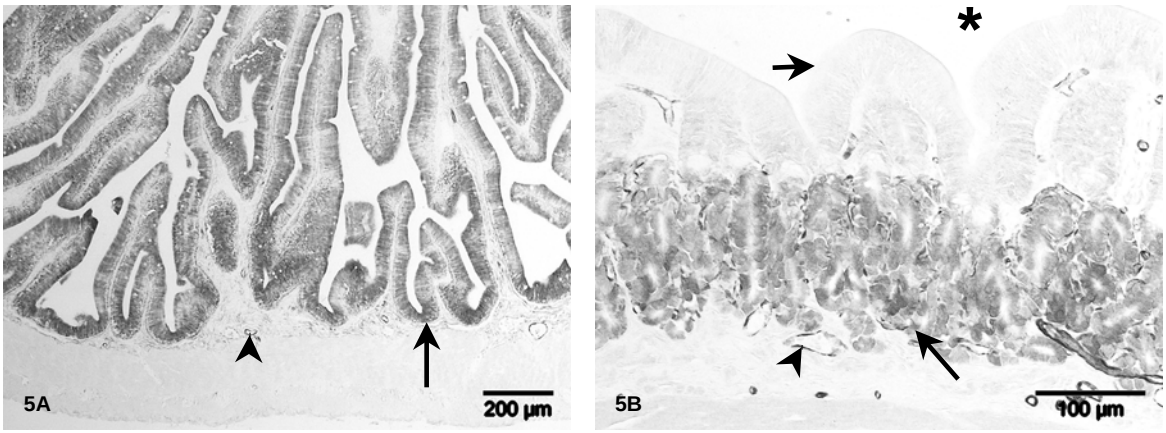

Fig. 5. Immunohistochemical localization of cytochrome P4501A in the digestive tract of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks. Strong immunoreactivity is visible in epithelial cells of the gut (arrow) and endothelial cells in all layers of the gut (arrowheads) (A). In the stomach, immunoreactivity is most prominent in the glandular epithelium (arrow) and the endothelium of small and larger vessels (arrowhead). The asterisk indicates the lumen, and the small arrow the surface epithelium of the stomach (B).

4. Discussion

The present experiments show that European flounder is relatively insensitive to exposure to TCDD under the presented experimental conditions. Juvenile rainbow trout (*O. mykiss*) showed 20% mortality, single cell necrosis and inflammation in the liver after intraperitoneal (ip) injection of 3.06 µg TCDD/kg bw (Van der Weiden et al., 1992). The LD_{50}s of TCDD in rainbow trout eggs were 0.331–0.489 µg/kg egg following injection and 0.346–0.519 µg/kg after waterborne exposure (Walker et al., 1992). In juvenile mirror carp (*C. carpio*) a decreased food intake, severe subcutaneous hemorrhages and apathy were reported after ip injection of 0.27 µg TCDD/kg bw, and 60% mortality was found after an ip dose of 2.93 µg TCDD/kg bw (Van der Weiden et al., 1994). However, in the present experiments oral exposure, which is clearly more relevant to the natural situation than injection, of European flounder to TCDD in a dose as high as 500 µg/kg bw did not induce lethal effects. Although strong induction of CYP1A in several organs, induction of PCNA immunoreactivity in the liver and a significant increase in hepatosomatic index after 4 weeks in the highest dose groups were noted, no marked pathology like liver necrosis and inflammation, the toxic effects found in most exposed animal species, was encountered. So these results suggest that, like in mammals, great differences in species sensitivity to TCDD can be expected. The oral exposure level of 500 µg/kg used in the present experiments was probably on the border of the real toxic dose considering the increase of the hepatosomatic index and the trend in thymus size reduction seen in animals exposed to this dose.

The significant induction of CYP1A immunoreactivity in the endothelium in all organs examined, and in the epithelial cells of the liver, digestive tract and mesonephros is in line with findings reported in other fish species exposed to TCDD (Smolowitz et al., 1991; Stegeman et al., 1991; Buchmann et al., 1993; Husøy et al.,1994). Exposure to 0.3125 µg TEQ/kg bw of an environmentally relevant mixture

of xenobiotics derived from harbor sludge (TEQ levels for PCDDs and PCDFs in sediments in the Dutch aquatic environment vary between sites, e.g. 34–36 ng TEQ/ kg dry weight in the Wadden sea, and 134–20557 ng TEQ/kg dry weight in the Chemieharbor (Evers et al., 1996)) also induced a significant increase in CYP1A immunoreactivity.

Table 3
Immunoreactivity against CYP1A in endothelial cells, epithelium of the digestive tract and mesonephros, and mononuclear cells in the hematopoietic tissue of the mesonephros of flounder (five animals per group) exposed to TCDD for 4 weeks (experiment 3c).

Organ and TCDD dose (µg/kg bw)	-	+/-	+	++
Endothelium[a]				
0	5	0	0	0
100***	0	0	0	5
500***	0	0	0	5
Epithelium of digestive tract[a]				
0	5	0	0	0
100**	1	0	1	3
500**	1	1	2	1
Epithelium of mesonephros[a]				
0	5	0	0	0
100*	1	0	2	2
500***	0	1	2	2
Mononuclear cells in the hematopoietic tissue of the mesonephros [b]				
0	5	0	0	0
100	4	0	0	1
500	2	0	3	0

[a] -: negative, +/-: some immunoreactivity, +: marked immunoreactivity, ++: strong immunoreactivity
[b] number of cells showing immunoreactivity; -: no immunoreactive cells, +/-: some immunoreactive cells, +: 1-10 immunoreactive cells per field (400x magnification), ++: > 10 immunoreactive cells per field (400x magnification)
* Significantly different from controls at $\underline{P} < 0.05$ (Fishers's Exact test, 2-sided).
** Significantly different from controls at $\underline{P} < 0.02$ (Fishers's Exact test, 2-sided).
*** Significantly different from controls at $\underline{P} < 0.001$ (Fishers's Exact test, 2-sided).

However, the interpretation of CYP1A induction in field research is complicated since CYP1A levels can be influenced by more factors than exposure to xenobiotics alone, e.g. water temperature (Sleiderink et al., 1995) or food composition (Jimenez and Burtis, 1988). The absence of morphological effects on the thyroid gland is not surprising since Besselink et al. (1997) found no changes in plasma thyroid hormone parameters after oral exposure of flounder to 100 µg TCDD/kg bw.

Remarkable is the induction of immunoreactivity in a distinct population of mononuclear cells in the hematopoietic tissue of the mesonephros and, in small numbers, also in the spleen. Indications for the presence of these immunoreactive mononuclear cells were also found in circulating blood, and data from experiments of European flounder exposed to 3,3',4,4',5-pentachloro-biphenyl (PCB-126) confirm

the presence of these cells in the circulation (unpublished data). These immunoreactive mononuclear cells were not convincingly present in the thymus. Due to the lack of specific markers for lymphoid cells in fish, a more specific characterization of the immunoreactive cell population was not possible. The induction of CYP1A in lymphoid cells in flounder is not unique; it has been reported in humans (Amsbaugh et al., 1986) and rats (Germolec et al., 1996). However, in literature dealing extensively with the immunohistochemical localization of CYP1A in scup (Smolowitz et al., 1991; Stegeman et al., 1991), zebrafish (Buchmann et al., 1993), and Atlantic cod (Husøy et al., 1994) after induction, immunoreactivity against CYP1A in cells of the hematopoietic system was not reported.

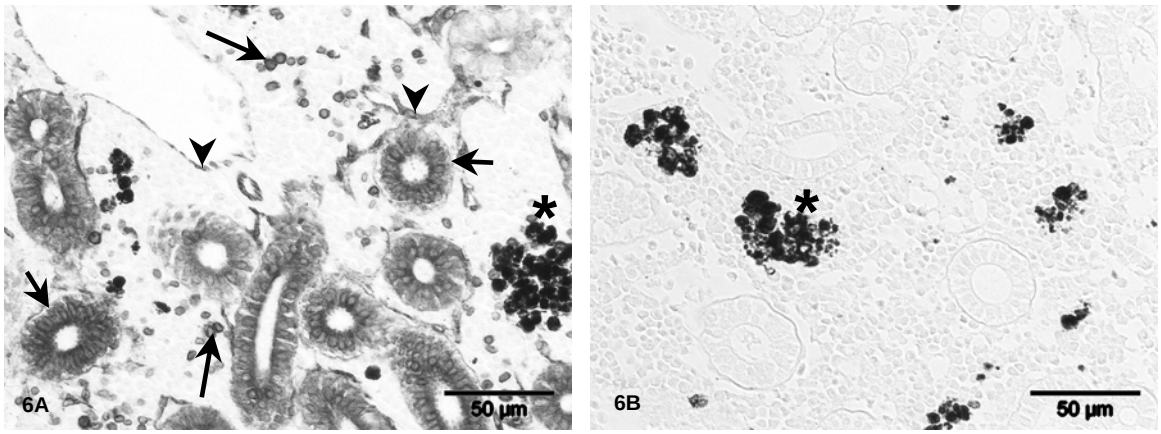

Fig. 6. Immunohistochemical localization of cytochrome P4501A in the mesonephros of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks (A). Strong immunoreactivity is visible in epithelial cells (small arrow) and endothelial cells (arrowhead). Remarkable is the presence of numerous immunreactive cells of the hematopoietic tissue (large arrows). Melanomacrophage centres are also clearly visible (asterisks). Note the absence of immunoreactivity in the mesonphros, containing several melanomacrophage centers (asterisks), of a control animal (B).

In humans, the detection of CYP1A-induction in peripheral blood lymphocytes, through measurement of CYP1A mRNA levels, has been described as a sensitive method to evaluate dose-response relationships of exposure to PCDDs and related chemicals (Vanden Heuvel et al., 1993). As a result of the findings of the present experiments, further research was initiated to investigate whether a similar 'non-invasive' technique can be applied in monitoring programs with fish. More research is also necessary to elucidate the possible consequences of induction of CYP1A in these immunoreactive cells with respect to functionality of the immune system since the presence of the Ah receptor, identified in cells of the immune system in man (Masten and Shiverick, 1995) and mouse (Lawrence et al., 1996), is probably important in mounting an inflammatory response in these mammalian species (Thurmond et al., 1999).

In mesocosm experiments, an increased prevalence of the viral lymphocystis disease was reported in European flounder after exposure to contaminated harbor sludge (Vethaak et al., 1996), which might be attributed to suppression of the immune system. Although atrophy of the thymus and suppression of the thymus-dependent immunity is the most important immunotoxic effect of TCDD and it is seen in all animal species tested (reviewed by Vos and Luster, 1989 and Holsapple et al., 1991), no significant thymus atrophy was found in the present study.

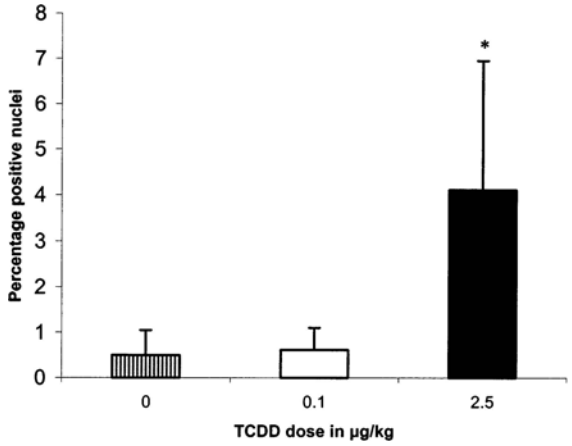

Fig. 7. Morphometric analysis of PCNA immunoreactivity (mean values ± standard deviation of seven animals per dose) in hepatocytes of flounder exposed to TCDD for 8 weeks (experiment 1).
*Significantly different from controls at $P < 0.01$ (Mann–Whitney test, 2-tailed).

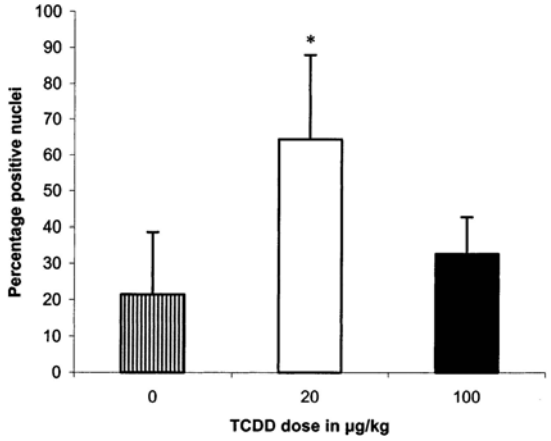

Fig. 8. Morphometric analysis of PCNA immunoreactivity (mean values ± standard deviation of six animals per dose) in hepatocytes of flounder exposed to TCDD for 8 weeks (experiment 2).
*Significantly different from controls at $P< 0.03$ (Mann–Whitney test, 2-tailed).

This could be explained by the dose used, which induced only borderline toxicity in the present experiments. Also, experiments by Vos and Moore (1974) have shown that TCDD-induced lymphoid involution is age related in mice and rats. This effect was more pronounced in the developing immune system than in the immune system of adult animals. Since all fish used in the present experiments were 3-years-old, the most sensitive period in thymus development might have been passed at the time of exposure to TCDD resulting in only a trend in thymus size decrease more than a significant size reduction. Immune function tests are necessary to evaluate the impact of TCDD on the specific resistance against relevant infectious diseases under field conditions, e.g. the viral lymphocystis disease.

The present experiments clearly show that immunohistochemistry used in surveys addressing exposure of fish to toxic xenobiotics, as an extension of histopathology, is a valuable tool that enables the researcher to detect enzyme induction, even in small cell-populations that might easily be overlooked in biochemical methods. The value of immunohistochemistry in research into the effects of xenobiotics in fish was also emphasized by other researchers (Goksøyr, 1995; Goksøyr and Husøy, 1998; Spies et al., 1996; Husøy et al., 1996; Woodin et al., 1997).

Table 4
Thymus volume and relative thymus volume of flounder exposed to TCDD for 4 weeks calculated from morphometric analysis (experiment 3c)[a]

TCDD dose in µg/kg bw	Absolute thymus volume (mm^3)	Thymus volume/body weight (×10−2)	Thymus volume/body length (×10−2)
0	1.18 ± 0.37	1.50 ± 0.50	6.10 ± 2.09
100	1.11 ± 0.63	1.10 ± 0.65	5.54 ± 3.62
500	0.84 ± 0.62	1.16 ± 1.09	4.36 ± 3.34

[a]Mean values ± standard deviation of six (0 and 100 µg TCDD/kg bw) or three (500 µg TCDD/kg bw) animals.
No significant differences.

Furthermore, significant induction of CYP1A in liver tissue was found, even at low dosages of TCDD or after exposure to an environmentally relevant mixture of xenobiotics (harbor spoil extract). The increased mitotic activity and induction of CYP1A in hepatocytes found in the present studies might indicate that TCDD and related chemicals could play a role in tumor promotion in the field.

