Chapter 12
Immunotoxic Effects of Organotin Compounds in Teleost Fish

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12.1 Evidence of Toxic Effects of Environmental Contaminants on the Immune System of Fish

A number of field studies have provided evidence that environmental toxicants can modulate immune parameters of exposed fish (for overviews see Snieszko 1974; Dunier and Siwicki 1993; Zeeman 1994; Zelikoff 1994; Rice 2001). Also, laboratory studies demonstrated that toxicants impact the immune system of fish (e.g., Weeks et al. 1988; Thuvander and Carlstein 1991; Kaattari et al. 1994; Rice and Schlenk 1995; Sanchez-Dardon et al. 1999; Carlson et al. 2004; Quabius et al. 2005). Consequently, it has been suggested to utilize immune parameters of fish as indicators of environmental pollution (Anderson 1990; Weeks et al. 1992; Wester et al. 1994).

In fish immunotoxicological studies, emphasis has been given to the measurement of single endpoints or functions such as depressed phagocytic activity of macrophages or the alteration of oxidative burst activity of immune cells. However, the ultimate concern is that toxic exposure might increase the susceptibility of fish to pathogen infections. Thus, it is important to elucidate the consequences of changes in molecular or cellular immune parameters for the overall immune system function, since, as pointed out by Rice (2001), alterations at the molecular and cellular level do not necessarily translate into immune modulation at the system level. Rather few studies directly correlated alterations of specific molecular and cellular immune parameters with altered immune system function and/or altered susceptibility to pathogens (Palm et al. 2003; Carlson et al. 2002; Burki et al. 2008). However, some more indirect evidence is available that toxic impact on immune

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parameters increases the susceptibility of fish to disease. One example comes from the decline of wild Pacific salmon populations in the USA and Canada. When juvenile chinook salmon (*Oncorhynchus tshawytscha*) collected from estuaries in the Puget Sound area and showing suppression of various immune parameters were infected with *Vibrio anguillarum* in the laboratory, they were more susceptible than fish from non- or less-polluted areas (Arkoosh et al. 1998). Significant differences were seen even 2 months after removal from the contaminated areas, suggesting that the chemical exposure had a lasting effect on disease susceptibility. This assumption is supported by the study of Milston et al. (2003) who showed that short-term contaminant exposure of chinook salmon during early life-history stages resulted in long-term impairment of humoral immune competence. Under complex field conditions, as in Puget Sound, it is difficult if not impossible to provide conclusive evidence that the contaminant-induced immunosuppression is causative to the observed decline of chinook salmon populations. However, in a demographic modeling study, Spromberg and Meador (2005) could show that immune suppression acting through reduction of age-specific survival would produce pronounced changes in the population growth rate. This result highlights the potential of immunotoxicants to adversely affect organism health and population growth of aquatic wildlife.

One factor that complicates the functional interpretation of toxicant-induced changes in single immunological endpoints of fish is the still rather limited knowledge of the immune systems of fish. Although often addressed as "primitive" immune system compared to mammals, the immune systems of fish are highly evolved. Importantly, since water is an excellent transmission medium for pathogens, much better than air, the efficiency of piscine immune systems has to be efficient enough to meet the demands of this challenging environment.

In the following section, we will introduce a few basic components and features of the immune system of teleost fish. For a comprehensive overview on the fish immune system, the reader is referred to the reviews of Iwama and Nakanishi (1996), Rice (2001) and Burnett (2005).

12.2 The Immune System of Teleost Fish

12.2.1 Immune Organs and Cells

While several features of the teleostean immune system are similar to those in the immune systems of higher vertebrates, there exist also a number of unique characters and processes such as the prominence of antipathogenic factors in the skin mucus (Suzuki et al. 2003) or the strong dependence on the innate immunity (Plouffe et al. 2005). In contrast to mammals, teleost fish lack bone marrow and lymph nodes. A clear separation of lymphoid and myeloid tissues is not realized but the "lymphopoietic" organs of teleost contain both lymphoid and myeloid cells in different stages of development. These organs include head kidney, excretory kidney,

