Effects of Sewage Treatment Plant Effluent on the Immune System of Rainbow Trout (*Oncorhynchus mykiss*)

Dissertation

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Contents

Abbreviations

AFB_1	aflatoxin B_1
A.s.s.	*Aeromonas salmonicida salmonicida*
CFU	colony forming units
DCF	2´,7´-dichlorofluorescein
DDT	dichloro-diphenyl-trichloro-ethane
DMBA	7,12-dimethyl-benz[a]anthracene
DMN	dimethylnitrosamine
DMSO	dimethylsulfoxid
11-KT	11-ketotestosterone
EROD	7-ethoxyresorufin-*O*-deethylase
FCS	fetal calf serum
GSI	gonado-somatic index
H_2DCFDA	2´,7´-dichlorodihydro-fluorescein diacetate
HBSS	hanks balanced salt solution
Ig	immunoglobulin
IL-1	interleukin-1
L-15 medium	Leibovitz´s L-15 medium
LPS	lipopolysaccharide
LSI	liver somatic index
mab	monoclonal antibody
MFO	mixed function oxygenase
MMCs	melano-macrophage centres
NCC	natural cytotoxic cell
OD	optical density
OP	organophosphorous pesticides
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate balanced salt solution
PCBs	polychlorinated biphenyls
PHA	phytohaemagglutinin
PMA	phorbol-myristate-acetate
SEM	standard error of the means
SD	standard deviation
SSI	spleen somatic index
STP	sewage treatment plant
STW	sewage treatment water
TMB	tetramethylbenzidine
U	units
vtg	vitellogenin

Species names

A. hydrophila	*Aeromonas hydrophila*
A. salmonicida	*Aeromonas salmonicida*
C. Carpio	*Cyprinus carpio*
C. aurata	*Carassius aurata*
E. tarda	*Edwardsiella tarda*
E. coli	*Escherichia coli*
F. heteroclitus	*Fundulus heteroclitus*
I. punctatus	*Ictalurus punctatus*
I. multifiliis	*Ichthyphthirius multifiliis*
L. rohita	*Labeo rohita*
L. salmonis	*Lepeophtheirus salmonis*
L. xanthurus	*Leistomus xanthurus*
M. lysodeikticus	*Micrococcus lysodeikticus*
M. saxatilis	*Morone saxatilis*
O. mykiss	*Oncorhynchus mykiss*
O. tshawytscha	*Oncorhynchus tshawytscha*
O. tau	*Opsanus tau*
O. niloticus	*Oreochromis niloticus*
O. latipes	*Oryzias latipes*
P. olivaceus	*Paralychthys olivaceus*
P. promelas	*Pimephales promelas*
P. americanus	*Pseudopleuronectes americanus*
S. trutta	*Salmo trutta*
S. vitreum	*Stizostedion vitreum*
T. adspersus	*Tautogolabrus adspersus*
T. maculatus	*Trinectes maculatus*
V. anguillarum	*Vibrio anguillarum*
Y. ruckeri	*Yersinia ruckeri*

General Introduction

Pollution of surface waters with man-made substances, including pharmaceuticals and pesticides has been observed throughout the world and shown to cause adverse effects in aquatic organisms (Bols et al., 2001; Bucher & Hofer, 1993; Burkhardt-Holm et al., 1997; Christensen, 1998; Dunier & Siwicki, 1993). Discharge of these substances into surface waters occurs mainly through sewage treatment plants (STP), but also through leakage of landfills (Heberer, 2002; Kuspis & Krenzelok, 1996; Andreozzi et al., 2003). Although pharmaceuticals in sewage treatment water (STW) and surface waters are usually found in very low concentrations, the presence of a broad array of substances has raised concerns about possible effects on all classes of organisms, that live in close interaction with water. In spite of the fact that residues of several pharmaceuticals have been demonstrated in surface and drinking waters throughout the last 10 years, very little is known about the possible effects of such a mixture contamination on aquatic organisms (Jones et al., 2001; Halling-Sorensen et al., 1998; Daughton & Ternes, 1999; Ternes, 1998). Impacts on fish populations have been observed due to alterations of the reproductive system (Jobling et al., 1996; Larsson et al., 1999; Matthiessen & Sumpter, 1998; Robinson et al., 2003; Spies & Rice, 1988), but less studied are adverse effects of mixture contamination on the immune system, the latter possibly leading to a decreased resistance against pathogens resulting in mortality (Luebke et al., 1997).