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Chapter 5

Toxicity of PCB-126 in European Flounder (*Platichthys flesus*) with emphasis on histopathology and cytochrome P4501A induction in several organs systems

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Abstract

A series of experiments was set up to elucidate the effects of pollution on marine and estuarine fish health, since the European flounder (*Platichthys flesus*) has shown a relatively high prevalence of (pre)neoplastic liver lesions and lymphocystis virus disease in Dutch coastal and estuarine waters. The hypothesis of a causal relationship between pollution and the above-mentioned diseases was supported by results from semi-field experiments. Therefore several laboratory experiments were carried out to further substantiate causality and to identify the xenobiotics that may play a major role in the field. The present study focuses on polychlorinated biphenyls (PCBs). European flounders were orally exposed to a single dose of 0, 0.5, 5 or 50 mg PCB-126/kg body weight under controlled laboratory conditions. The effects on liver, gills, gastrointestinal tract, gonads, spleen and mesonephros were examined histologically after 16 days. Induction and localization of cytochrome P4501A (CYP1A) immunoreactivity, and effects on hepatocyte proliferation were visualized immunohistochemically. Effects on thymus size were examined by morphometric analysis of serial sections. Three out of five animals of the highest dose group showed haemorrhages in the fins and tail after 16 days. All animals showed reduced activity in the later stages of the experiment, and some animals of the highest dose group discontinued feeding 14 days after exposure. Strong and exposure-related induction of CYP1A immunoreactivity was noted in hepatocytes, endothelium in all organs examined, and epithelium of the digestive tract and mesonephros at PCB-126 levels of 0.5, 5 and 50 mg/kg. In addition, the strong induction of CYP1A immunoreactivity in a distinct population of haematopoietic cells in the mesonephros and in circulating blood is remarkable, and has not been described previously in other fish species. Furthermore, a morphometrically determined significant reduction in relative thymus size was noted in animals exposed to 50 mg PCB-126/kg. Although the functional implications for the immune system of this reduction need to be further investigated, an impact on the specific resistance against infectious diseases as observed in the field, e.g. viral lymphocystis disease, is not implausible. In addition, a significant increase in absolute liver weight, in hepatosomatic index, and in number of proliferating hepatocytes [measured as immunoreactivity against proliferating cell nuclear antigen (PCNA)] was noted in animals of the highest dose group. From these findings we suppose that PCB-126 (and related chemicals) may play a role in the promotion of tumor development in the liver of European flounders as observed in the field. The results of the present experiment show relatively stronger effects than effects previously reported from experiments with TCDD, suggesting that the TEF of 0.005 assigned to PCB-126 from early life stage mortality experiments in rainbow trout (Oncorhynchus mykiss), underestimates the toxic potential of PCB-126.

1. Introduction

European flounder (*Platichthys flesus*) is a euryhaline benthic species that lives in close contact to sediments in coastal and estuarine areas that are often heavily polluted. Therefore this species is theoretically a pre-eminent candidate to contract xenobiotic-related diseases, and field surveys have indeed shown relatively high prevalences of several diseases in European flounder, e.g. liver tumors, lymphocystis disease and skin ulcers in relation to pollution (Vethaak and Jol 1996; Vethaak and Wester 1996). Semi-field (mesocosm) experiments, combining moderate control of

experimental variables with moderate relevance for the feral populations, indicated a causal relationship between exposure to polluted sediment and water, and an increased prevalence of (pre)neoplastic liver lesions and lymphocystis infections in European flounder (Vethaak et al., 1996). To validate the postulated causal relationship between pollution and fish disease, a series of laboratory experiments was performed with single, potentially involved, xenobiotic substances. Previous experiments in this series with European flounder showed gill lesions and effects on the immune system after aqueous exposure to TBTO (Grinwis et al., 1998). Oral exposure to TCDD showed strong induction of cytochrome P4501A (CYP1A) immunoreactivity in epithelia (e.g. in liver, kidney and digestive tract), in the endothelium of all organs examined, in cells of the hematopoietic tissue, as well as an increase in hepatosomatic index and immunoreactivity against proliferating cell nuclear antigen (PCNA) in the liver (Grinwis et al., 2000).

The present study focuses on PCBs. Commercially produced PCBs were initially used in so-called closed systems as dielectric fluids in transformers and later in capacitors, heat-transfer systems and hydraulic equipment for mining purposes, but also in open systems in for instance paper, paints, lubricants, and pesticides (de Voogt and Brinkman 1989). Polychlorinated biphenyls (PCBs) and the structurally related polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) are environmental pollutants of considerable importance because of their high persistence, strong lipophilic properties, and their toxic effects reported in mammalian and non-mammalian species (reviewed by Safe 1990). Ross and co-workers even suggested that PCBs were largely responsible for the immunotoxic effects in seals as opposed to the effects of PCDDs and PCDFs based on toxic equivalence (TEQ) profiles derived from these immunosuppressed marine mammals (Ross et al., 1995). Like 2,3,7,8-tetrachorodibenzo-*p*-di-oxin (TCDD), most of the effects of the planar PCBs that are structurally related to TCDD are mediated through the cytosolic aryl-hydrocarbon (Ah) receptor. This receptor has been identified in many mammalian and non-mammalian species (Denison et al., 1986), including in cells of the immune system of man (Masten and Shiverick 1995) and mouse (Lawrence et al., 1996). On a qualitative basis, the type of toxic effects is similar to that of TCDD, and PCBs are assigned toxicity equivalence factors (TEF) to express their toxic potential in relation to TCDD. 3,3',4,4',5-Pentachlorobiphenyl (PCB-126) is the most toxic planar PCB, with a TEF of 0.005 in early life stage mortality experiments in fish (Walker and Peterson 1991; Zabel et al., 1995) and 0.1 in mammals (Van den Berg et al., 1998).

The present study was designed to assess target organs of PCB-126 toxicity. The dosing of PCB-126 was guided by previously reported experiments with 2,3,7,8-TCDD in European flounder (Grinwis et al., 2000) and by the above-mentioned TEF of 0.005.

2. Materials and methods

2.1. *Fish species and maintenance*

The experiment was performed using 2-year-old European flounder (*Platichthys flesus*) with a length ranging from 16 to 18 cm, weighing between 40 and 60 g. The animals were caught with a dip-net as young animals (0+ age group) at a relatively unpolluted site in an estuary near Southampton (United Kingdom), and subsequently

raised at the Netherlands Institute for Fisheries Research (RIVO-DLO, IJmuiden) (Grinwis et al., 1995). An acclimatization period of more than 4 weeks prior to the experiment was taken into account.

The fish were kept in glass aquaria (40x40x40 cm) filled with fresh water (Dutch Standard Water (DSW)) and 2.5 l of silver sand (M32, van Roon-Vreeswijk, Nieuwegein, The Netherlands). The water was renewed semi-statically twice a week, and aerated continuously. Water temperature was kept at 19.2°C and a 12-12 h light-dark regimen was maintained during the experiments. The animals were fed defrosted Artemia salina (SELCO, ArtemiaSystems, Baas-Rode, Belgium) daily except during weekends.

The present study was approved by the ethical committee for animal experiments of the Utrecht University, and complies with Dutch laws.

2.2. *Experimental design*

European flounders were treated with 0, 0.5, 5 or 50 mg 3,3',4,4',5-pentachlorobiphenyl/kg body weight (PCB-126; Lot R126A,2; Promochem, Wesel, Germany) with a purity of 99.1% dissolved in corn oil. All fish were exposed by single oral administration of a 250 µl gelatine capsule (Boon et al., 1992), similar to Grinwis et al. (2000). The control group was given a capsule with solvent only. After administration of the capsule, the animals were individually kept in aquariums for approximately 30 min to check for regurgitation of oil. The fish were not sedated prior to administration of the capsule.

2.3. *Sampling*

Initially, a 4-week experiment (as in Grinwis et al., 2000) was planned. However, because of the unexpected occurrence and severity of clinical symptoms, all animals were killed by an overdose of tricaine methanosulphonate (MS222®, Sandoz, Basel, Switzerland) after 16 days and examined for external and internal gross lesions. Their body length and weight and liver weight were measured. The liver, gill, gastrointestinal tract, the head (including the caudal edge of the opercula, and the major part of the thyroid gland), gonads, the spleen and mesonephros were fixed in a solution of 4% phosphate buffered formaldehyde for histological examination.

2.4. *Histological techniques*

Histopathology was performed on the above-mentioned organs and tissues. All fixed materials were routinely processed and paraffin embedded. Sections of 3-5 µm were stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The latter staining was used for the identification of glycogen.

Immunohistochemistry was performed on the liver using a monoclonal antibody (MoAb) against CYP1A (kindly provided by J.J. Stegeman, Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Mass., USA) with indirect immunoperoxidase staining (Grinwis et al., 2000). Staining intensity of the liver tissue with the CYP1A MoAb was measured using an IBAS 2000 (Kontron, Munich, Germany) image analysis system. For this measurement, the monochrome picture of a liver section at a magnification of x300 was translated into a "grayscale" (0=black and 220=blank slide). The gray values of ten fields per section were measured,

avoiding artefacts, sinusoids, bile ducts, and pancreatic tissue. No counterstaining of the slides was used, to avoid interference with the morphometric analysis. The relative immunoreactivity as displayed in Fig. 4 was calculated by subtraction of the measured gray-value from the gray-value of a blank slide (220).

Liver sections were also stained with a monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Glastrup, Denmark) in an indirect immunoperoxidase staining (Grinwis et al., 2000). Observers scoring the histological sections were not aware of which exposure group the samples originated.

Due to its irregular shape and small size, weighing of the thymus, a procedure often used in mammals to detect thymotoxicity, is not practically feasible in flounder. In addition, a clear distinction between cortex and medulla as seen in mammals is lacking in the thymus of this species. Therefore we used morphometric analysis of serial sections of the thymus in our experiments (Grinwis et al., 1998) from which the thymus volume was calculated. To obtain these sections, a transverse block of each fish of about 0.5 cm thick was taken cranial from the caudal edge of the opercula. This block was decalcified for 5 days in a formic acid solution after fixation in 4% buffered formaldehyde before it was paraffin embedded. Serial sections of 5 µm were made at intervals of 30 lm until the whole thymus was sectioned. The surface areas of the slides of the thymus were 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 either side of the body was measured, and the mean values were used in our results. The thymus volume was also expressed relative to body length and body weight.

2.5 *Statistical analysis*

Statistical analysis was performed using SPSS for Windows 9.0 software (SPSS Chicago, Ill., USA). Data were analyzed using an analysis of variance (ANOVA) followed by an independent t-test if a significant difference between groups was observed in the ANOVA. If parametric testing was not appropriate, the Kruskal-Wallis test was used, followed by the Mann-Whitney test if a significant difference between groups was observed. Semi-quantitative data were tested for significance with the Fisher's exact test. Data were considered significantly different if $P < 0.05$.

3. Results

3.1. *General (toxicological) parameters*

The animals of most exposure groups showed a slight, not exposure related, reduction in body weight (Table 1). Only the 5.0 mg/kg group showed a small, not significant, increase in body weight after 16 days (Table 1). One week after exposure, all animals of the 5.0 and 50 mg/kg groups were less active (notably reduced swimming behavior during renewal of water) than animals in the 0.5 mg/kg and control group. After 11 days all PCB-exposed animals showed reduced activity, and after 14 days, three out of five animals in the 50 mg/kg group discontinued feeding, were lethargic and showed small hemorrhages in the tail and fins. No other gross lesions were observed during the study or at necropsy. A significant increase in liver weight of 87% in animals treated with 50 mg/kg was noted compared to controls. The hepatosomatic index increased significantly in the highest dose group by 75% when

compared to controls (Table 1).

3.2. *Histopathology*

H&E stained sections of one animal exposed to 50 mg/ kg showed granulomatous lesions in the submucosa of the gill cavity and in the spleen with a different morphology from the common melanomacrophage centers (MMCs). Another animal exposed to 50 mg/kg revealed some small granulomas in the hepatopancreas adjacent to the exocrine pancreas tissue. Also, several telangiectasia or peliosis-like distended sinusoidal structures were found in the liver of this animal. Both of these animals showed hemorrhages in tail and fins macroscopically. A trend for exposure related increased lipid storage in hepatocytes (seen as cytoplasmic vacuolization) was noted.

In all animals exposed to PCB-126, a strong and dose-related increase in CYP1A immunoreactivity was noted in hepatocytes of treated animals (50 mg/kg, Fig. 3A) as compared to controls (Fig. 3B), in the endothelium of vessels in all organs examined, in pillar cells in the gills (Fig. 1), and in epithelial cells of the digestive tract and mesonephros (Fig. 2, Table 2). The induction of CYP1A immunoreactivity in the endothelium of the hepatic sinusoids was not as marked as in the endothelium of larger vessels in the liver (e.g. portal vein branches). In the stomach, induction of CYP1A immunoreactivity was mainly visible in the glandular epithelium and less intense in the surface (mucous) epithelium. The epithelium of the proximal renal tubules displayed stronger CYP1A immunoreactivity than the distal parts and collecting ducts. Strong immunoreactivity in a distinct population of mononuclear cells of the hematopoietic system was detected in animals of all groups exposed to PCB-126 (Fig. 2, Table 2). These immunoreactive cells were also present in circulating blood and could therefore be seen in vessels in several organs, e.g. in the liver (Fig. 3A). Macrophages, as seen in for instance MMCs, did not demonstrate CYP1A immunoreactivity. Morphometric analysis showed that the increase of CYP1A immunoreactivity in hepatocytes was significantly stronger in all exposure groups compared to the controls.

Table 1
General toxicity parameters (body weight loss, condition index, liver weight, and hepatosomatic index) of flounders exposed to PCB-126 for 16 days[a]

PCB dose in mg/kg	Body weight loss[b] (g)	Condition index[c]	Liver weight (g)	Hepatosomatic index[d]
0	1.6 ± 10.1	1.09 ± 0.03	0.32 ± 0.07	0.66 ± 0.10
0.5	1.6 ± 8.0	1.03 ± 0.06	0.42 ± 0.12	0.88 ± 0.24
5	-0.3 ± 7.9	0.98 ± 0.1*	0.42 ± 0.10	0.85 ± 0.2
50	2.1 ± 8.0	1.07 ± 0.09	0.60 ± 0.05**	1.15 ± 0.09**

[a]Mean values.SD of five animals per dose
[b]Body weight loss=body weight at D0-bodyweight at D16
[c]Condition index=100[body weight (g)/length $(cm)^3$]
[d]Hepatosomatic index=100[liver weight (g) /body weight (g)]
* *P*<0.05 (Student's t-test, 2-tailed) compared to controls
***P*<0.01 (Student's t-test, 2-tailed) compared to controls

Also, the CYP1A induction in the 50 mg/kg (highest dose) group was significantly stronger than in the 0.5 mg/kg group (Fig. 4). The immunoreactivity of CYP1A in all

organs examined of the control animals was weak or absent (illustrated by Fig. 3B). Immunohistochemical PCNA labeling in nuclei of hepatocytes indicated stimulation of the mitotic activity in liver cells (Fig. 5). Despite a wide range in the number of positive nuclei between animals of the same exposure group, the increase was statistically significant in the highest dose group when compared to controls.