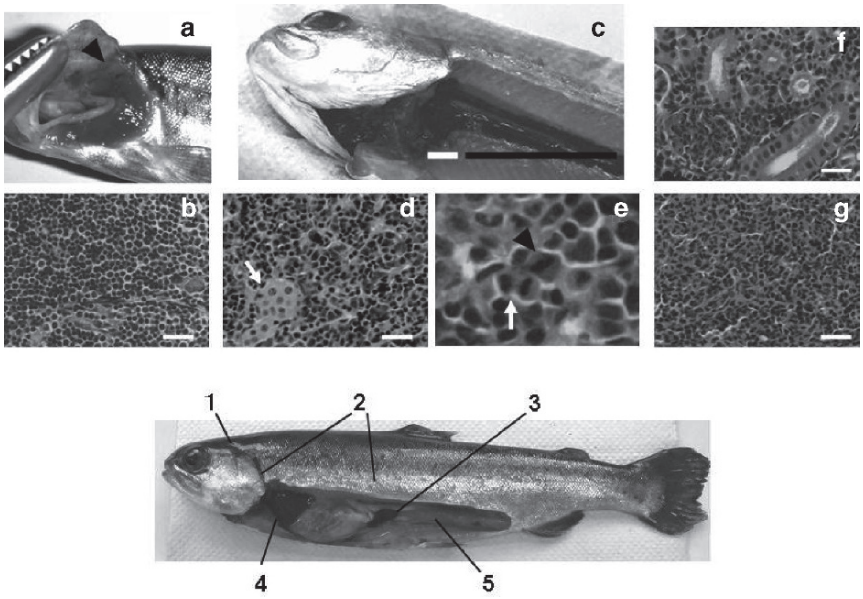

Fig. 12.1 Immune-related organs in rainbow trout (*Oncorhynchus mykiss*). Location of thymus (1 and a; *arrowhead*) and thymus histological appearance (b) occupied with lymphoblasts (thymocytes). Location of kidney (2 and c; the *white* and *black lines* indicate head – *anterior* – kidney and trunk – excretory – kidney. The head kidney (d) histologically consists of hematopoietic tissue with mitotically active (e; *arrowhead*) precursors of erythrocytes and leukocytes (e; *arrow*), lymphoid parts, as well as endocrine (interrenal and chromaffin) cells (d; *arrow*). The trunk kidney consists of hematopoietic tissue, and excretory tissue with renal tubules and glomeruli (f). Spleen (3) serves to remove blood cells from the circulation, as antigen-trapping and presenting organ, as well as hematopoietic organ. Histologically, erythrocyte-rich and lymphocyte-rich fractions are clearly visible (g). (4) Liver, containing resident phagocytes. Gut-associated lymphoid tissue (GALT) is present along the intestine (5). Hematoxylin-Eosin staining, scale bar; 50 µm

thymus and spleen (Fig. 12.1). Further, with pathogens entering the fish usually from the water, intestinal mucosa as well a skin and branchial epithelia function as major immune barriers. Teleosts possess a reticuloendothelial systems (RES) encomprising phagocytes in various locations such as wandering serosal phagocytes in the body cavity, endothelium fixed phagocytes in the liver (although the existence of Kupffer cells homologues is discussed controversially – see Hinton et al. 2001), or microglia in the neuronal system.

Teleost immune cells comprise – according to the present state of knowledge – three types of granulocytes including neutrophils, basophils and eosinophils (the latter are considered to be functionally analogous to the mammalian mast cells), monocytes/macrophages, T- and B-lymphocytes, as well as thrombocytes. In addition, natural cytotoxic cells (NCC) – which are considered to be analogous to natural killer cells in mammals – and cytotoxic T cells requiring MHC class I molecules have been characterized (Fischer et al. 2006). The immune cell composition of lymphoid

organs and blood appears to be highly variable both between individual fishes and between fish species.