The aim of this doctoral thesis was to gain an insight into effects of sewage treatment plant effluent on the immune system of teleost fish and to evaluate test parameters in fish immunotoxicology, also for their later use in monitoring of environmental pollution. Therefore, rainbow trout (*Oncorhynchus mykiss*) were exposed to wastewater effluent in an acute (27 days) exposure with high effluent concentrations, as well as in a chronic (32 weeks) exposure experiment, with lower, environmentally relevant effluent concentrations. Beside investigation of immune parameters, effects of chronic exposure to effluent on general physiology and endocrine parameters were also assessed. Effects of exposure to effluent on the trout immune system are moreover compared to results from exposure experiments with the model substances and known immunosuppressors cortisol and rifampicin.

Pollution of surface waters

With the expansion of heavy industry in the 19th and 20th century, surface waters were commonly polluted from point sources. High concentrations of chemicals, acutely toxic to aquatic organisms, caused visually obvious damage to aquatic ecosystems, including fish kills. With the past three decades of emerging environmental concern, and resultant pollution control and remedial measures, most of the surface waters in Central Europe are considered to be relatively clean, with regard to nutrients and acutely toxic substances. Widespread mortality due to lethal concentrations of industrial chemicals seldom occurs today. Despite these dramatic improvements, pollution of surface waters continues to be an ongoing concern, nowadays due to contamination of waters with low concentrations of a variety of substances, such as hormonally active compounds, residues of pharmaceuticals and health care products, as well as sub-lethal concentrations of industrial chemicals (Guillette & Guillette, 1996; Jobling & Sumpter, 1993; Jobling et al., 1995; Jobling et al., 1996; Christensen, 1998). Occasionally, reductions in certain fish populations are observed e.g. in Central Europe, including Switzerland and Germany. The reasons for these declines in fish populations have not yet been elucidated, but impairment of reproduction through contamination of habitats with endocrine-disrupting compounds (Larsson et al., 1999) and subtle changes in the ability to fight disease due to immunologically active pollutants, are commonly addressed suspects (Bly et al., 1997; Daughton & Ternes, 1999; Bucher & Hofer, 1993; Wahli et al., 2002).

The most widespread source of pollution of surface waters with the substance classes mentioned above are sewage treatment plants. Pharmaceuticals are either excreted, or directly disposed of by humans into sewage. The presence of pharmaceuticals in surface water, largely from human waste, has been well documented (Daughton & Ternes, 1999; Halling-Sorensen et al., 1998; Hirsch et al., 1999; Jones et al., 2001; Ternes, 1998). Some pharmaceuticals, like e.g. diclofenac and ethinylestradiol have even been found to reach ground water (Heberer, 2002; Hirsch et al., 1999; Sacher et al., 2001). Several pharmaceutical compounds have been shown to be highly recalcitrant with regards to microbial degradation (Henschel et al., 1997; Kümmerer et al., 1997; Steger-Hartmann et al., 1997; Ternes, 1998; Andreozzi et al., 2003). Municipal waste is also known to contain the residues of compounds contained in many household products, such as various derivatives of alkylphenols (Lee et al., 1998; Bennie, 1999) and measurable levels of natural hormones (Desbrow et al., 1998).