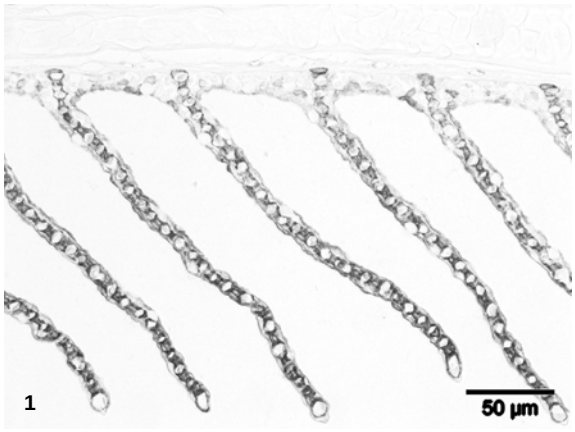

Fig. 1 Immunohistochemical localization of cytochrome P4501A in the gills of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in pillar cells.

Morphometric analysis of the thymus volume of animals exposed to 50 mg PCB-126/kg showed a significant reduction of 47% compared to control animals when the thymus volume was related to body length or body weight (Table 3).

4. Discussion

Sixteen days exposure of European flounder to 50 mg PCB-126/kg body weight did induce alterations in some general toxicological parameters: in three out of five animals, behavior was changed (e.g. lethargy, anorexia) and small hemorrhages in tail and fins were noted.

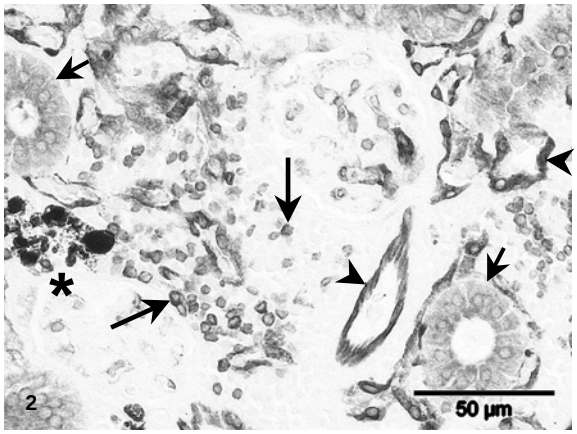

Fig. 2 Immunohistochemical localization of cytochrome P4501A in the mesonephros of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in epithelial cells (small arrows) and endothelial cells (arrowheads). Remarkable is the presence of numerous immunoreactive cells of the hematopoietic tissue (large arrows). Melanomacrophage centres are also clearly visible (asterisk).

By contrast, no changes in behavior and no externally visible abnormalities were noted in previously reported experiments in which the same fish species was orally

exposed to a single dose of up to 500 µg TCDD/kg body weight (Grinwis et al., 2000). Significant increases in liver weight, hepatosomatic index, and CYP1A immunoreactivity in hepatocytes in animals in the highest dose group were noted.

In addition, an increase in mitotic activity in hepatocytes, indicated by increased immunoreactivity against PCNA, was detected in hepatocytes. Although high variability in positive nuclei was present, the increase in the highest dose group was statistically significant compared to controls.

Table 2 CYP1A
Immunoreactivity in endothelial cells, epithelium of the digestive tract and mesonephros, and mononuclear cells in hematopoietic tissue of the mesonephros of flounders (five animals per dose) exposed to PCB-126 for 16 days

Organ and PCB-126 dose (mg/kg)	−	±	+	++
Endothelium[a]				
0	5	0	0	0
0,5**	0	1	1	3
5**	0	0	1	4
50**	0	0	0	5
Epithelium of digestive tract[a]				
0	5	0	0	0
0,5*	1	0	4	0
5*	1	1	1	2
50** #	0	0	0	5
Epithelium of mesonephros[a]				
0	5	0	0	0
0,5	2	1	2	0
5*	1	0	2	2
50**	0	0	2	3
Mononuclear cells in the hematopoietic tissue of the mesonephros[b]				
0	5	0	0	0
0,5	3	0	1	1
5	2	0	0	3
50**	0	0	1	4

[a] ± negative, +/± some immunoreactivity, + marked immunoreactivity, ++ strong immunoreactivity
[b] Number of cells showing immunoreactivity; ± no immunoreactive cells, +/± some immunoreactive cells (per tissue section), + 1±10 immunoreactive cells per field (400x magnification), ++ >10 immunoreactive cells per field (400x magnification)
* Significantly different from controls at P<0.05 (Fisher's Exact test, 2-sided)
** Significantly different from controls at P<0.01 (Fisher's Exact test, 2-sided)
# Significantly different from 0.5 group at P<0.01 (Fisher's Exact test, 2 sided)

The use of more animals in the experimental groups would probably reduce this variation, but the increase in hepatocellular proliferation is in agreement with findings reported in literature in rodents and could indicate that PCB-126 plays a role in the promotion of tumor development in the liver of European flounder as observed in the field. The significant exposure-related induction of CYP1A and the pattern of induction, e.g. induction in epithelia (in the liver, digestive tract and mesonephros), and endothelium in all organs examined, in the present experiment is in agreement with literature data on experiments in which fish were exposed to planar PCBs or TCDD (Smolowitz et al., 1991; Stegeman et al., 1991; Buchmann et al., 1993; Husøy et al., 1994, 1996). Strong induction of CYP1A was also seen in a distinct population of mononuclear cells, possibly of lymphoid origin, in the mesonephros, as was also noted after exposure to TCDD (Grinwis et al., 2000). These immunoreactive cells

also appeared in small numbers in circulating blood, but due to the lack of specific markers for lymphoid cells in European flounder, a more specific characterization of this immunoreactive cell population was not possible. The induction of CYP1A in (presumptive) lymphoid cells is not unique for this species; it has also been noted in humans (Amsbaugh et al., 1986) and rats (Germolec et al., 1996). However, CYP1A-immunoreactivity in cells of the hematopoietic system was not reported in literature dealing extensively with the immunohistochemical localization of CYP1A after induction with xenobiotics in scup (Smolowitz et al., 1991; Stegeman et al., 1991), zebrafish (Buchmann et al., 1993), and Atlantic cod (Husøy et al., 1994). In humans, the detection of CYP1A-induction in peripheral blood lymphocytes, through measurement of CYP1A mRNA levels, has been described as a sensitive method to evaluate dose-response relationships of exposure to PCDDs and related chemicals (vanden Heuvel et al., 1993). As a result of the findings of the present experiment, further research has been initiated to investigate whether a similar “non-invasive” technique can be applied in monitoring programs with fish. Most literature data indicate that immunotoxicity, including thymotoxicity (as most of the other effects) of polyhalogenated aromatic hydrocarbons (PHAHs) in laboratory rodents is Ah-receptor mediated (Silkworth and Grabstein 1982; Clark et al., 1983; Vecchi et al., 1983; Silkworth et al., 1984; Kerkvliet et al., 1990a, 1990b), and the presence of the Ah receptor, identified in cells of the immune system in man (Masten and Shiverick 1995) and mouse (Lawrence et al., 1996), is probably important in mounting an inflammatory response in these mammalian species (Thurmond et al., 1999).

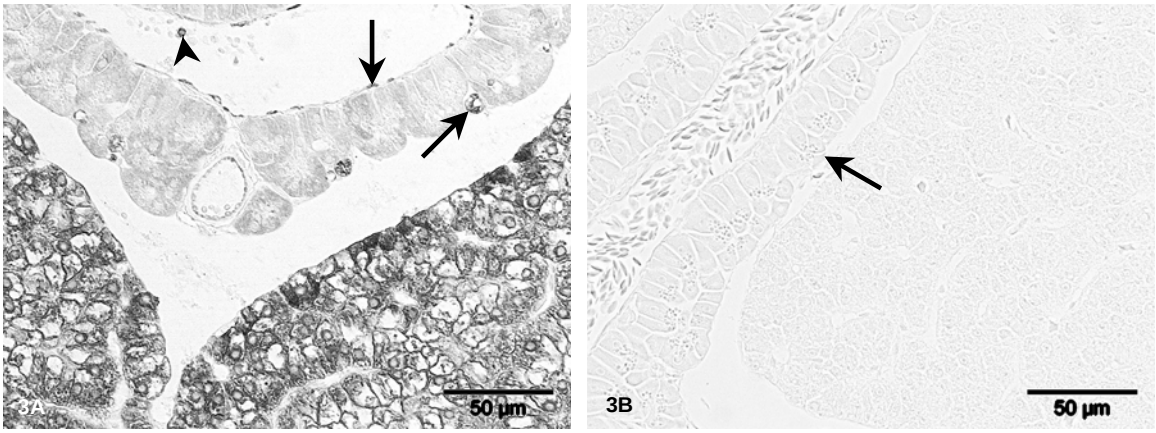

Fig. 3 A Immunohistochemical localization of cytochrome P4501A in the liver of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in hepatocytes, and in the endothelium of arterioles and portal vein branches (arrows). Note the presence of an immunoreactive mononuclear cell in sinusoids of the liver (arrowhead). B Immunoreactivity in hepatocytes is weak, and absent in exocrine pancreatic tissue (arrow) of a control animal.

Therefore, immunotoxic effects of PCB-126 can also be expected in European flounder, since this study showed that exposure of European flounder to PCB-126 significantly induced thymus atrophy and CYP1A in cells of the haematopoietic tissue. Since functional immune effects are reported to be more sensitive than morphological thymus effects (Vos et al., 1978; Silkworth and Antrim 1985; Holsapple et al., 1986; Tucker et al., 1986; Kerkvliet and Brauner 1990), an impact on the specific resistance against infectious diseases as observed in the field, e.g. viral lymphocystis disease, is not implausible. However, the functional implications of the reduction in thymus size and the possible consequences of induction of CYP1A

immunoreactivity in cells of the hematopoietic system need to be addressed in immune function and host resistance experiments.

In the present experiment, dosing of PCB-126 was guided by the results from previous experiments in which European flounders were exposed to a maximum single oral dose of 500 µg 2,3,7,8-TCDD/kg (Grinwis et al., 2000), and the TEF of 0.005 of PCB-126 as was determined in rainbow trout early life stage mortality (Walker and Peterson 1991; Zabel et al., 1995).

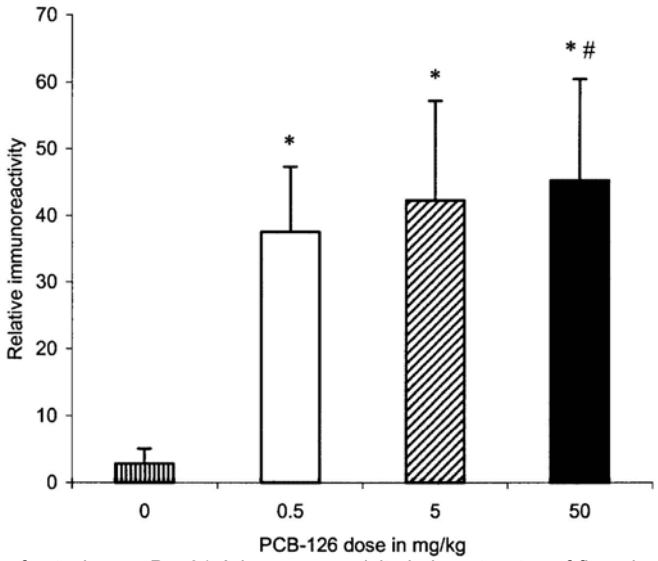

Fig. 4 Morphometric analysis of cytochrome P4501 A immunoreactivity in hepatocytes of flounders exposed to PCB-126 for 16 days. Relative immunoreactivity=mean of (gray value of a blank slide)-(measured gray value) ± SD of five animals per group.
* Significantly different from controls at P<0.001 (Student's t test, 2-tailed).
# Significantly different from 0.5 group at P<0.05 (Student's t test, 2-tailed)

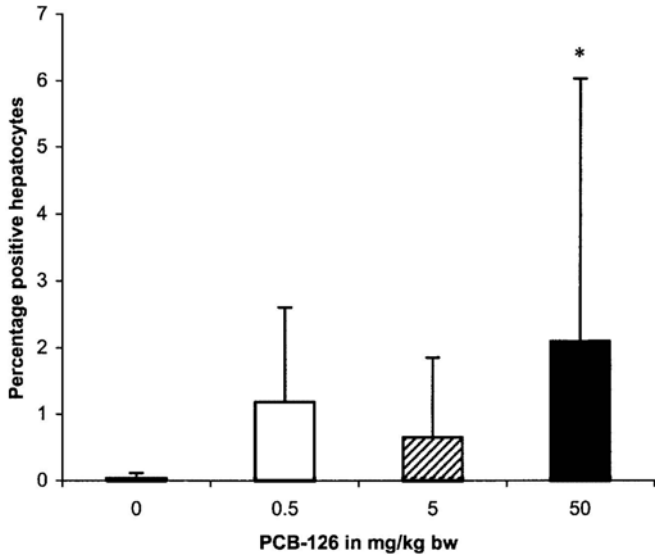

Fig. 5 Morphometric analysis of PCNA immunoreactivity (mean value of five animals per dose ± SD) in hepatocytes of flounders exposed to PCB-126 for 16 days.
*Significantly different from controls at P<0.05 (Mann-Whitney test, 2-tailed)

In the present study, administration of a 50% lower maximum dose of PCB-126 than the maximum TCDD dose (on TEF basis) in previous experiments resulted in

stronger effects than induced after exposure to TCDD: clinical signs were noted (and were absent after TCDD exposure), stronger induction of CYP1A, significant reduction of the relative thymus size (which was not found after exposure to TCDD), and slightly stronger liver effects. These results indicate that the above-mentioned TEF of 0.005 for PCB-126 possibly underestimates the toxic potency of this planar PCB.

Table 3
Absolute and relative thymus volume of flounders exposed to PCB-126 for 16 days, calculated from morphometric analysis[a]

PCB dose in mg/kg	Absolute thymus volume (mm^3)	Thymus volume/ body weight ($x10^{-2}$)	Thymus volume/ body length ($x10^{-2}$)
0	1.75 ± 0.61	3.60 ± 0.94	10.6 ± 3.37
50	0.95 ± 0.39*	1.84 ± 0.89**	5.60 ± 2.35*

[a] Mean values ± SD of five animals per dose
* Significantly different from controls at P<0.04 (Student's t test, 2-tailed)
** Significantly different from controls at P<0.02 (Student's t test, 2-tailed)

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Chapter 6

Cytochrome P4501A induction in peripheral blood leukocytes in European flounder (*Platichthys flesus*): an intravital biomarker of exposure?

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Submitted for publication

Abstract

Cytochrome P4501A (CYP1A) is a frequently used indicator for exposure to aryl-hydrocarbon receptor (AhR) agonists. Typically, induction in fish is determined by measurement of CYP1A-protein content or by measuring the activity of the CYP1A-catalyzed ethoxyresorufin-O-deethylase (EROD), both usually obtained from killed animals. We previously reported CYP1A induction in cells of hematopoietic tissue as well as in circulating leukocytes in European flounder (*Platichthys flesus*). In humans CYP1A induction can be easily determined in peripheral blood leukocytes and is suggested as a biomarker for exposure to dioxin-like compounds. In the current experiment European flounder was orally exposed by gelatin capsule to 0, 0.005, 0.05, 0.5 and 5 mg PCB-126. Animals were killed after two and sixteen days. General histopathology, immunohistochemistry and immunocytochemistry against CYP1A were performed to assess CYP1A induction and to evaluate whether peripheral blood could be used as a biomarker for environmental exposure to AhR agonist compounds. We were able to detect immunoreactivity in mononuclear leukocytes in peripheral blood after 16 days and significant CYP1A immunoreactivity in endothelium and hepatocytes of the highest dose group (5 mg/kg) compared to control animals. The use of peripheral blood leukocytes as an intravital biomarker of exposure to AhR agonists would help researchers, especially in laboratory and semi-field experiments, to monitor animals in time and potentially could reduce the number of animals used in these experiments. Our findings show some potential for this biomarker. However, more research is needed into the applicability of CYP1A mRNA quantification in circulating mononuclear leukocytes in flounder, and whether inter-individual variability in flounder leukocytes hampers the applicability of this technique as a biomarker as it does in humans.