12.2.2 The Innate, Non-specific Immune System

The innate immune response provides fairly rapid defense mechanisms against pathogens, which are immediately activated after recognition of pathogen associated molecular patterns (PAMP) that are common to many pathogens, for instance, bacterial lipopolysaccharide (LPS). The key role in innate immune system activation is played by pattern recognition receptors (PRR) of the host which recognize either the foreign molecules or endogenous, host-derived alarm molecules (Magnadóttir 2006). Main components of the innate immune system in fish include physical barriers such as skin or endothelia, humoral factors (anti-bacterial peptides, lysozyme, acute phase proteins or complement factors) and phagocytic cells such as granulocytes, monocytes/macrophages and NCC. Humoral factors such as lysozyme are found in plasma, skin mucus as well as in eggs, and are a frequently measured parameter in immunotoxicity studies with fish. Complement factors are synthesized in the liver and probably also in extrahepatic sites (Løvoll et al. 2007; Boshra et al. 2004), and they have a number of functions including opsonization of the pathogen or activation of the acquired immune system. The main functions of the phagocytic cells are to phagocytose tissue debris and microorganisms, to secrete immune response regulating factors and to bridge innate and adaptive immune responses (Secombes and Fletcher 1992). A key feature of phagocytic cells such as macrophages and granulocytes is the respiratory burst activity, i.e. the generation of reactive oxygen intermediates in order to kill pathogenic microorganisms (Sharp and Secombes 1993). Measuring the respiratory burst activity is often used as an indicator of the immunological capacity of the fish, and has been shown to be modulated by a wide variety of environmental toxicants (Rice 2001).

12.2.3 The Acquired, Specific Immune System

The importance of the acquired immune system in bony fish is believed to be secondary to the innate immune system. Cells involved in the specific immune system are T- and B-lymphocytes, which mediate the cellular and humoral response, respectively. Although the characterization of piscine T-lymphocytes is not as far progressed as in mammals, it is clear that fish possess both antigen-presenting T-helper cells (CD4-like) and cytotoxic T-cells (CD8-like) (Fischer et al. 2006). Fish B-lymphocytes produce immunoglobulins which are primarily tetrameric IgMs (Warr 1983), instead of the pentameric immunoglobulins of mammals. The kinetics of antibody production is much slower (weeks to months) in teleosts than

in mammals, and the antibody response shows a clear temperature dependency. Toxicant effects on the acquired immune system are often measured as alteration of mitogen-stimulated lymphoproliferation or a change of antibody production.

12.3 Evaluation for Immunotoxic Effects of Tributyltin (TBT) Using the Rainbow Trout Model

Reported immunotoxicies of TBT in fish include thymus atrophy (Wester and Canton 1987; Grinwis et al. 1998), reduction of leukocyte numbers (Grinwis et al. 1998) and impairment of leukocyte functions, for instance, decrease of phagocytic activities (Wishkovsky et al. 1989; Rice and Weeks 1991; Rice et al. 1995; Harford et al. 2005; Nakayama et al. 2005, 2007) and reduced lymphocyte functions (O'Halloran et al. 1998; Regala et al. 2001; Harford et al. 2007). In vitro studies with isolated fish leukocytes indicated that low doses of TBT and short-term exposures to TBT stimulate the production of reactive oxygen species, whereas higher doses or longer exposure period suppress reactive oxygen formation (Rice and Weeks 1991). Possibly, these effects are mediated via an effect of TBT on cellular Ca^{2+} levels (Elferink et al. 1986; Raffray et al. 1993; Rice et al. 1995). Further in vitro immune cell effects reported for TBT include the suppression of mitogen-stimulated lymphocyte proliferation (O'Halloran et al. 1998). A limitation in our current knowledge on TBT-induced immunological alterations in fish is that published studies related observed effects to external TBT concentrations, but not to internal body burdens. Knowledge of the relationship between accumulated TBT doses in the organism and immunotoxic responses would help to compare results between studies and to extrapolate from laboratory-derived effect thresholds to the field situation. In order to better understand the relationship between immunotoxic effects of TBT and accumulated TBT concentrations, we experimentally set out for two types of exposure experiments using the rainbow trout model: an immersion exposure with TBT, and intraperitoneal (ip) injection of TBT. In both approaches, the actual amounts of TBT in target immune tissues, especially in blood were analytically determined in order to link the internal doses to the effects.