Aquatic immunotoxicology

In recent years, adverse effects of natural and synthetic hormones, as well as hormonally active industrial compounds, on the reproductive system of aquatic organisms have gained a great deal of attention. The investigation of endocrine disruption has led to the establishment and widespread use of "biomarkers", such as the activation of estrogen-regulated gene transcription and subsequent production of the egg yolk precursor vitellogenin in male fish (Barron et al., 2000; Matthiessen & Sumpter, 1998; Harries et al., 1997; Hemming et al., 2001). Biomarkers have been defined as a biological response to a chemical or chemicals, that give a measure of exposure, and sometimes, also of toxic effects (Peakall & Walker, 1994). Evidence of environmental relevance of such biomarkers, like e.g. impacts at the population level, are generally lacking. Concerns about the effects of hormonally active pollutants on reproduction in fish have now been followed by concerns about a possible disruption of immune reactions, which might result in impaired disease resistance. Knowledge about the immune system of teleosts lags behind that of mammals. However, dramatic increases in knowledge of fish immunology over the last decade have demonstrated a multifaceted immune system that may be as or more complex than that of mammals, which makes investigating effects of pollution on immune parameters a difficult task. Examinations on the immune system of fish have produced very heterogeneous results and depend on a variety of factors, such as species investigated, gender, diurnal variations associated with reproductive and seasonal cycles (Yamaguchi et al., 2001; Vladimirov, 1968), as well as stress and environmental factors, like temperature (Le Morvan et al., 1998). In the case of immune challenges, which are often used to test effects of pollution on disease resistance, the influence of pollutants on different immune parameters can vary strongly with the pathogen used in the study and route of antigen administration (reviewed by Sharma & Zeeman, 1980). Therefore, clear statements about pollution effects can only be made for one species at a time, using data, which has been gained within comparable experimental set ups, employing similar immune parameters. The establishment of methods, detecting adverse effects on immune parameters, as tools for exploring possible mechanisms of chemical impact are moreover impeded by the complexity of many of the methods.

Fish immunology

Bony fish possess an immune system, that is highly developed and the basic mechanisms of immunity in fish and mammals are quite similar (Press, 1998). Fish do not possess bone marrow, however, it is widely accepted that a lymphatic system is differentiated from the blood vascular system (Press, 1998). The major lymphoid organs in fish are thymus, kidney and spleen. A gut-associated lymphoid tissue, which contains significant populations of leucocytes has also been shown in several teleost species as well as a mucosal immune system in gut and skin. Like mammals, fish possess leucocytes, which can be classified as lymphocytes, monocytes / macrophages and granulocytes. Description of specific cell surface markers is still rare and consequently knowledge about development and function of fish leucocytes lacks behind that of mammals. While for certain fish species, specific monoclonal antibodies exist against immunoglobulin, enabling the identification of B-cells (Thuvander et al., 1990; Sizemore et al., 1984; Miller et al., 1987; Scapigliati et al., 1999), and against surface markers on granulocytes (Kuroda et al., 2000) (however, the identity of those markers is unknown) and monocytes (Köllner et al., 2001), T-cells have only been defined indirectly as Ig-negative cells. The presence of T-cells is suggested through several functional assays (specific T-cell mediated cytotoxicity, T-cell dependent antibody response, secretion of T-cell produced cytokines) (Fischer et al., 2003; Miller et al., 1985; Blohm et al., 2003) and the presence of T-cell receptor and CD8 genes, the expression of which can be demonstrated at the mRNA level (Partula et al., 1995; Partula, 1999; Wilson et al., 1998; Fischer et al., 2003). Granulocytes can, in most fish species, be divided into neutrophils, eosinophils and basophils, however this differentiation is almost solely based upon morphological characterisation. The functions of fish granulocytes (especially neutrophils) have been described as phagocytosis, chemotaxis and bactericidal activity with the help of reactive oxygen and nitrogen species (respiratory burst). Fish have moreover been shown to possess natural cytotoxic cells (NCCs), which are functionally similar to mammalian natural killer cells and have been shown to kill target cells, including various pathogenic protozoa, without the requirement for previous exposure (Evans et al., 2001). A special characteristic of fish is the presence of so-called melanomacrophages (macrophages, containing heterogenous inclusions, the most frequent of which are melanin, hemosiderin and lipofuscin (Wolke, 1992)), which form centres in spleen, kidney and liver. The function of melanomacrophage centres (MMCs) is not clearly elucidated yet, but their ability to retain antigens for long periods suggests a role in immune

reactions (Wolke, 1992). A list of the different leucocytes in fish, their function and surface markers, demonstrated so far, is given in table 1.

Table 1: Leucocytes in teleosts (Iwama & Nakanishi, 1996; Press, 1998)

Cell type	Function	Surface marker
granulocytes (neutrophil, eosinophil and basophil)	phagocytosis chemotaxis production of reactive oxygen-species release of immunopharmacological substances	antibody-receptors (Fc) complement-receptors
monocytes/ macrophages	accessory cells in lymphocyte reaction (antigen processing and presentation) production of IL-1-like activity phagocytosis chemotaxis release of reactive oxygen and nitrogen species	antibody-receptors (Fc) complement-receptors MHC II molecules
B-lymphocytes	specific antibody response	immunoglobulin
T-lymphocytes	cytotoxic activity	T-cell receptor CD8
unspecific killer cells	cytotoxic activity	?