1. Introduction

European flounder (*Platichthys flesus*) is one of the fish species used in monitoring programs in European waters and is included in the guidelines for monitoring biological effects of contaminants in estuaries and coastal waters by the OSPAR Joint Assessment and Monitoring Program (JAMP; JAMP 1998). Because European flounder is a common species on soft substrata, where it lives in close contact to sediment (encountering potentially high levels of pollution) this species is prone to develop diseases related to exposure to environmental contaminants. Findings of fields surveys and a semi-field study in the Netherlands have indicated that the occurrence of certain diseases (lymphocystis virus infection and in particular liver tumors) in Dutch flounder populations is at least in part due to the presence of chemical contaminants in the estuarine and coastal environment (Vethaak et al., 1996; Vethaak and Jol, 1996; Vethaak and Wester, 1996).

3,3',4,4',5-Pentachlorobiphenyl (PCB-126) is the most toxic PCB and is an important environmental pollutant since it is highly persistent and has toxic effects reported in numerous mammalian and non-mammalian species (reviewed by Safe, 1990). Like the structurally related 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, most effects of PCB-126 are mediated through the aryl-hydrocarbon (Ah) receptor that has been identified in the cytoplasm of hepatocytes of many mammalian and non-mammalian

species (Denison et al., 1986), and in cells of the immune system of man (Masten and Shiverick, 1995) and mouse (Lawrence et al., 1996).

Previous experiments with PCB-126 in European flounder have shown induction of cytochrome P4501A (CYP1A) in several organs and circulating mononuclear blood cells, and a significant reduction of both absolute and relative (related to length and weight) thymus volume (Grinwis et al., 2001). Although CYP1A-immunoreactivity of hematopoietic cells has not been reported in the literature on the immunohistochemical localization of CYP1A after induction with xenobiotics in Atlantic cod (*Gadus morrhua*) (Husøy et al., 1994), scup (*Stenotomus chrysops*) (Smolowitz et al., 1991; Stegeman et al., 1991), and zebrafish (*Brachydanio rerio*) (Buchmann et al., 1993), it is not unique for flounder. It has also been noted in humans (Amsbaugh et al., 1986) and rats (Germolec et al., 1996). Measurement of CYP1A mRNA levels in peripheral blood lymphocytes in humans has been described as a sensitive method to evaluate dose-response relationships of exposure to PCDDs and related chemicals (vanden Heuvel et al., 1993).

The present study was performed to evaluate the value of immunocytochemistry of mononuclear leukocytes in peripheral blood of PCB-126-exposed European flounder as a non-lethal way of detecting exposure to Ah-receptor agonists. Typically, in monitoring projects and laboratory research, fish are sacrificed and hepatocytic microsomes are used to determine the level of CYP1A induction through measurement of ethoxyresorufin-O-deethylase (EROD) activity. With the use of immunocytochemistry, experimental animals don't need to be sacrificed, can be followed in time and such a method can thus lead to reduction of the number of test animals.

2. Materials and methods

2.1. *Experimental fish*

Captive-bred European flounder (*Platichthys flesus*), obtained as fingerlings (1-group) from the Port Erin Marine laboratory, the School of Biological Sciences, University of Liverpool (UK), and grown for 2 years at the National Institute for Coastal and Marine Management (RIKZ) field station at Jacobahaven, the Netherlands, were used as experimental animals. The approximately 3-year old animals were ranging from 61-247 gram body weight. The fish were kept in glass aquaria in a flow-through system containing 10kg of relatively clean sandy sediment (drawn from the Eastern Scheldt) on a 70x100 cm bottom, and 160 l seawater. The water was renewed by continuous flow-through with salt water of 32 promille from the relatively clean Eastern Scheldt at a rate of 175 l per day. Water temperature was 16 $\pm$ 1°C and the animals were subjected to a 12-12 hours dark-light regimen. Fish were fed pelleted feed (Trouvit; Seafarm, Kamperland, the Netherlands) at an estimated 1% of the total body weight three times a week, and inspected daily for behavioral changes and signs of disease. Seawater quality was checked twice a week and concentrations of dissolved oxygen and pH remained within a normal range for the duration of the experiment. Accumulating organic debris was removed from the tank bottom regularly.

The present study was approved by the ethical committee for animal welfare in experiments of the RIKZ, and complies with Dutch laws.

2.2. *Chemicals and experimental design*

Five controls and 10 animals per dose were treated with 0, 0.005, 0,05 or 5 mg 3,3',4,4',5-pentachlorobiphenyl/kg body weight (PCB-126; Lot R126A,2; Promochem, Wesel, Germany) with a purity of 99.1% dissolved in corn oil. All fish were exposed by single oral administration of a 250 µl gelatin capsule (Boon et al., 1992), similar to Grinwis et al. (2000). The control group was given a capsule with solvent only and the fish were not sedated prior to administration of the capsule. Animals were killed by an overdose of MS222® (ethyl 3-amino benzoate methanesulfonate, Sigma-Aldrich, Steinheim, Germany) in two batches. The first batch of five animals of every exposure group, was sampled two days after exposure. The second batch, which included the remaining five animals of all exposure groups and the five control animals, was killed 16 days after exposure. The animals were examined for external and internal gross lesions. Body weight and liver weight were measured and blood samples were taken from animals killed on D16 and collected in heparin coated glass tubes. Two air-dried blood smears were made on glass slides. The liver, gill, gastrointestinal tract, the head (including the caudal edge of the opercula, and the major part of the thyroid gland), gonads, the spleen and mesonephros of all animals (sampled on D2 and D16) were fixed in a solution of 4% phosphate buffered formaldehyde for histological examination.

2.3 Histological techniques

Histopathology was performed on the above-mentioned organs and tissues. All fixed materials were routinely processed and paraffin embedded. Sections of 3-5 µm thick were stained with haematoxylin and eosin (H&E). One blood smear of all sampled animals was stained with May-Grünwald Giemsa.

Immunohistochemistry was performed on the paraffin sections using a monoclonal antibody (MoAb) against CYP1A (kindly provided by J.J. Stegeman, Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Mass., USA) with indirect immunoperoxidase staining, using the avidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA; method after Grinwis et al., 2000). To test for false positive results, negative controls were included in which the primary antibody was replaced by a primary monoclonal mouse anti chlamydia antibody (Clone C5, Argene Biosoft, Varilhes, France).

2.4 Immunocytochemistry

Leukocytes from all animals killed on D16 were separated from other blood components by using a Percoll gradient (Amersham Biosciences; density 1.13 ± 0.005 g/ml). Solutions with two densities, 1.02 and 1.07, were made from the Percoll stock using Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) and modified 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). 4.5 ml of the solution with 1.02 density was put onto 4.5 ml of the solution with density 1.07 in a 15 ml Greiner tube. Subsequently, a maximum of 2 ml of full blood was applied on top of the density gradient. After 30 minutes centrifugation at 3000 rpm and 4°C, a band containing the leukocytes could be removed (at the 1.02-1.07 junction) for

further processing. The fraction containing the leukocytes was washed once with 10-15 ml fcRPMI, centrifuged for 10 minutes at 1500 RPM and 4°C. The pellet was resuspended in 0.5 ml fcRPMI and smears were made from the cell suspension and air-dried. One smear of each animal was stained with May-Grünwald Giemsa. A second smear was fixed, together with the full-blood smears, in acetone for immunostaining.

Immunostaining of the smears was similar to the procedure used for paraffin embedded sections. Briefly, smears were pre-incubated with horse serum in PBS (1:10) for 15 minutes at room temperature. Subsequently the primary antibody was applied in a 1:800 dilution in 10% horse serum, and incubated overnight at 4°C. After incubation, smears were rinsed in PBS/Tween (three times five minutes) and incubated with biotinylated horse anti-mouse IgG (Vetcor Laboratories) diluted 1:125 in PBS and 1% horse serum for 30 minutes at room temperature. Next, the sections were rinsed in PBS/Tween (three times five minutes), and incubated with avidin-biotin complex, freshly prepared according to the manufacturer's instructions for 30 minutes at room temperature. After rinsing the slides with PBS (three times five minutes), immunoreactive CYP1A was visualized using 0.3% H_2O_2 and 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) diluted in 0.05 mol/l Tris/HCl buffer during a 30 minutes incubation step.

The immunostained sections and smears were stained in several batches. To prevent possible technique-induced staining differences, every batch contained similar numbers of smears or sections of all exposure groups.

Observers scoring the histological sections and smears were not aware from which exposure group the samples originated.

Table 1
General toxicity parameters (body weight increase, liver weight, and hepatosomatic index) of flounders exposed to PCB-126 for 16 days[a]

PCB dose in mg/kg	Mean body weight increase[b] (g)	Liver weight (g)	Hepatosomatic index[c]
0	5.6	2.05 ± 0.61	1.03 ± 0.11
0.005	16.9	1.49 ± 0.43	0.92 ± 0.22
0.05	12.4	1.89 ± 0.89	1.18 ± 0.56
0.5	-6.7	1.53 ± 0.57	1.16 ± 0.40
5	5.3	1.89 ± 0.69	1.32 ± 0.30

[a] Values of five animals per dose
[b] Body weight increase =body weight at D16-bodyweight at D0
[c] Hepatosomatic index=100[liver weight (g) /body weight (g)]

2.5 Statistical analysis

Statistical analysis was performed using SPSS for Windows 9.0 software (SPSS Chicago, Ill., USA). Data were analyzed using an analysis of variance (ANOVA) followed by an independent t-test if a significant difference between groups was observed in the ANOVA. Semi-quantitative data were tested for significance with the Fisher's exact test. Data were considered significantly different if $P < 0.05$.

3. Results

3.1 General (toxicological) parameters

On D2, the 0.005 and 0.05 mg/kg groups showed a slight decrease in body weight whereas animals in the higher dose-groups showed a slight increase in body weight. On D16, all dose-groups including the controls, except the 0.5 mg/kg group, showed an increase in body weight (Table 1) but, as on D2, standard deviations were high and these effects were not statistically significant. The increase in hepatosomatic index in the 3 highest dose-groups was not statistically significant (Table 1). No mortality occurred during the experiment and behavioral changes or external lesions were not noted in any of the experimental animals. Macroscopic external and internal lesions were absent at necropsy.

3.2 Histopathology

Several lesions were noted in standard H&E-stained sections, none of which appeared exposure-related. Most animals, also those of the control group, showed moderate to marked vacuolation of the epithelium of small bile ducts. These vacuolar changes were also seen, with lower prevalence, in larger bile ducts on D2 and D16 (Fig. 1A). This vacuolation occurred without concurrent cell-death, inflammation or proliferation of bile duct epithelium or hepatocytes. In some animals, small numbers of accumulations of protozoan parasites (xenomas) were encountered in the hepatopancreas (Fig. 1B). In one animal of the 0.05 mg group, a focus of swollen hepatocytes containing optically empty vacuoles (suggestive of fat-storage) without cellular or nuclear atypia was present in the liver, compressing the surrounding hepatocytes. Characteristics of this lesion are consistent with focal hepatocellular vacuolation and hypertrophy (Fig. 1 C). Ovaries in most animals contained atretic and often calcified follicles, sometimes accompanied by a granulomatous reaction (Fig 1D).

Dose related induction of immunoreactivity against CYP1A was noted in paraffin embedded tissue sections of renal tubular epithelium, hematopoietic tissue, hepatocytes, gastro-intestinal epithelium, and endothelium in most organs and tissues examined (Table 2, Fig. 2). Individual leukocytes in accumulations of blood in larger blood vessels also showed CYP1A immunoreactivity (Table 2). No difference in induction pattern was noted between D2 and D16 samples. Statistically significant differences in the induction of CYP1A immunoreactivity were only noted in the endothelium in the highest dose group and in hepatocytes in the 0,5 and 5 mg/kg group (Table 2).

3.3 Immunocytochemistry

May-Grünwald Giemsa stains of smears of cell-suspensions confirmed the successful isolation of leukocytes from full blood following the procedure described above. Typically, immunostaining of leukocytes of animals killed on D16 was more prominent in smears of full blood than in the samples of isolated leukocytes and was present in both controls as well as in animals exposed to PCB-126 (Fig. 2D; Table 3), most markedly in the 0.5 mg/kg group. Differences were not statistically significant.

Table 2
CYP1A immunoreactivity in endothelial cells, epithelium of the digestive tract and mesonephros, and mononuclear cells in hematopoietic tissue of the mesonephros of flounders (five animals per dose) exposed to PCB-126 for 16 days

Tissue and PCB-126 dose (mg/kg)	−	±	+	++
Endothelium[a]				
0	4	1	0	0
0.005	5	0	0	0
0.05	2	1	2	0
0.5	3	0	2	0
5*	0	0	2	3
Epithelium of digestive tract[a]				
0	3	1	1	0
0.005	4	1	0	0
0.05	3	1	0	1
0.5	0	2	3	0
5	0	1	4	0
Epithelium of mesonephros[a]				
0	3	1	1	0
0.005	2	3	0	0
0.05	0	4	1	0
0.5	1	4	0	0
5	0	2	1	2
Mononuclear cells in the hematopoietic tissue of the mesonephros[b]				
0	2	3	0	0
0.005	2	2	1	0
0.05	2	3	0	0
0.5	3	1	1	0
5	0	2	2	1
Peripheral blood leukocytes				
0	3	0	2	0
0.005	2	1	2	0
0.05	1	1	2	1
0.5	0	2	2	1
5	0	2	2	1
Hepatocytes				
0	3	2	0	0
0.005	2	3	0	0
0.05	3	1	1	0
0.5	1	0	4	0
5**	0	0	3	2

[a] ± negative, +/± some immunoreactivity, + marked immunoreactivity, ++ strong immunoreactivity
[b] Number of cells showing immunoreactivity; ± no immunoreactive cells, +/± some immunoreactive cells (per tissue section), + 1±10 immunoreactive cells per field (400x magnification), ++ >10 immunoreactive cells per field (400x magnification)
* Significantly different from controls at $P<0.01$ (Fisher's Exact test, 2-sided)
** Significantly different from controls at $P<0.02$ (Fisher's Exact test, 2-sided)

4. Discussion

The present study was conducted to evaluate the potential of peripheral blood mononuclear leukocytes in detecting exposure to Ah-receptor (AhR) agonists like PCB-126. In general, CYP1A immunoreactivity after exposure to PCB-126 in cells and tissues in the present experiment were comparable to studies reported in literature in which fish were exposed to planar PCBs or TCDD (Smolowitz et al., 1991; Stegeman et al., 1991; Buchmann et al., 1993; Husøy et al., 1994, 1996;

Grinwis et al., 2000, 2001). The induction of CYP1A immunoreactivity in circulating leukocytes, which has been reported in our previous histological work (Grinwis et al., 2001), was confirmed in both tissue sections and blood smears.

Table 3
Semi-quantitative immunoreactivity of isolated peripheral-blood mononuclear leukocytes of animals exposed to PCB-126 for 16 days.