12.3.1 Exposure of Rainbow Trout Tributyltin via Water Alters the Number of Neutrophils and Their Respiratory Burst Activity

Our first aim was to determine toxic effects of TBT on phagocyte numbers in rainbow trout. After immersion exposure of trout to 20 µg tributyltin chloride (TBTCl)/l for 5 days, we measured the numbers of head kidney neutrophils and their respiratory burst activities using flow cytometry. The advantage of this method is the

discrimination of head kidney leukocyte populations (lymphocytes + thrombocytes, monocytes/macrophages and granulocytes) by size (forward scatter: FSC, X-axis) and granularity (side scatter: SSC, Y-axis). The typical FSC/SSC cytograms of head kidney leukocytes are shown in Fig. 12.2a for the control and Fig. 12.2b for the TBT exposure group. In these cytograms, an increased percentage of neutrophils (marked with dotted line) prepared from the TBT exposure group compared to non-treated group is clearly shown. Moreover, the respiratory burst activity, measured

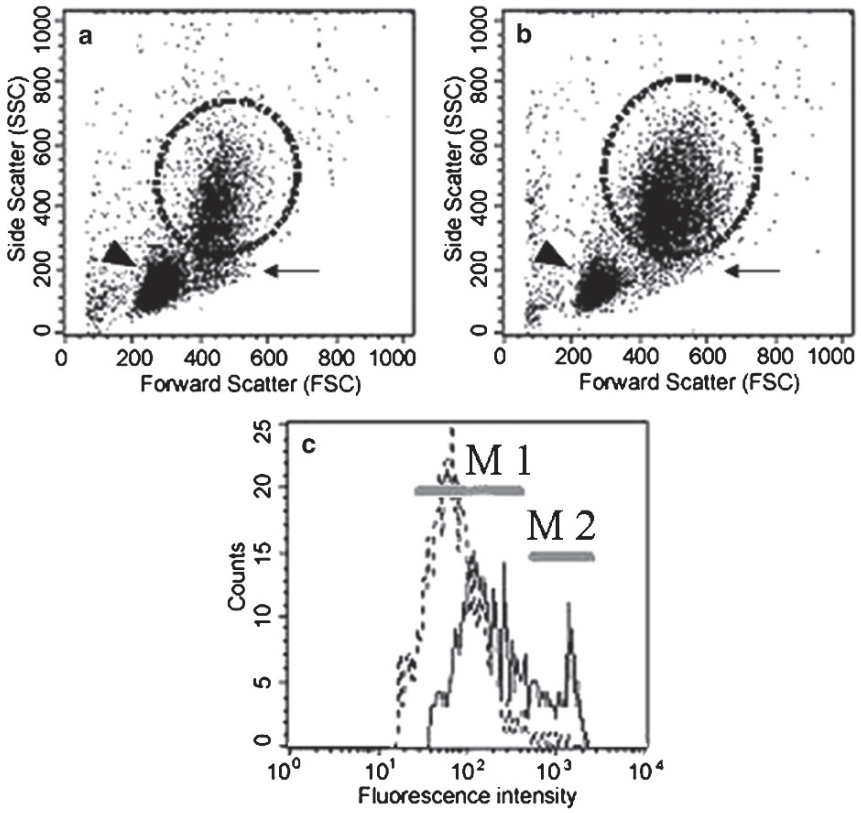

Fig. 12.2 Flow cytometric analysis (a, b) and detection of respiratory burst activity (c) of head kidney leukocytes from rainbow trout exposed to TBT 20 µg/l for 5 days (a, b) Typical FSC/SSC cytogram of one trout from the control group (a) and one from the TBT-treated group (b) shows the distribution of lymphocytes (*arrowheads*), granulocytes (almost neutrophils; *dotted lines*) and monocytes/macrophages (*arrows*). Note the increase of neutrophile granulocytes (b; *dotted line*) in TBT exposed trout. (c) The respiratory burst activity of head kidney leukocytes obtained from trout exposed to TBT 20 µg/l (*dotted line*) decreased, especially the population in control trout (*solid line*) expressing a high activity (10^3 fluorescence intensity; M2-area). Additionally, the mean fluorescence intensity of lower 10^2 fluorescence intensity (M1-area) in TBT exposed trout shifted toward the weaker intensity.

by means of fluorescence intensity, in head kidney leukocytes of TBT exposure group is decreased (Fig. 12.2c). Especially, the number of leukocytes which have a high respiratory burst activity, determined by a high fluorescence intensity around 1,000 (M2), clearly decreased in the exposure group. Probably, the population expressed lower respiratory burst activity, shown as lower fluorescence intensity around 100 (M1), representing a neutrophil population with disturbed functional activities. These results demonstrate firstly, that water-borne exposure of rainbow trout to 20 µg TBTCl/l for 5 days elicited a temporal increase of the neutrophil population together with a concomitant decrease of other leukocyte populations (lymphocytes and monocytes) in the head kidney and secondly, these neutrophils although being increased in number, show reduced respiratory burst activities.