Similar to the mammalian immune system, communication between immune cells in fish has been suggested through the finding of complement factors, eicosanoids and cytokines. Several cytokines and one cytokine receptor have been demonstrated by biological activity, antigenic cross-reactivity or on the gene level, through cDNA cloning and sequencing of cytokine genes (Graham & Secombes, 1988, 1990a, 1990b; Zou et al., 1999a, 1999b, 2000a, 2000b; Verburg-van Kemenade et al., 1995; Secombes et al., 1998, 1999, 2001; Blohm et al., 2003). Recently, a monoclonal antibody against carp (*Cyprinus carpio*) IL-1β has been developed (Mathew et al., 2002).

As in mammals, the fish immune system can be roughly divided into an unspecific, innate part (table 2) and specific, adaptive reactions (table 3). In spite of displaying adaptive, humoral immune responses, fish appear to rely primarily on their innate, unspecific immune reactions. Macrophages and granulocytes, and unspecific humoral substances, such as lytic enzymes (e.g. lysozyme), complement factors, C-reactive protein and lectin in skin, gill and gut mucus build a first line of defence, to prevent bacterial invasion (Jones, 2001; Alexander & Ingram, 1992; Ellis, 2001). Mucus has even been shown to contain IgM antibodies along with the above mentioned unspecific immune cells and enzymes (Loghothetis & Austin, 1994; Hatten et al., 2001).

Table 2: Innate, unspecific immune reactions in teleosts (Iwama & Nakanishi, 1996)

Cellular reactions		
Participating cells and tissues	**Pathogen/cells affected**	**Reaction**
mobile, phagocytotic cells (macrophages and granulocytes)	bacteria	phagocytosis and production of reactive nitrogen and oxygen species
eosinophilic granulocytes	bacteria, helminths	secretion of immunopharmacological substances
non-specific cytotoxic cells	virus-infected cells, protozoa	induction of necrosis or apoptosis
Humoral reactions		
Participating substance	**Pathogen/cells affected**	**Reaction**
lysozyme	gram-positive bacteria	splitting of N-acetylmuraminic acid and N-acetyglucsoamine bond in cell wall stimulation of phagocytosis as opsonin
complement	virus bacteria parasites	viricidal, bactericidal and parasiticidal building of membrane attack complex → cytolysis of target cell opsonic activity chemoattractant activity inactivation of bacterial exotoxins
interferon	virus	inhibition of virus replication stimulation of macrophages
C-reactive protein	bacteria (*Saprolegnia sp*., *S. pneumonia*)	recognition and precipitation of c-polysaccharide activation of the classical complement pathway
transferrin	bacteria	chelating iron, making it unavailable for bacterial use
lectins (haemagglutinins)	bacteria (*Aeromonas hydrophila, A. salmonicida, Vibrio anguillarum, V. ordalii, Renibacterium salmoninarum, Yersinia ruckeri, Edwardsiella tarda*)	agglutination of cells and/ or precipitation of glycoconjugates opsonic activity and activation of classical complement pathway? (not clearly shown in fish)
haemolysin	?	?
proteinase	gram-negative bacteria	trypsin-like activity
α_2-macroglobulin	*A. salmonicida*	inhibition of the proteolytic activity of *A. salmonicida* protease
chitinase	?	hydrolysis of N-acetylglucosamine tetramers and higher oligosaccharides, including chitin
α-precipitin	?	reacts with carbohydrates and glycoproteins of several fungi species

As soon as bacteria enter an organism, macrophages and neutrophils form the next line of defence through phagocytosis and with the help of reactive nitrogen and oxygen species as well as internal enzymes. When the first line defence fails to prevent the establishment of a pathogen in the organism, a specific antibody response occurs.

Fish have been shown to produce antibodies, with specificity and measurable affinity for the immunising antigen and these antibodies have biological properties, such as agglutination, precipitation, complement fixation, opsonisation and skin sensitisation (Press, 1998). However, in most fish species only immunoglobulin of the IgM type has been found so far and isotype switching has not been demonstrated. High titers of anti-hapten antibodies have also been demonstrated in fish, however the reason for the presence of these so-called natural antibodies is not clear yet (Press, 1998). Similarities of epitopes with common bacteria in water and environmental agents have been suggested as the cause for these natural antibodies. Immunoglobulin M in fish usually forms tetramers, but monomers and dimers have also been described. In fish, triggering of the specific humoral response is very slow and the production of specific antibodies can take more than 14 days, as shown for immunisation of rainbow trout (*O. mykiss*) with the bacteria *Aeromonas salmonicida* (Köllner & Kotterba, 2002).