PCB dose in mg/kg	CYP1A immunoreactivity[a]		
	-	±	+
0	2	2	1
0.005	2	2	1
0.05	2	2	1
0.5	1	0	4
5	3	1	1

[a] -: no; ±: moderate; +: frequent immunoreactive mononuclear leukocytes in peripheral blood.

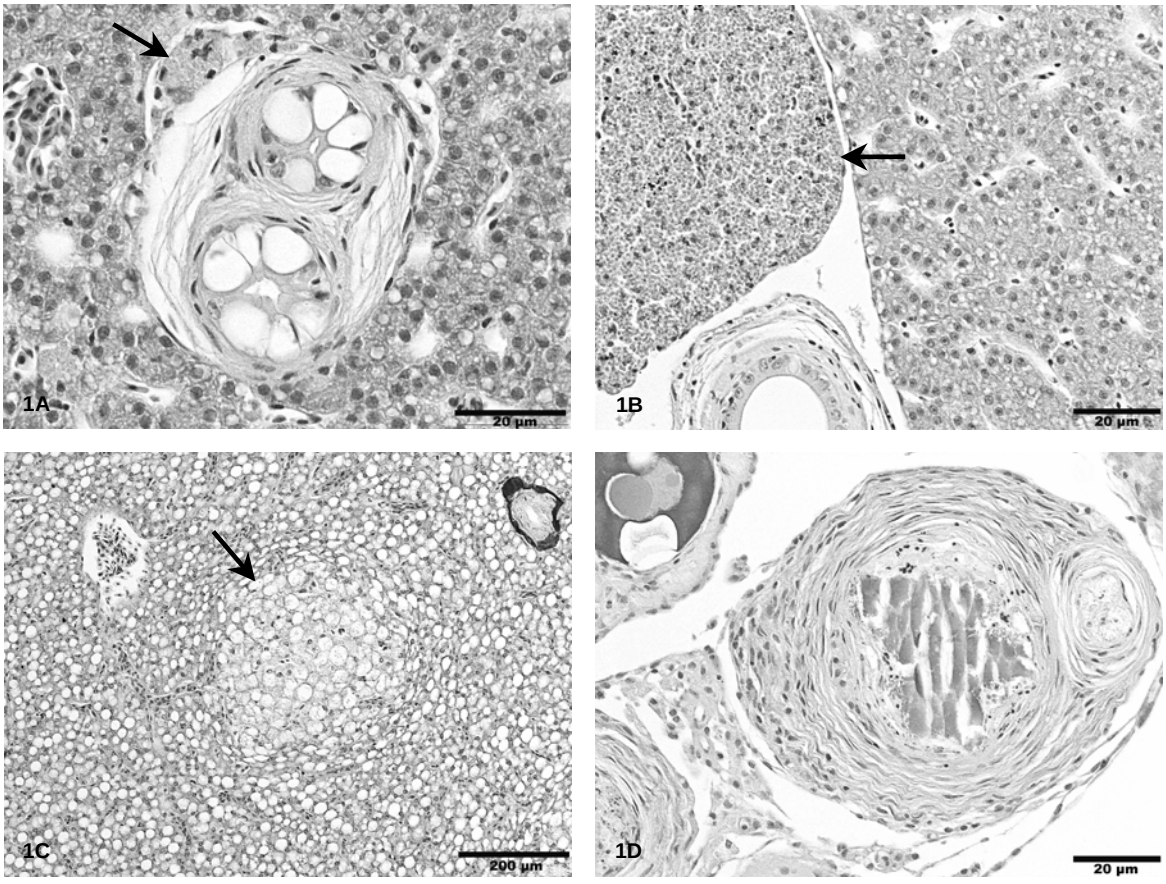

Fig. 1.
Marked vacuolation of epithelial cells of a larger bile duct with some periductular fibrosis and a small accumulation of pigment-containing macrophages (arrow) is visible in Fig. 1A. In Fig. 1B a large accumulation of microsporidian protozoa, probably *Glugea stephani* in the hepatopancreas of one of the fish is visible (arrow). A significant inflammatory response is absent. Fig. 1C shows a fatty focus in the liver. A group of swollen, vacuolated hepatocytes compresses surrounding hepatocytes (arrow). Fig. 1D shows an ovary containing a granulomatous focus with central calcification and concentric fibrosis. None of these changes were dose related. *H&E*.

Thus, the present study has shown that peripheral blood samples can be used to support the detection of exposure to Ah-receptor agonists in intact fish. However, as is shown in Table 2, immunoreactivity was also detected in control animals and

results of the present method were not statistically significant. High inter-individual variation in EROD levels has been reported in European flounder under field conditions (Eggens et al., 1995; George et al., 2004), and might have been of influence. Particularly since our experimental animals have had similar life history conditions suggesting that the variability is indeed related to inter-individual variation. The isolation method used to separate leukocytes from other blood components needs to be refined since isolation of these cells in the present study resulted in loss of immunoreactivity, and peripheral blood leukocyte numbers in our fish were typically low hampering detection of CYP1A induction. Since leukocytes isolated from spleen and mesonephros of European flounder, following a similar method, were successfully used for immune function tests in a previously reported study (Grinwis et al., 1998) a major effect of the isolation technique on the leukocytes is therefore unlikely as an explanation for the difference in immunoreactivity between isolated leukocytes and leukocytes in full blood.

Other methods for the detection of exposure to Ah-receptor agonists in liver tissue and lymphocytes include measurement of CYP1A mRNA levels. This technique has also been used in livers in several fish species (Zapata-Pérez et al., 2002; Rees et al., 2003) including European flounder (Vethaak et al., 1996; Dixon et al., 2002; George et al., 2004) but not in leukocytes. In rats CYP1A mRNA induction was detected at lower TCDD doses than induction of EROD activity, indicating a greater sensitivity of the reverse transcription-polymerase chain reaction (RT-PCR) compared to measuring EROD activity (vanden Heuvel et al., 1994). Whether this difference in sensitivity holds true for European flounder needs to be examined since no difference was found between EROD and CYP1A mRNA induction by TCDD in human and monkeys cells (Silkworth et al., 2005) indicating a species dependent variation. An additional advantage of measurement of CYP1A mRNA levels is the absence of interference with protein levels and/or functions caused by posttranslational modifications or interferences (Staskal et al., 2005). Also, mRNA levels provide a way to measure the competency of ligands with different AhR binding affinities to activate Ah-gene expression (Chen et al., 2004). Therefore, quantitatively measuring CYP1A mRNA levels in circulating lymphocytes might provide additional information not provided by measuring EROD activity. However, expression of CYP1A in blood mononuclear cells in humans measured by EROD expression as well as CYP1A mRNA levels showed high inter-individual variation and might therefore not be suitable to use as a biomarker of exposure to dioxin-like compounds in man (van Duursen et al., 2005). Also in European flounder, high interanimal variability in CYP1A mRNA levels in livers has been shown (Vethaak et al., 1996; Dixon et al., 2002). Whether similar variability is also present in circulating mononuclear cells in European flounder needs to be examined before a conclusion can be drawn towards the use of CYP1A mRNA levels in peripheral blood as a biomarker of exposure to AhR agonists.

The presence of moderate to strong vacuolation of bile duct epithelium in both control and exposed animals has neither been encountered in our previous experiments with PCB-126, nor in experiments with European flounder and TCDD and TBTO. No attempts were made to determine the contents of the vacuoles that, from the morphology, probably contain lipids or, less likely, water (fatty or hydropic change respectively). Because these changes were not anticipated we did not freeze liver samples to perform an oil-red-O, or other lipid stain, that could have given a clue towards the nature of the storage in these cells.

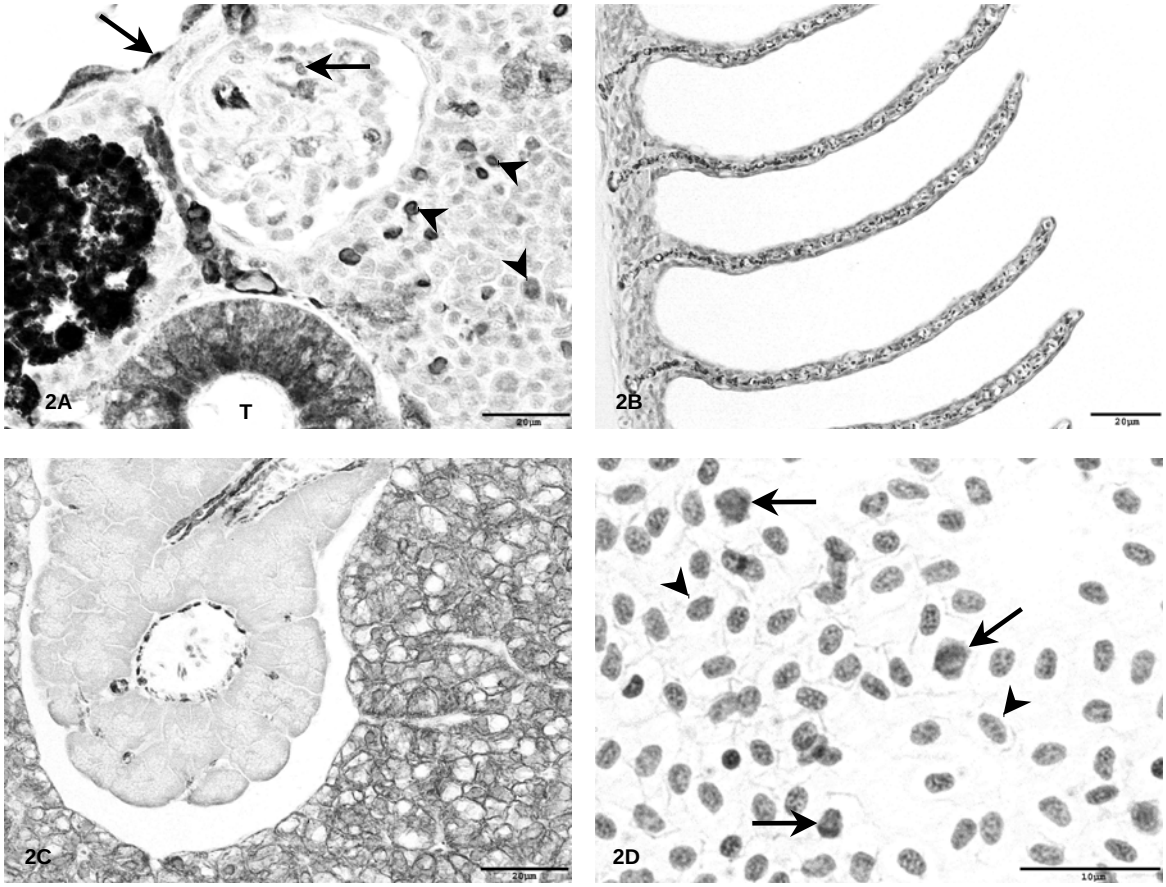

Fig. 2.
Immunohistochemical detection of cytochrome P4501A expression in the mesonephros (2A), pillar cells of the gills (2B) and hepatopancreas (2C) of European flounder exposed to 5 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in the endothelium of glomeruli and veins (arrows), epithelium of renal tubules (T) and several cells of the hematopoietic tissue (arrowheads). In the left side of Fig. 2A a melanomacrophage center is visible. Fig. 2D depicts a smear of peripheral blood of an animal exposed to 5 mg PCB-126/kg for 16 days, containing several immunoreactive mononuclear leukocytes (arrows). Note the negative staining cytoplasm of the erythrocytes (arrowheads).

Since reactive changes and/or indications of increased cell death in the bile duct epithelium were absent, it is questionable whether these morphological changes have any functional effect. The origin of the cause of this vacuolation remains unclear. Similar vacuolation of biliary epithelium was reported in European flounder captured in Dutch coastal and estuarine waters (Vethaak and Wester 1996), and European flounder used in a large scale mesocosm experiment (Vethaak et al., 1996). Particularly, results from the mesocosm experiment showed an association of the vacuolar changes with pollution and a significant association with hepatic foci of cellular alteration (Vethaak et al., 1996). The presence of atretic and often calcified follicles in ovaries, sometimes accompanied by a granulomatous reaction, is perhaps related to the inability of these animals to spawn in their artificial environment.

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Chapter 7

Summary and concluding remarks

Summary.

Triggered by the concern over relative high prevalences of liver tumors, skin ulcers and the viral lymphocystis disease in European flounder (*Platichthys flesus*) living in Dutch coastal and estuarine waters, an integrated study was initiated to investigate a possible causal relationship between chemical environmental pollution and the above mentioned diseases. The integrated study was a joint effort of the Dutch National Institute for Coastal and Marine Management (RIKZ), the Dutch National Institute for Public Health and the Environment (RIVM) and the Department of Pathobiology (Pathology Division) of the Veterinary Faculty of the Utrecht University. Systematic and routine epizootiological studies in Dutch coastal fish populations were initiated in the early 1980s. These field surveys showed relatively high prevalences of three grossly visible diseases in European flounder: skin ulcers, lymphocystis disease, and (histologically confirmed) liver tumors (Vethaak and Jol, 1996; Vethaak and Wester, 1996; *chapter 1*). In the following large scale semi-field (mesocosm) experiment, European flounder were directly or indirectly (through the water) exposed to contaminated dredged sediment from Rotterdam harbor. In this semi-field study a positive association between chemical contaminants (e.g. PAHs, planar PCBs) and (pre)neoplastic liver lesions and lymphocystis disease was noted (Vethaak et al., 1996; *chapter 1*). It was also concluded that disease etiology is generally multifactorial including infectious pathogenic agents and environmental factors such as chemical pollution (Vethaak and ap Rheinallt, 1992).

This thesis describes the laboratory experiments that were part of the integrated study. The following working hypothesis for the laboratory experiments was formulated from the results of field and semi-field experiments:

Exposure to major environmental chemical contaminants cause adverse health effects in European flounder including:

- *immunodeficiency, making individuals more susceptible to infectious diseases*
- *induction of (pre)neoplastic liver lesions.*

The choice of chemicals to which the test fish were exposed was based on analyses of sediments and fish, combined with data on the effects of the chemicals in mammalian (mainly rodents) and non-mammalian (mainly fish) species and their persistence in the environment. The xenobiotics used in our laboratory experiments were bis (tri-*n*-butyltin)oxide (TBTO), 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD) and 3,3',4,4',5 pentachlorobiphenyl (PCB-126). These toxic compounds are major contaminants in aquatic environments. In an attempt to stay as close to the natural situation as possible within practical limits, aqueous (TBTO) or oral (TCDD and PCB) exposure routes were used. Next to short-term exposures, an 8 months study with TBTO was performed.

Experimental design

Laboratory experiments were incorporated in the integrated study design as an essential element to allow assessment of causal relationships between single chemicals and diseases in fish, since from both field and semi-field experiments only a (possible) correlation could be established between diseases in flounder and environmental pollution (*chapter 1*). The advantages of laboratory experiments are

evident (e.g. controlled environmental conditions, “standardized” test animals, controlled exposure) but several other aspects need to be realized when interpreting the results from these experiments. From a risk assessment perspective, it is important to recognize that free-living animals are exposed to complex mixtures of potentially toxic substances instead of single chemicals. This can lead to augmentation of toxic effects resulting in underestimating the toxic potential of single chemicals as was found for instance in the potentiation of carbon tetrachloride (CCl_4) hepatotoxicity in rats after enzyme induction by pretreatment with the polychlorinated biphenyl mixture Aroclor 1254 (Carlson, 1975). Also a change of effects was shown in channel catfish (*Ictalurus punctatus*) where TBT resulted in modulation of PCB-126 induced CYP1A activity (Rice and Roszell, 1998). The presence of other stressors or risk factors, both anthropogenic (e.g. habitat destruction, net injuries) and natural (e.g. presence of pathogenic organisms, movement, temperature, nutrition), will also influence the effects of contaminants on fish health in their natural habitat, but this was not investigated in our laboratory experiments.