The concentration selected for our experiment – 20 µg TBTCl/l – is rather high and lethal in the long term. Thus, our finding that TBT affected the composition of the leukocyte population in the head kidney and their respiratory burst activity may be questioned. However, results from longer-term exposure experiments with lower TBT concentrations (Oliveira-Ribeiro et al. 2002; Schwaiger et al. 1992) also reported changes in the cellular composition of immune organs such as increased karyorrhexis of lymphoid cells and erythrophagia in spleen. These results support our observations that immune cells are a target of the toxic action of TBT.

The same analyses for composition of head kidney leukocytes were made for trout exposed by immersion to TBTCl at 5 µg/l for 28 days, the results showed no remarkable changes of leukocyte composition, except an increased neutrophil population in the blood after 7 days immersion. To date, TBT accumulation in blood is well studied (Oshima et al. 1997; Shim et al. 2002). In the next section, internal doses of TBT and its effects on a humoral factor in blood are discussed.

12.3.2 Relationship Between TBT Concentration in Blood and Lysozyme Activity in Plasma

The aim of this study was to evaluate the relationship between TBT concentration in the blood and plasma lysozyme activity. TBT was analysed using a gas chromatograph equipped with a mass spectrometer and lysozyme was analysed by decreased turbidity of *Micrococcus* bacterial solution. To this end, rainbow trout were exposed to TBTCl at 0 (control), 5 and 10 µg/l for 5 days. As shown in Fig. 12.3, TBT accumulated in a dose-dependent manner in the blood of exposed fish and the levels of TBT in 5 and 10 µg/l 5day exposure groups were recorded at 5.8 and 10 mg/l blood, respectively. This correlated with a significantly decreased plasma lysozyme activity of fish exposed to 10 µg/l for 5 days, whereas in the 5 µg/l exposure group, lysozyme activity was not affected. The different effect might be due to the border between non-effective and effective internal doses of TBT in blood or due to the short exposure time.

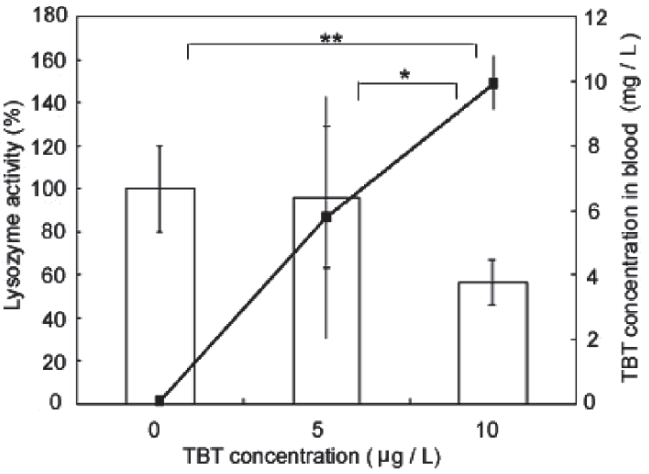

Fig. 12.3 The effects of different concentrations of TBT exposure for 5 days on lysozyme activity and TBT concentration in blood. Lysozyme activities (*columns* □) and TBT concentration in blood (*line* ■) are shown. Lysozyme activity and TBT concentration measurements were made on blood samples from the same individuals. The mean values ± SD are shown the average of fish in each experimental group. The ** and * show significant differences between 0 and 10 µg/l exposure groups, and 5 and 10 µg/l exposure groups of lysozyme activity, for $p < 0.01$ and $p < 0.05$, respectively (Nakayama et al. 2005 with some modifications)

Oshima et al. (1997) reported that a cultured Japanese flounder (*Paralichthys olivaceus*) sold in a market possessed very high accumulated TBT concentrations in the blood. While the experimental levels used in our study were more than 1,000 times higher than TBT concentrations usually detected in an aquatic environment (maximum ppt levels in water), the TBT concentrations recorded in the blood from the experimental fish were only about twofold higher than those detected from the former market fish. Thus, since internal TBT concentrations of the laboratory and the field-caught fish are rather similar, the immunotoxic effects observed in our experimental fish may point to the presence of immunotoxic effects in the cultured flounder. The available evidence from short term laboratory exposures indicate that internal blood TBT concentrations of <~5 mg/l blood do not affect plasma lysozyme activity while fish accumulating 10 mg/l blood or more show significant immunosuppressive changes including modulated leukocyte populations, decreased respiratory burst activities (as shown in Fig. 12.2) as well as down regulated lysozyme activities in plasma.