Exogenous modification of the fish immune system, specifically immunosuppression, has been mainly attributed to exposure to chemicals, temperature differences and stress. Studies on adverse effects of chemicals and stress on the fish immune system are reviewed in the following section.

Table 3: Adaptive, specific immune reactions in teleosts (Iwama & Nakanishi, 1996)

Cellular immunity	
Function	**Evidence in fish**
specific cytotoxicity	surface immunoglobulin negative cells with cytotoxic T-cell-like activity
cell communication through cytokines	cytokines with functional similarity and cross-reactivity with mammalian cytokines
antigen-presentation	MHC I and II
Humoral immunity	
Function	**Evidence in fish**
specific antibody reaction	immunoglobulin in fish
immunological memory	faster reaction against second infection with a pathogen, but no isotype switch proven so far

Effects of immunologically active substances in fish

In recent years, the effects of several different substances on the immune system of fish have been investigated. A wide range of substance classes has been covered, including industrial chemicals, pesticides, heavy metals, hormones and pharmaceuticals.

Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)

Polycyclic aromatic hydrocarbons (PAHs) are immunotoxic, carcinogenic chemicals that are widely distributed in the environment. Therefore, many studies on immunotoxicity in fish have focused on this chemical class. In spot (*Leistomus xanthurus*) and hogchoker (*Trinectes maculatus*), as well as in mummichog (*Fundulus heteroclitus*) from the Elizabeth River in Virginia, which is known to be highly contaminated with PAHs, a variety of immune parameters were investigated (Weeks & Warinner, 1986; Kelly-Reay & Weeks, 1994). In these studies, spot and hogchocker displayed reduced macrophage migration and phagocytotic activity; however, chemiluminescence response (resulting from reactive oxygen species production) in mummichog from Elizabeth River was significantly increased. PAHs have also been shown to suppress proliferative responses of mitogen-stimulated leucocytes in spot (*L. xanthurus*) (Faisal & Huggett, 1993), carp (*C. carpio*) (Reynaud et al., 2003) and rainbow trout (*O. mykiss*) (Karrow et al., 1999), to cause a significant reduction of melano-macrophage centres in liver tissue from winter flounder (*Pseudopleuronectes americanus*) (Payne & Fancey, 1989) and to reduce pronephros leucocyte oxidative burst, as well as plasma lysozyme levels in rainbow trout (*O. mykiss*) (Faisal & Huggett, 1993; Reynaud et al., 2003; Payne & Fancey, 1989; Karrow et al., 1999; Karrow et al., 2001).

Polychlorinated biphenyls (PCBs) have been shown to cause reduced oxidative burst activity in channel catfish (*Ictalurus punctatus*) phagocytes (Rice & Schlenk, 1995), lower cytotoxic leucocyte activity in tilapia (*Oreochromis niloticus*) (Smith et al., 1999), suppressed antibody production in chinook salmon (*O. tshawytscha*) (plaque-forming cell responses of head kidney and splenic leucocytes) (Arkoosh et al., 1994), as well as increased disease susceptibility of channel catfish to *A. hydrophila* (Jones et al., 1979) and of rainbow trout (*O. mykiss*) to *Yersinia ruckeri* (Mayer et al., 1985). In chinook salmon, decreased antibody production was also shown after exposure to 7,12-dimethyl-benz[a]anthracene (DMBA) (Arkoosh et al., 1994) and cytotoxic leucocyte activity in tilapia (*O. niloticus*) was significantly decreased after *in vivo* exposure to benzo[a]pyrene, DMBA and dimethylnitrosamine (DMN) (Smith et al., 1999).

In general, PAHs and PCBs have been shown to suppress several immune reactions in different fish species, including macrophage migration, phagocytosis and oxidative burst, lysozyme activity, leucocyte proliferation, plaque-forming cell response and cytotoxic leucocyte activity. These chemical classes can therefore generally be considered as immune toxic in fish and can be found in the environment in concentrations high enough to display adverse effects on the fish immune system, potentially decreasing resistance against opportunistic pathogens.