Test species

European flounder has been chosen as the experimental animal based on findings from field research. Because it is known that sensitivity to the effects of chemicals can differ greatly between species, a fish species was used for performing laboratory experiments that showed diseases potentially related to pollution under natural conditions. This choice would facilitate translation of laboratory results into implications for the real world.

Our studies demonstrated that European flounder is a suitable test species for the use in laboratory experiments. Several effects with possible implications for the susceptibility of flounder to infectious diseases and tumor induction were caused by exposure to TBTO (*chapters 2 and 3*), TCDD (*chapter 4*) and PCB-126 (*chapters 5 and 6*). Therefore the laboratory experiments with flounder reported in this thesis produced valuable additional information in the framework of the integrated study design. However, since relatively high levels of xenobiotic substances were used in our laboratory experiments, one can question whether flounder is a very sensitive and suitable species to perform risk assessment in relation to contaminant-associated diseases in the real world. Again, the limitations of the present experiments need to be taken into account.

Immune system

Several of our laboratory experiments produced effects that can have implications for the immune status of the experimental animals.

Short-term exposure to bis (tri-*n*-butyltin)oxide (TBTO), an organotin compound almost exclusively used as a biocide in anti-fouling paints, resulted in a number of effects on the immune system (*chapter 2*):

- significant decrease in percentage of lymphocytes (29%) and total lymphocyte number (58%) in the spleen;
- strong and significant decrease of the non-specific cytotoxic cell (NCC) activity (up to 78%);
- reduction of the thymus size (14% and 21% of the absolute and relative thymus size, respectively).

The thymotoxicity was confirmed in a unique chronic exposure experiment (41% and 23% reduction of the absolute and relative thymus size respectively.; *chapter 3*) in which mature flounder were exposed to TBTO for 8 months. Since TBTO levels in both the short-term and the long-term (chronic) experiments were in the same order of magnitude as high field values, relevance of these results for feral flounder populations in highly polluted habitats, such as in the vicinity of harbors and marinas, is likely.

Also in the experiments with 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD) and the structurally related 3,3',4,4',5 pentachlorobiphenyl (PCB-126) effects with possible repercussions for the effectiveness of the immune system were encountered. Oral exposure of European flounder to TCDD resulted in a trend in thymus size reduction (29% of the absolute and relative thymus size; *chapter 4*), and oral exposure to the dioxin-like PCB-126, the most toxic PCB-congener, resulted in a statistically significant reduction of relative thymus size (~46% of the absolute and relative thymus size; *chapter 5*). In both the TCDD and PCB-126 experiments strong induction of CYP1A was recorded in several organs and cell types, including cells of the hematopoietic system and circulating leukocytes (*chapters 4, 5 and 6*). In literature an association between CYP1A induction and immunotoxicity is suggested, and mechanisms of immunotoxicity of AhR agonists in mice are at least partly mediated by this receptor as is the induction of CYP1A (reviewed by Whyte et al., 2000). Because the TCDD and PCB-126 doses that induced these effects were relatively high and the animals were only dosed once, relevance of our results to natural populations is difficult to interpret.

Unfortunately, our attempts to develop a reproducible and practical infection model with the lymphocystis virus were unsuccessful (*chapter 1*). It would have been an invaluable tool to ascertain the impact of the examined xenobiotics on the efficacy of the immune system and would have facilitated translation of results of our laboratory experiments to natural conditions.

Liver lesions

Field and semi-field experiments not only showed a possible relationship between environmental pollution and infectious diseases. Also compelling evidence was produced, especially in the semi-field experiments, that contaminated sediments were capable of inducing liver tumors and presumed preneoplastic liver lesions (in particular evidence for a cause-and-effect relationship with polycyclic aromatic hydrocarbons (PAHs)). No (liver) tumors or preneoplastic lesions were found in our laboratory experiments. This is probably related to the relative short exposure periods and, in case of the PAH experiments, to the exposure route. However, for two reasons a potential influence of the examined chemicals on the early onset of liver neoplasia can still be expected from the results described in this thesis.

First, several chemicals acquire their toxic characteristics only after metabolic activation by the phase 1 enzyme CYP1A (e.g. bioactivation of BaP in a carcinogenic metabolite), and a positive relationship between CYP1A and ethoxyresorufin-O-deethylase (EROD) levels and carcinogenesis has frequently been reported in literature (reviewed by Whyte et al., 2000).

Second, tumor occurrence might be indirectly influenced by affecting the immune system. Natural killer (NK) cells, belonging to the group of large granular

lymphocytes, play a role in eliminating tumor cells and virus-infected cells. Short-term exposure to TBTO significantly affected the activity of the piscine homologue of the NK cell, the NCC (*chapter 2*). This phenomenon might well facilitate the surviving of tumor cells due to diminished or absent lysis of tumor cells by NCCs in flounder exposed to TBTO.

Also other effects on the liver of flounder were found in our laboratory experiments. Short-term TBTO exposure and exposure tot TCDD ands PCB-126 resulted in an increased hepatosomatic index (*chapters 2, 4, and 5*), and induction of CYP1A in hepatocytes was noted after exposure to TCDD and PCB-126 (*chapters 4, 5 and 6*).

Histopathology

Histopathology proved to be a very useful tool in assessing (toxic) effects of environmental pollutants in our experiments. In addition to routine histopathology, we incorporated and refined the morphometric analysis of histological sections of the thymus in our experiments. Morphometry proved to be invaluable for the detection of a reduction in (relative) thymus size caused by exposure to TBTO and PCB-126 (*chapters 2, 3 and 5*). Immunohistochemistry was an essential addition to routine histopathology for the detection of CYP1A induction in mononuclear cells in the hematopoietic system and in circulating leukocytes as well as in several other cell types (*chapters 4, 5 and 6*). A similar technique enabled us to evaluate the use of immunocytochemistry as a biomarker of AhR agonist exposure in European flounder (*chapter 6*). This would not have been possible if only enzyme induction would have been measured in tissue homogenates, as was the case in the field studies and mesocosm experiment.

Concluding remarks

European flounder proved to be a good choice for the main purpose of our laboratory experiments: filling the gap between correlative relationships (field surveys) and circumstantial evidence (semi-field study) on one side and causal relationships (laboratory studies) on the other side, linking chemical pollution and diseases in fish. Results from experiments with TBTO under controlled laboratory conditions using levels that are comparable to high levels in the field indicate that TBTO is indeed a risk factor that may contribute to an increased occurrence of infectious and non-infectious diseases in wild populations. The relevance of the effects recorded in our experiments with TCDD and PCB-126 are more difficult to interpret due to aspects related to the experimental design (e.g. exposure levels, frequency and time), but a contribution of these substances to chemical-induced diseases in the real world is likely. Experiments using juvenile animals or even eggs, that are generally more susceptible to toxic effects, and long-term exposure in the case of carcinogenesis might offer a different perspective. Establishing a functional infection model is an essential prerequisite for validation of the immune status.

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Nederlandse samenvatting

Inleiding.

De aanleiding voor het onderzoek dat in dit proefschrift is beschreven kwam voort uit problemen bij de bot (*Platichthys flesus*), in Nederlandse kustwateren. Bij deze platvissoort kwamen relatief veel ziektes voor waaronder huidzweren, wratachtige woekeringen die door het lymphocystis virus worden veroorzaakt en knobbels in de lever. Deze leverknobbels bleken uit ander onderzoek enerzijds door ontstekingen te worden veroorzaakt en anderzijds door goedaardige en kwaadaardige tumoren of verdachte voorstadia van tumoren. Om het vermoeden van de betrokkenheid van milieuvervuiling bij het ontstaan van deze ziektes na te gaan werd een geïntegreerd onderzoeksprogramma opgezet. Hierbij waren het Rijksinstituut voor Kust en Zee (RIKZ), het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) en de vakgroep Pathobiologie van de faculteit Diergeneeskunde betrokken.

Eerder onderzoek

Uit eerder verricht veldonderzoek bleek het lastig om oorzakelijke verbanden te leggen tussen milieuverontreiniging en de hierboven beschreven ziektes. Het sterke punt van veldonderzoeken ligt in de grote relevantie van de resultaten van dit type onderzoek voor in het wild levende (vis)populaties. Echter, vanwege het grote aantal oncontroleerbare factoren die de uitkomst van veldonderzoek kunnen beïnvloeden blijkt het vaak erg lastig om oorzakelijke verbanden te leggen. Daarom werd besloten om de natuurlijke veldsituatie na te bootsen in een semi-veld of mesocosmos experiment. Hierbij werden vissen in een groot bassin van 40 bij 40 bij 3 meter blootgesteld aan vervuild havenslib. Uit dit semi-veldonderzoek kwamen sterke aanwijzingen naar voren voor een relatie tussen levertumoren en gehaltes van kankerverwekkende stoffen (zoals benzo-a-pyreen) en een verband tussen blootstelling aan verontreinigd slib en lymphocystis infecties (**hoofdstuk 1**). Mede omdat in het vervuilde slib een groot aantal verschillende milieuverontreinigende stoffen voorkomt, bleek het ook met semi-veldonderzoek lastig om oorzakelijke verbanden te leggen. Daarom werd besloten om laboratoriumonderzoek op te nemen in het geïntegreerde onderzoeksproject.

In dit proefschrift wordt het laboratoriumonderzoek beschreven dat, door de hoge mate van controle over de omstandigheden tijdens het onderzoek, bij uitstek geschikt is om oorzakelijke verbanden te leggen. Voor de interpretatie van de resultaten van het laboratoriumonderzoek zijn echter zowel het semi-veld- als het veldonderzoek onontbeerlijk.

Op basis van de resultaten van veld- en semi-veldonderzoek werd de volgende **stelling** voor het laboratoriumonderzoek opgesteld:

Blootstelling aan belangrijke milieuverontreinigende stoffen hebben negatieve effecten op de gezondheid van de bot waaronder:

- *onderdrukking van het immuunsysteem waardoor de dieren gevoeliger worden voor infecties*
- *inductie van tumoren en voorstadia van tumoren.*

Alvorens met het daadwerkelijke onderzoek kon worden begonnen werd de anatomie en histologie van de bot in kaart gebracht. Daarbij werd vooral aandacht besteed aan het immuunsysteem. Zo werden laboratorium testen die voor zoogdieren zijn ontwikkeld om de nadelige effecten op het immuunsysteem in kaart te brengen aangepast voor gebruik bij de bot. Ook is er geprobeerd om met het lymphocystis virus een infectiemodel op te zetten met als doel om de effectiviteit van het immuunsysteem te onderzoeken. Helaas is het niet gelukt een werkbaar infectiemodel te ontwikkelen (**hoofdstuk 1**).

Experimenten

De eerste experimenten zijn op het RIVM uitgevoerd met botten die als jonge dieren waren gevangen in schone Engelse kustwateren. Een tweede serie experimenten vond plaats in het veldstation van het RIKZ in Kamperland waarbij gewerkt werd met in gevangenschap gekweekte botten. De keuze van chemische stoffen voor de experimenten was gebaseerd op het voorkomen van deze stoffen in het milieu en de effecten ervan bij andere diersoorten.

In **hoofdstuk 2 en 3** worden experimenten met bis(tri-*n*-butyltin)oxide (**TBTO**) beschreven. TBTO is een door de mens gemaakte verbinding die vooral wordt toegepast in verf waarmee de rompen van schepen worden bewerkt om het aangroeien van algen en schelpdieren te voorkomen. Van deze stof is bekend dat het onder andere effecten heeft op het immuunsysteem. Voor de risico-inschatting van TBTO voor de mens wordt met name het effect op de thymus (zwezerik; van groot belang bij de ontwikkeling van het immuunsysteem) gebruikt.

In hoofdstuk 2 wordt een drietal kortdurende experimenten (tot maximaal 28 dagen) beschreven waarbij botten via zoetwater werden blootgesteld aan TBTO. Na 7 tot 12 dagen trad er sterfte op, werden er microscopisch veranderingen aan de kieuwen waargenomen en werd een relatieve verkleining van de thymus vastgesteld. Ook bleek de activiteit te zijn onderdrukt van een bepaald type witte bloedcel die tumorcellen en cellen die geïnfecteerd zijn met virussen onschadelijk maakt (de niet-specifieke cytotoxische cel). Omdat vissen in de natuur meestal langdurig blootstaan aan verontreinigingen is ook een chronisch experiment uitgevoerd. Hierbij werden botten gehouden in zeewater en gedurende 8 maanden blootgesteld aan TBTO. Het meest opvallende effect was, net als in het acute experiment, een verkleining van de thymus.

In **hoofdstuk 4,5 en 6** worden experimenten beschreven met **TCDD** (dioxine) en de op dioxine lijkende **PCB-126**.

Dioxines behoren tot een grote groep stoffen die gevormd worden als ongewenste bijproducten van diverse industriële processen waarbij chloor betrokken is (zoals het bleken van papier en verbranding van chloorhoudend afval). Dioxines zijn slecht afbreekbaar en stapelen zich op in vet(weefsel). De gevoeligheid voor TCDD kan sterk variëren. Zo is de dodelijke dosis TCDD voor een cavia meer dan 500 keer zo klein als voor een hamster. Dioxines hebben bij verschillende diersoorten effecten op de lever en het immuunsysteem. Het merendeel van deze effecten vindt plaats via de zgn Ah-receptor. Eén van de enzymen die door dioxines ook via de Ah-receptor, worden geactiveerd is cytochroom P4501A (CYP1A). Dit enzym wordt in veldonderzoek met vissen veel gebruikt om blootstelling aan dioxineachtige stoffen vast te stellen en speelt onder andere een rol bij het omzetten van benzo-a-pyreen in een kankerverwekkende stof. De hoeveelheid van dit enzym is in onze experimenten

zichtbaar gemaakt in microscopische preparaten door gekleurde antilichamen en is mede gebruikt om de effecten van TCDD en PCB-126 in kaart te brengen.

PCB's zijn chemische verbindingen die worden gebruikt in "gesloten systemen" zoals transformators en hydraulische apparaten en in het verleden ook in "open systemen" zoals verven, papier en pesticiden. Net als bij dioxines vinden de meeste effecten van PCB-126 ook via de Ah-receptor plaats en zijn effecten op het immuunsysteem beschreven.