12.3.3 Enhanced Susceptibility to a Pathogen Correlated with TBT Intraperitoneal Exposure

While the available data clearly indicate that TBT has immunotoxic potency for different fish species, it is less clear whether these TBT-induced changes of

immune parameters translate into altered resistance or susceptibility towards pathogenic microorganisms. To address this question, we treated rainbow trout with intraperitoneal (ip) injection of TBT and assessed the consequences on the susceptibility of trout to the bacterial pathogen, *Aeromonas salmonicida*, the causative agent of furunculosis. Rainbow trout reared at 13° were treated with a single ip injection of 2.5 mg TBTCl/kg body weight 7 days prior bacterial challenge. TBT distribution in the tissues was as follows: blood 498 µg/l, Liver 4,037 µg/kg, kidney 2,624 µg/kg and spleen 2,087 µg/kg tissue. Seven days after TBT injection, fish were challenged intraperitoneally with 10^5 colony forming units (CFU) of *A. salmonicida* per fish, and the resulting cumulative mortality was monitored over a 7-day-period. Initial mortalities occurred 4 days after infection with *A. salmonicida* in fish that previously had received TBT treatment. Macroscopic observation in the dead fish revealed an enlarged spleen, liquefied kidney tissue and dilated intestine. Cumulative mortality in the TBT treatment reached 90% by day 7 post-challenge. In contrast, *A. salmonicida* challenge resulted in no mortality in control fish (corn oil injection instead of TBT), although enlarged spleen and hyperemia in the muscle tissue surrounding the injection site were present. These findings suggest that the immunotoxicity of TBT, as shown in the immersion experiment (see 12.3.2.) translates into enhanced pathogen susceptibility of rainbow trout. This observation is particularly remarkable since the tissue TBT levels of rainbow trout from the laboratory challenge experiment were clearly lower than TBT tissue burdens often found wild fish (Oshima et al. 1997; Hassani et al. 2006, summarized by Liu et al. 2006). This observation highlights the risk of TBT accumulation for the disease resistance of wild fish populations (Fig. 12.4).

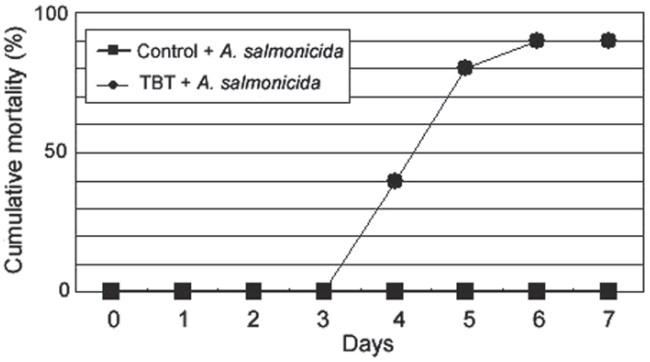

Fig. 12.4 Cumulative mortality. Prior to the bacterial challenge with *Aeromonas salmonicida* (10^5 CFU per fish) at 13°C, treated fish received a single ip injection of TBT 2.5 mg/kg body weight for 7 days (Control fish received corn oil only). Afterwards, fish were challenged with a bacterial pathogen, *Aeromonas salmonicida*, therefore, 0 day indicates 7 days following the TBT administration. After the pathogen challenge, the cumulative mortalities of both control and TBT-injected fish infected with *A. salmonicida* for 7 days are shown (unpublished data)

12.4 Conclusions

Both the literature and our own experimental data provide clear evidence that TBT is immunotoxic to fish, even at low, environmentally relevant concentrations. The available data also provide evidence that this immunotoxic activity can compromise the fish's ability to resist pathogens, thus posing an ecological risk. Future studies on the immunotoxicity of TBT should aim to develop understanding of the immunotoxic mechanisms by which TBT disturbs the resistance to pathogens. Furthermore, greater attention should be given to monitor for indications of compromised immune status in fish from TBT-contaminated areas.

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