Pesticides

A general statement about the effects of pesticides on fish immune system investigated so far is difficult to make, however suppressing effects seem to prevail. Reduced serum antibody numbers have e.g. been shown for lindane in carp (*C. carpio*) and rainbow trout (*O. mykiss*) (Cossarini-Dunier et al., 1987; Dunier & Siwicki, 1994), endrin in rainbow trout (Bennett & Wolke, 1987a), DDT in goldfish (*Carassius auratus*) (Sharma & Zeeman, 1980), Bayluscide in African catfish (*Claries lazera*) (Faisal et al., 1988) and tributyltin in channel catfish (*I. punctatus*) (Rice et al., 1995). The organochlorine pesticide lindane has moreover been observed to suppress B-cell proliferation, lysozyme levels, phagocytic activity of blood neutrophils and chemiluminescent response in rainbow trout, as well as cytotoxic leucocyte activity in tilapia (*O. niloticus*) (Smith et al., 1999; Dunier et al., 1994; Dunier & Siwicki, 1994). However, high concentrations of lindane have also been shown to increase *in vitro* oxidative burst activity in rainbow trout (*O. mykiss*) head kidney macrophages (Betoulle et al., 2000). Although the rainbow trout immune system was affected by lindane exposure, similar studies in carp did not result in immunosuppressive effects on antibody production, changes in spleen weight (Cossarini-Dunier et al., 1987), skin graft rejection, or changes in phagocytosis (Dunier & Siwicki, 1994). The known endocrine disruptor tributyltin has also been shown to decrease macrophage chemiluminescence reaction in oyster toadfish (*Opsanus tau*), hogchoker (*T. maculatus*) and croaker toadfish (Rice & Weeks, 1990; Wishkovsky et al., 1989). Trichlorphon has been found to reduce neutrophil phagocytotic activity and lysozyme activity in carp (*C. carpio*) (Cossarini-Dunier et al., 1990).

Organophosphorous pesticides (OPs) were introduced in replacement for the persistent organochlorine pesticides, especially after the tendency of DDT and its metabolites to bioaccumulate in ecosystems and to cause adverse health effects, particularly to top predators, led to the legal ban or restriction of their use in the 1970s (Peakall et al., 1975). Over the last 20 years, experimental evidence has accumulated that OPs can interfere with the immune

system and exert immunotoxic effects in laboratory animals (Vial et al., 1996). Exposure of Japanese medaka (*Oryzias latipes*) to the OP malathion had no effect on haematocrit or leucocrit values or mitogen-induced T-cell proliferation, but caused a dose dependent decrease in plaque-forming cell numbers, indicating a significant decrease in humoral immune response (Beaman et al., 1999).

It should be noted that contradictory results, described in the literature, might be due to differences in substance concentrations and route of application (*in vivo* versus *in vitro*), as well as diverse sensitivity of the fish species studied. In general, *in vivo* application as well as exposure of wildlife to low pesticide concentrations should be regarded as more relevant for considerations in the field of environmental toxicology than *in vitro* tests and exposure to high concentrations. A review of the literature currently available on immunotoxicity in fish suggests that pesticides should be considered as potential immunosuppressive contaminants of surface waters.

Therapeutic substances and hormones

The effect of internal as well as external cortisol on the fish immune system has been thoroughly studied in relation to its function as a stress mediator and a known immunosuppressant. Exposure routes mainly used in investigations are oral application, injection and induction of internal cortisol through stress. Treatment of fish with cortisol results in a reduction in leucocyte proliferation (Le Morvan-Rocher et al., 1995; Espelid et al., 1996; Verburg-van Kemenade et al., 1999; Ellsaesser & Clem, 1987; Choi Sang & Oh, 2003), reduced numbers of antibody producing cells (Carlson et al., 1993; Mazur & Iwama, 1993), decreased antibody levels (Wechsler et al., 1986), and lower numbers of peripheral blood lymphocytes and eosinophilic granulocytes (Espelid et al., 1996; Ellsaesser & Clem, 1987). As has been shown for flounder (*Paralychthys olivaceus*) and carp (*C. carpio*), cortisol induces its depressing effects on B-lymphocyte numbers through induction of apoptosis in these cells (Verburg-van Kemenade et al., 1999; Choi Sang & Oh, 2003). The finding that cortisol elicits a depressing effect on fish lymphocytes, is in line with the mechanism of cortisol shown in mammalian models. As a possible result of the negative impact of cortisol on lymphocytes, reduced resistance of carp (*C. carpio*) and channel catfish (*I. punctatus*) against the protozoan parasite *Ichthyphthirius multifiliis* and coho salmon (*O. kisutch*) against sea louse (*Lepeophtheirus salmonis*) has also been shown after exposure to exogenous cortisol (Houghton & Matthews, 1990; Davis et al., 2003). However, investigations on the effects of stress on immune parameters in different fish species and the role of cortisol in these stress