In de door ons uitgevoerde experimenten werd éénmalig TCDD of PBC-126 ingegeven in een gelatine capsule. Het bleek dat zowel TCDD als PCB-126 een relatieve vergroting van de lever veroorzaken en sterke inductie van het enzym CYP1A. Daarnaast werd door PCB-126 een verkleining van de thymus veroorzaakt, een effect dat na blootstelling aan TCDD minder duidelijk was. Ook bleken de botten aan het eind van het onderzoek met PCB-126 minder te eten, waren de dieren minder actief en vertoonden enkele dieren kleine onderhuidse bloedinkjes. Een opmerkelijke vaststelling was de inductie van CYP1A in bepaalde cellen (waarschijnlijk witte bloedcellen) in weefsel dat vergelijkbaar is met het beenmerg van zoogdieren. Deze enzyminductie was niet eerder in onderzoek met vissen waargenomen. Dit enzym bleek ook in cellen in het bloed van de botten aantoonbaar te zijn. Tot nu toe wordt CYP1A in onderzoeken met vissen meestal bepaald in de lever waarvoor de dieren moeten worden opgeofferd. In het experiment dat beschreven is in **hoofdstuk 6** is bekeken of het mogelijk is om met behulp van bloedonderzoek vast te stellen of vissen in aanraking zijn gekomen met dioxine-achtige stoffen door het aantonen van CYP1A inductie. Als deze methode werkt zou het mogelijk kunnen zijn om een beeld te krijgen van de blootstelling aan dioxineachtige stoffen zonder daarvoor de vissen te moeten doden. Uit het onderzoek bleek dat enzyminductie in bloedcellen vastgesteld kon worden. Maar omdat ook bij dieren die niet waren blootgesteld aan PCB-126 het enzym in bloedcellen werd aangetroffen is het niet duidelijk of deze methode echt kan worden toegepast. Uit wetenschappelijke literatuur blijkt ook bij mensen een grote variatie in het CYP1A gehalte in witte bloedcellen voor te komen. Hierdoor wordt aan het gebruik van CYP1A inductie in witte bloedcellen als graadmeter voor blootstelling aan dioxineachtige stoffen bij mensen getwijfeld.

Conclusies

De bot blijkt goed bruikbaar in laboratoriumonderzoek naar de relatie tussen milieuverontreiniging en ziektes bij vissen.

Uit experimenten met TBTO werden voornamelijk effecten op het immuunsysteem vastgesteld bij concentraties in dezelfde orde van grootte als concentraties die kunnen worden aangetroffen in relatief vervuilde gebieden in de natuur. Hieruit werd geconcludeerd dat het aannemelijk is dat TBTO een risicofactor is die kan bijdragen aan het vaker voorkomen van infecties bij de bot.

De betekenis van de uitkomsten van de experimenten met TCDD en PCB-126 is moeilijker te interpreteren. Dit hangt samen met de opzet van de experimenten waarbij, in vergelijking met de natuurlijke situatie, relatief grote hoeveelheden TCDD en PCB-126 werden toegediend, er slechts éénmalig werd gedoseerd en de blootstellingsduur relatief kort was. Toch lijkt het aannemelijk dat ook dit type stoffen een bijdrage levert aan het voorkomen van ziektes in de natuur.

Experimenten met zeer jonge vissen of eieren (die over het algemeen gevoeliger zijn voor de effecten van schadelijke chemische stoffen) en meer chronische experimenten (om ontwikkeling van tumoren te onderzoeken) geven mogelijk een ander inzicht. Daarnaast is de ontwikkeling van een bruikbaar infectiemodel noodzakelijk om de betekenis van de effecten op het immuunsysteem goed te kunnen interpreteren.

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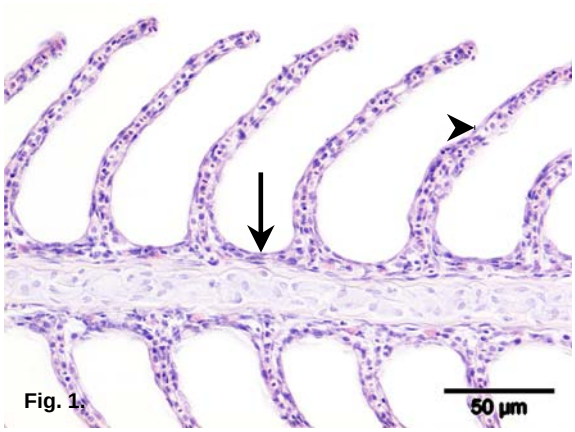
Curriculum vitae

Guillaume Cornelis Maria (Guy) Grinwis was born in Utrecht, the Netherlands, on May 26th in 1962. After graduation from college de Klop in Utrecht in 1980, obtaining a VWO diploma, he started studying veterinary medicine at the Utrecht University. He graduated in November 1987 as a veterinarian, and entered civil service in March 1988, which he left as an officer in June 1989. He subsequently worked as a private veterinary practitioner in several companion animal clinics throughout the Netherlands until September 1990. In October 1990 he joined the Pathology department of the Veterinary faculty at the Utrecht University where he specialized in veterinary pathology, leading to certification by the Royal Netherlands Veterinary Association in September 1995. His PhD research into health effects of some major aquatic pollutants in European flounder was performed in cooperation with Dutch National Institute for Coastal and Marine Management (RIKZ) and the Dutch National Institute for Public Health and the Environment (RIVM). He is currently employed as a staff veterinary pathologist at the department of Pathobiology, at the Veterinary Faculty of the Utrecht University and is involved in teaching veterinary students, post-graduate training of residents in veterinary pathology and research. He also participates in the veterinary pathology diagnostic service of the department with special interest in eye pathology.

Color illustrations

Chapter 2. *Short-term toxicity of TBTO in flounder*

Chapter 3. *Chronic aqueous exposure of European flounder to bis(tri-*n*-butyltin)oxide*



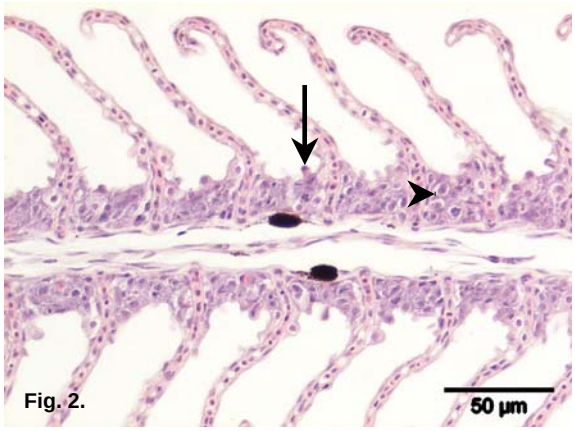
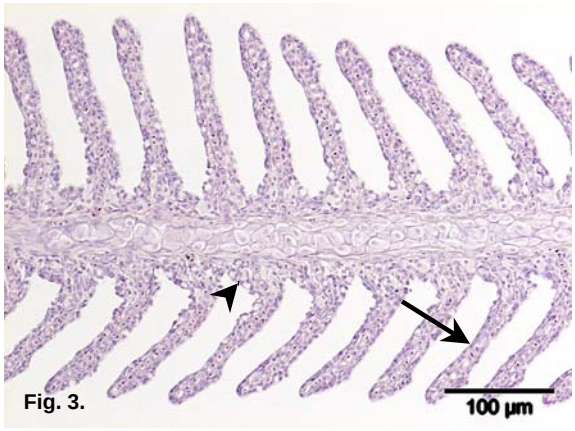


Fig. 1. Gill of a control animal with normal primary (arrow) and secondary (arrowhead) lamellae. *H&E*.
Fig. 2. Gill of an animal exposed to 32 µg TBTO/l for 6 days showing budding (arrow) of epithelial cells; some minor epithelial hyperplasia is also present (arrowhead). *H&E*.



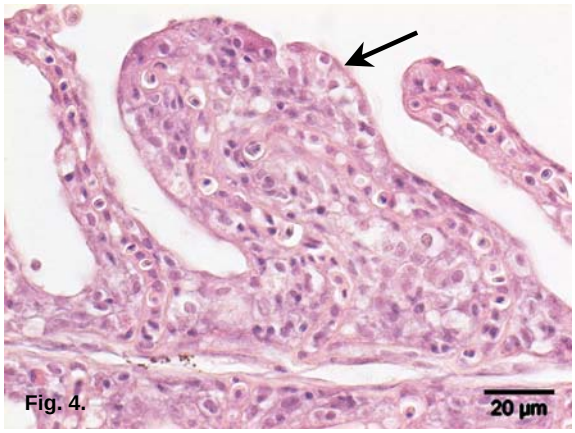


Fig. 3. Gill of an animal exposed to 32 µg TBTO/l for 6 days with mild (arrow), and marked (arrowhead) respiratory epithelial hyperplasia. Also budding of epithelial cells is visible. *H&E*.
Fig. 4. Gill of an animal exposed to 32 µg TBTO/l for 6 days with respiratory epithelial proliferation and fusion of secondary lamellae (synechia; arrow). *H&E*.

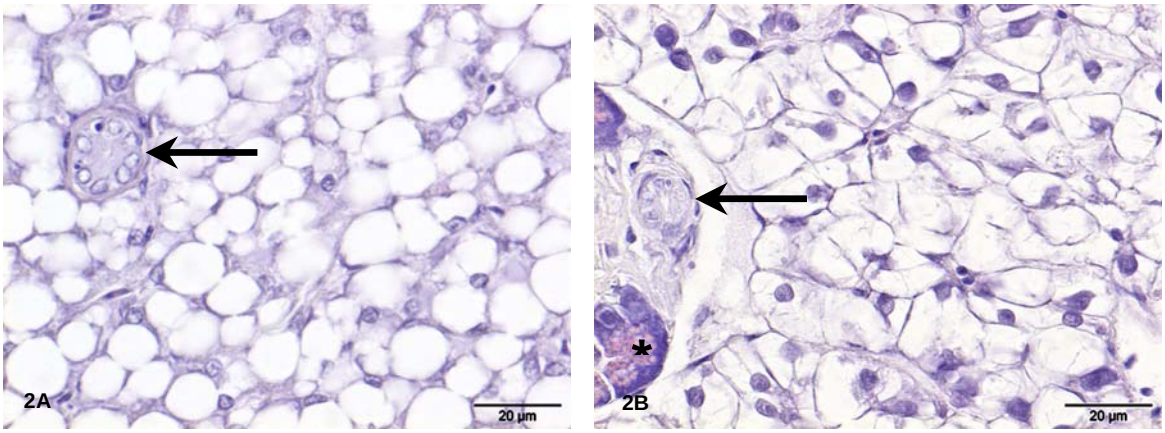


Fig. 2. Hepatopancreas of animals exposed to TBTO for 8 months. Hepatocytes in Fig. 2A contain large clearly demarcated empty vacuoles and peripherally located nuclei characteristic of fat accumulation (steatosis). In contrast, the cytoplasm of the hepatocytes in Fig. 2B show distinctive features of glycogen storage with pale cytoplasm containing reticular structures and centrally located nuclei. Flounder showed a dose related shift from hepatic glycogen storage to fat accumulation. Arrows indicate bile ducts, the asterisk shows a part of the exocrine pancreas. *H&E*.

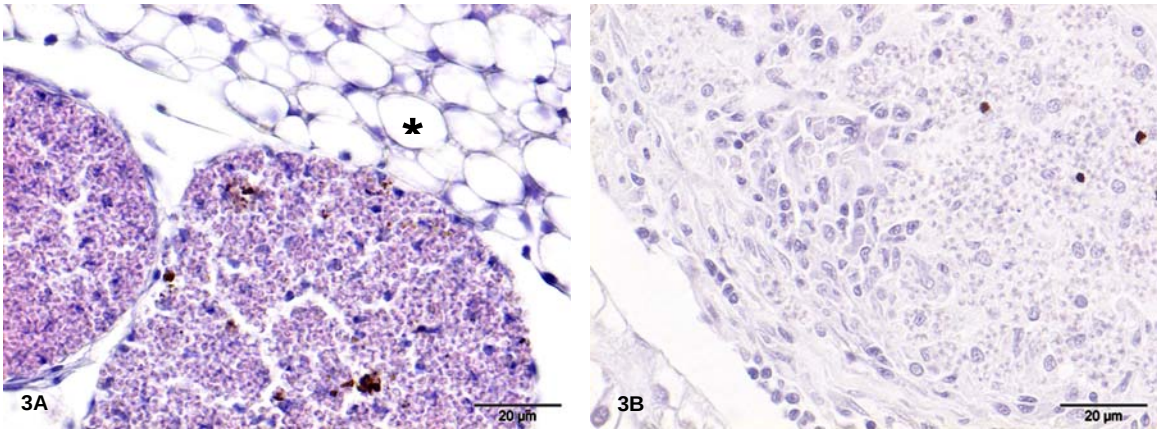


Fig. 3. Accumulations of microsporidian protozoa (xenomas), probably *Glugea stephani*, in the liver of flounder exposed to TBTO for 8 months. Most protozoa appear to be situated inside phagocytes. Note the absence of a marked tissue reaction in Fig. 3A whereas in Fig. 3B clear fibrosis and infiltration of mononuclear inflammatory cells are visible. Asterisk indicates fatty aspect of hepatocytes. *H&E*.

Color illustrations

Chapter 4. *Toxicity of TCDD in European flounder (Platichthys flesus) with emphasis on histopathology and cytochrome P450 1A induction in several organ systems*

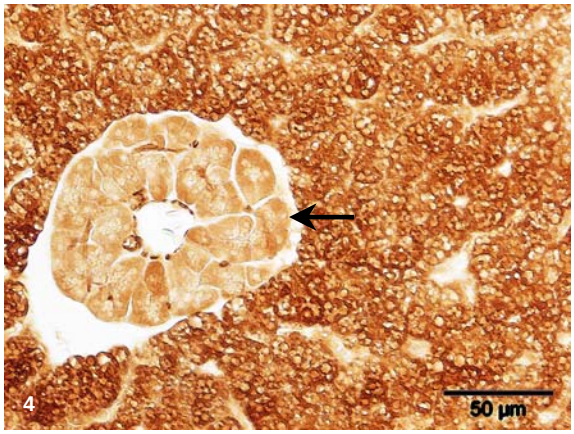


Fig. 4. Immunohistochemical localization of cytochrome P4501A in the liver of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks. Strong immunoreactivity is visible in hepatocytes and in the exocrine pancreas tissue (arrow).

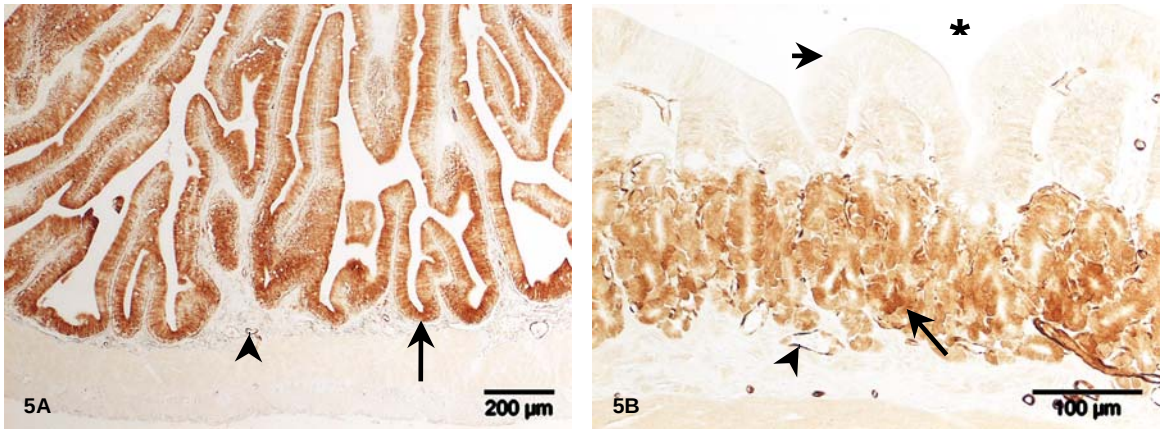


Fig. 5. Immunohistochemical localization of cytochrome P4501A in the digestive tract of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks. Strong immunoreactivity is visible in epithelial cells of the gut (arrow) and endothelial cells in all layers of the gut (arrowhead) (A). In the stomach, immunoreactivity is most prominent in the glandular epithelium (arrow) and the endothelium of small and larger vessels (arrowhead). The asterisk indicates the lumen, and the small arrow the surface epithelium of the stomach (B).