reactions do not provide consistent results, the findings varying with the type and severity of stress applied, fish species, immune parameters investigated and, in the case of challenge experiments, with the pathogen type and species (Davis et al., 2003; Narnaware & Baker, 1996; Espelid et al., 1996). Narnaware and Baker (1996) moreover showed that depressing effects of injection-stress in rainbow trout (*O. mykiss*) were opposed by additional injection of cortisol. The inconsistent results found in the literature, concerning effects of stress on fish immune parameters and the role of exogenous cortisol in stress reactions might reflect the sensitivity of hormonal regulatory mechanisms, depending strongly on endogenous concentrations, site of action and receptor up- and down-regulation.

The idea that close interactions between the endocrine and the immune system also exist in fish has been supported by Slater et al. (1995), who demonstrated the presence of an androgen receptor in rainbow trout (*O. mykiss*) leucocytes. They therefore suggested, that androgens act directly on salmonid leucocytes through this androgen receptor, while inducing immunosuppression during sexual maturation (Slater & Schreck, 1993). Steroid hormones, including estradiol, progesterone and 11-ketotestosterone have been shown to suppress carp (*C. carpio*) and goldfish (*C. auratus*) macrophages activity *in vitro* (Yamaguchi et al., 2001).

The effects of antibiotics on immune parameters in fish have also been investigated, whereby *in vitro* exposure to trimethoprin / sulfadiazine (TS) increased phytohaemagglutinin (PHA)- and lipopolysaccharide (LPS)-stimulated lymphocyte proliferation, while oxolinic acid (OA), oxytetracycline (OTC) and florfenicol (FF) inhibited proliferation in a dose-dependent manner, with FF being the most effective antibiotic tested in this study (Lundén & Bylund, 2000). After oral administration of a therapeutic dose, all the antibiotics tested, except for TS, lead to suppressed mitogenic response of the head kidney cells, with the suppression being more severe in T-cells than in B-cells (Lundén & Bylund, 2000).

Metals

One of the metals most thoroughly studied in the field of fish immunotoxicology is cadmium. However, studies on effects of cadmium on different immune reaction in fish have revealed contradictory results. Cadmium has e.g. been shown to either suppress or increase macrophage activity in rainbow trout (*O. mykiss*), depending on the type of stimulation (un-stimulated vs. phorbol-myristate-acetate (PMA)-stimulated macrophages) (Zelikoff et al., 1995; Elsasser et al., 1986). Contradictory results have also been found for the effects of cadmium on antibody response in fish. Antibacterial antibody levels in serum were lower in cadmium-exposed cunners (*Tautogolabrus adspersus*) compared to control fish, but enhanced

in striped bass (*Morone saxatilis*) and rainbow trout (*O. mykiss*) (Robohm, 1986; Thuvander, 1989). However, protective immunity in rainbow trout vaccinated against *V. anguillarum* was not influenced by exposure to cadmium (Thuvander, 1989). Cadmium has moreover been shown to reduce leucocyte proliferation and total leucocyte counts, reflected in a decrease in lymphocyte and thrombocyte numbers in goldfish (*C. aurata*) (Murad & Houston, 1988). Cadmium-chloride (CdCl) was shown to significantly decrease cytotoxic leucocyte activity in tilapia (*O. niloticus*) after *in vivo* exposure (Smith et al., 1999).

For other metals, like aluminium, chromium, copper, lead and mercury mainly suppressive effects on immune parameters have been described, including reduced macrophage activity (Elsasser et al., 1986), lower serum antibody levels (O´Neill, 1981a; O´Neill, 1981b; Anderson et al., 1989), reduced lymphocyte numbers and a higher susceptibility to diseases (Hetrick et al., 1979; Rodsaether et al., 1977; Gill & Pant, 1985).