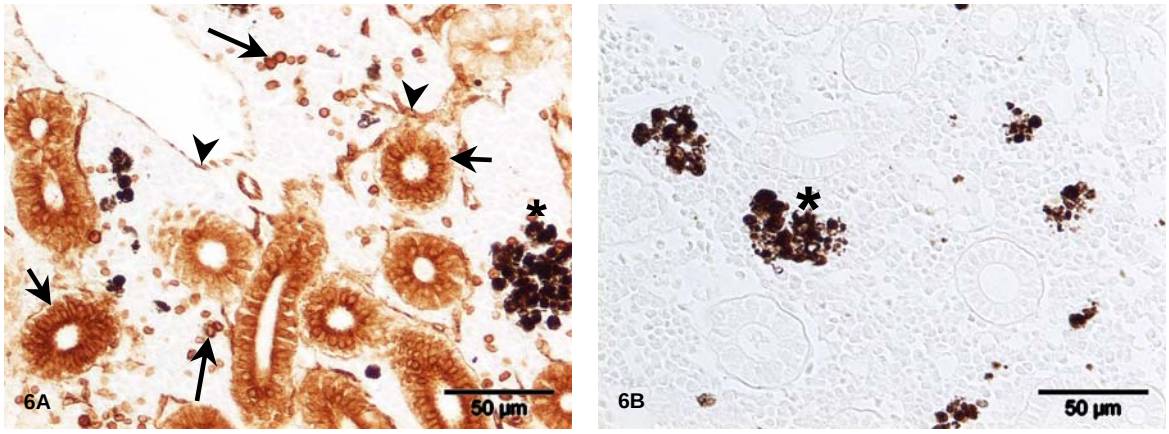


Fig. 6. Immunohistochemical localization of cytochrome P4501A in the mesonephros of European flounder exposed to 500 µg TCDD/kg bw for 4 weeks (A). Strong immunoreactivity is visible in epithelial cells (small arrow) and endothelial cells (arrowheads). Remarkable is the presence of numerous immunreactive cells of the hematopoietic tissue (large arrows). Melanomacrophage centres are also clearly visible (asterisks). Note the absence of immunoreactivity in the mesonphros, containing several melanomacrophage centers (asterisks), of a control animal (B).

Color illustrations

Chapter 5. *Toxicity of PCB-126 in European Flounder (Platichthys flesus) with emphasis on histopathology and cytochrome P4501A induction in several organs systems*

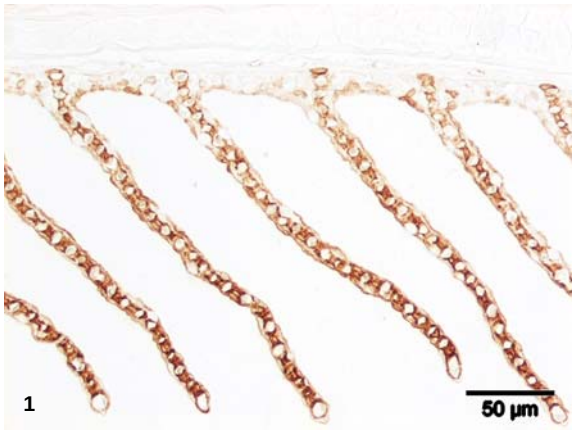


Fig. 1 Immunohistochemical localization of cytochrome P4501A in the gills of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in pillar cells.

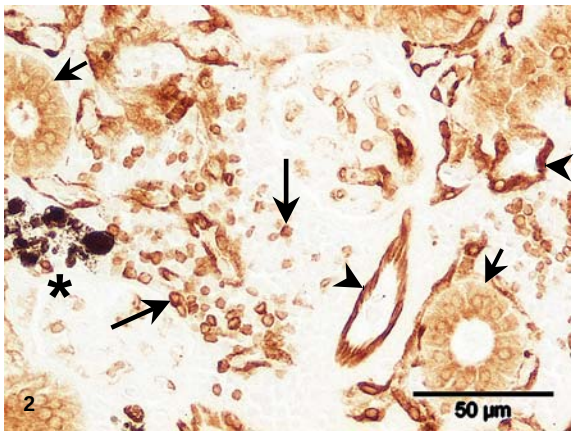


Fig. 2 Immunohistochemical localization of cytochrome P4501A in the mesonephros of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in epithelial cells (small arrows) and endothelial cells (arrowheads). Remarkable is the presence of numerous immunoreactive cells of the hematopoietic tissue (large arrows). Melanomacrophage centres are also clearly visible (asterisk).

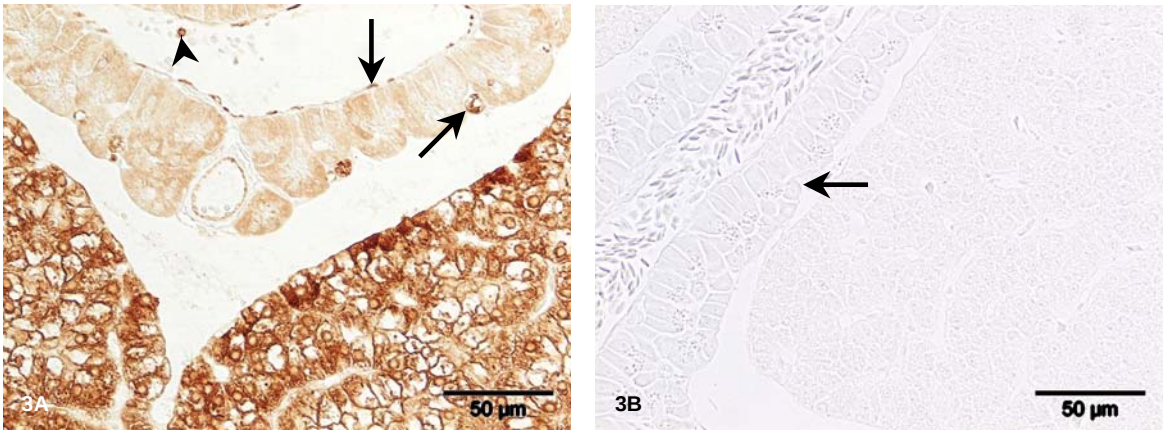


Fig. 3 A Immunohistochemical localization of cytochrome P4501A in the liver of European flounder exposed to 50 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in hepatocytes, and in the endothelium of arterioles and portal vein branches (arrows). Note the presence of an immuno-reactive mononuclear cell in sinusoids of the liver (arrowhead). B Immunoreactivity in hepatocytes is weak, and absent in exocrine pancreatic tissue (arrow) of a control animal.

Color illustrations

Chapter 6. *Cytochrome P4501A induction in peripheral blood leukocytes in European flounder (Platichthys flesus): an intravital biomarker of exposure?*

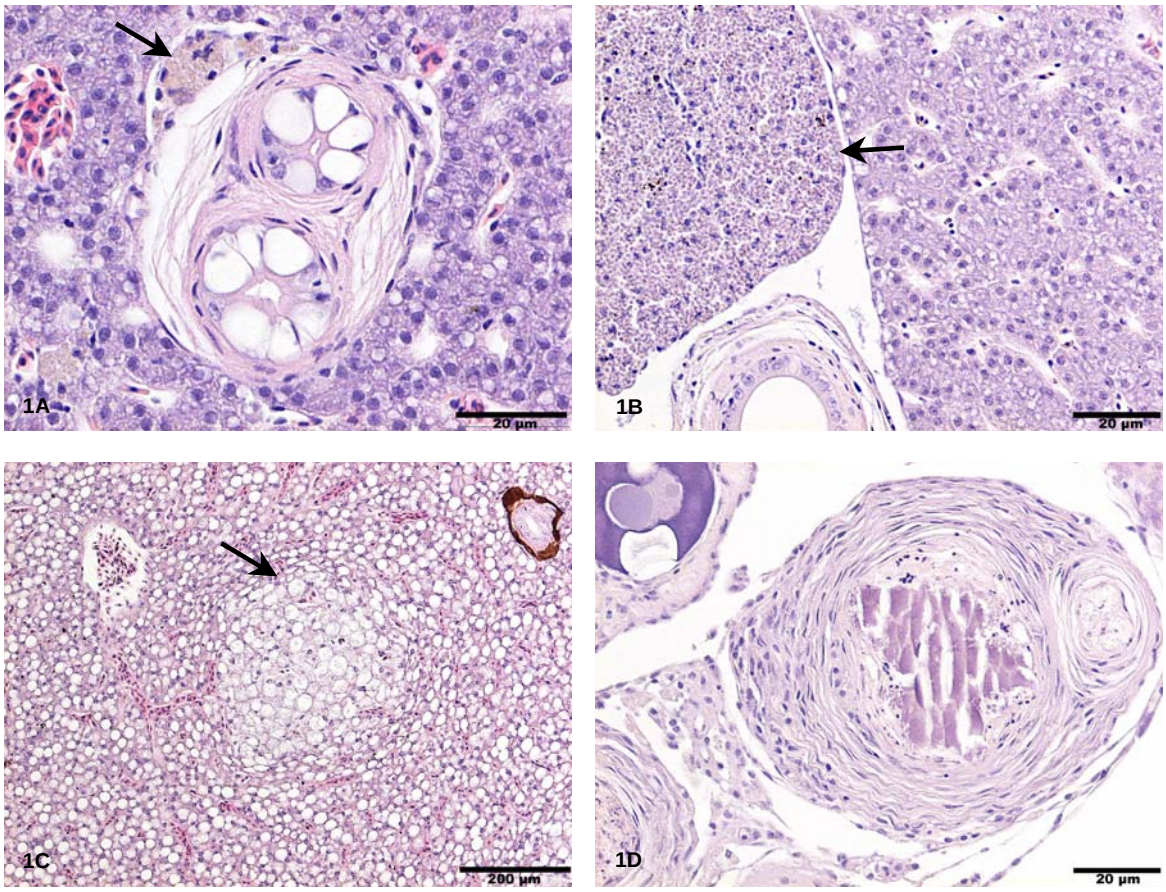


Fig. 1.
Marked vacuolation of epithelial cells of a larger bile duct with some periductular fibrosis and a small accumulation of pigment-containing macrophages (arrow) is visible in Fig. 1A. In Fig. 1B a large accumulation of microsporidian protozoa, probably *Glugea stephani* in the hepatopancreas of one of the fish is visible (arrow). A significant inflammatory response is absent. Fig. 1C shows a fatty focus in the liver. A group of swollen, vacuolated hepatocytes compresses surrounding hepatocytes (arrow). Fig. 1D shows an ovary containing a granulomatous focus with central calcification and concentric fibrosis. None of these changes were dose related. *H&E*.

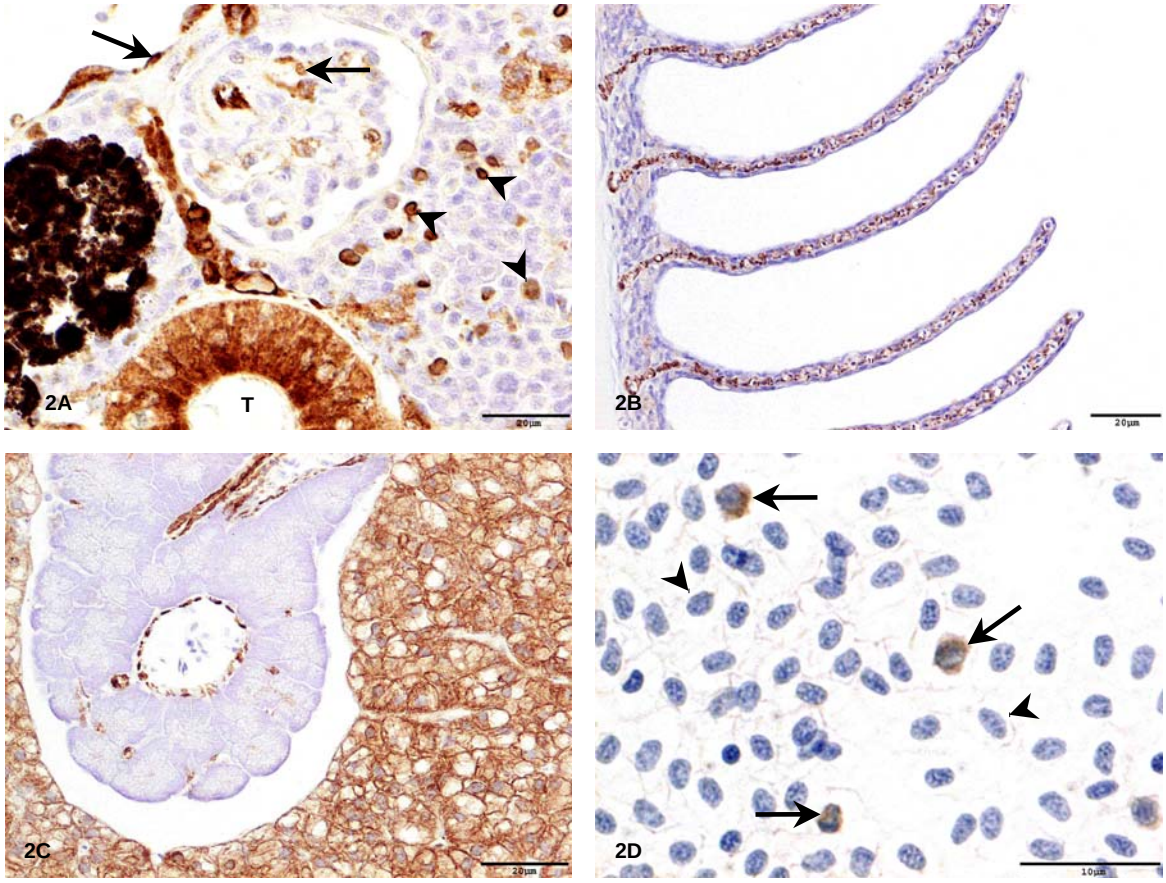


Fig. 2.
Immunohistochemical detection of cytochrome P4501A expression in the mesonephros (2A), pillar cells of the gills (2B) and hepatopancreas (2C) of European flounder exposed to 5 mg PCB-126/kg for 16 days. Strong immunoreactivity is visible in the endothelium of glomeruli and veins (arrows), epithelium of renal tubules (T) and several cells of the hematopoietic tissue (arrowheads). In the left side of Fig. 2A a melanomacrophage center is visible. Fig. 2D depicts a smear of peripheral blood of an animal exposed to 5 mg PCB-126/kg for 16 days, containing several immunoreactive mononuclear leukocytes (arrows). Note the negative staining cytoplasm of the erythrocytes (arrowheads).