In general, metals have to be regarded as potentially immunosuppressive in fish, however concentrations used in some of the laboratory studies were relatively high and might not be found in effective concentrations in mixed effluent pollution, like sewage treatment plant effluent.

Mycotoxins

In vitro exposure of rainbow trout (*O. mykiss*) peripheral blood leucocytes to aflatoxin B_1 (AFB_1) has been shown to decrease lymphocyte proliferation and immunoglobulin production in response to the mitogen lipopolysaccharide (Ottinger & Kaattari, 1998). A single injection of Indian major carp (*Labeo rohita*) with AFB_1 reduced bacterial agglutination titre, serum bactericidal activity against *E. tarda*, serum lysozyme level and disease resistance against *A. hydrophila* and *E. tarda* (Sahoo & Mukherjee, 2002). *In vivo* exposure to trichothecene (T_2) mycotoxin was shown to decrease cytotoxic leucocyte activity in tilapia (*O. niloticus*) (Smith et al., 1999).

In general, it can be said that some immune parameters have been shown to be sensitive to a variety of contaminants. For example phagocyte oxidative burst in several fish species is affected by tributyltin (Rice et al., 1995), metals (Zelikoff, 1993; Dunier & Siwicki, 1993), planar PCBs (Rice & Schlenk, 1995) and PAHs (Kelly-Reay & Weeks, 1994). With the present state of knowledge it is difficult to deduce functional patterns for different environmental contaminants concerning their effects on the fish immune system. There are very few compounds for which the exact molecular target and mechanism of immunotoxic

actions are known and indeed for many compounds there are probably multiple targets and mechanisms of action. As can be seen from investigations on metals, the results from different experiments do not necessarily suggest a common effect mechanism or are even contradictory. Therefore, further studies on effects of environmental contaminants on fish immune parameters should ideally be complemented with more detailed investigations into mechanisms, in order to supply vital information on exposure-effect patterns. More detailed insights into mechanisms of action could facilitate interpretation of effects on immune parameters measured in the course of monitoring environmental pollution and therefore enhance their relevance.

Experiments with water containing a mixture of pollutants

To date, only limited data is available on the effects of complex mixtures on immune stress of aquatic organisms. Studies have shown adverse immunological effects of pulp and paper mill effluents (Aaltonen et al., 2000a; Aaltonen et al., 1997; Jokinen et al., 1995; Fatima et al., 2001; Ahmad et al., 1998; Fournier et al., 1998; Fatima et al., 2000) and sewage sludge (Secombes et al., 1991; Secombes et al., 1992; Secombes et al., 1995) on the piscine immune system. To my knowledge, only two studies so far, have focused on effects of municipal effluent on immune parameters. In a cage experiment, Price and coworkers (1997) exposed carp (*C. carpio*) to river water receiving sewage treatment effluent for 47 days. The exposed fish displayed a significant reduction in proliferative responses of T- and B-lymphocytes, as well as a decrease in serum lysozyme activity, when compared with fish from a reference (high water quality) site. Exposure of goldfish (*C. auratus*) to 10 and 20 % treated sewage in a laboratory scale experiment for 30 days, resulted in a decrease in cardiac blood erythrocyte, granulocyte and lymphocyte numbers, as well as lower phagocytic activity of blood cells (Kakuta, 1997). In the same study, exposure to 5 % treated sewage moreover led to lower survival rates after challenge with *A. salmonicida*.

The assessment of numbers of melanomacrophage centres (MMC) in spleen and liver tissue has been suggested as a useful parameter to examine effects of complex pollution of surface water on the fish immune system (Wolke et al., 1985). Wolke and coworkers investigated MMC numbers, area, and pigment distribution in tissue samples from winter flounders (*P. americanus*), collected from Georges Bank (clean), the south shore of Long Island, New York, from Montauk to New York City (clean and polluted), and the Arthur Kill, New Jersey (polluted). The mean number and area of MMCs were greater in the spleens of fish from

polluted sites. Hemosiderin was also more prevalent. In the liver, only the size of aggregates was greater in fish from polluted sites. Gonadal maturity, sex, and the presence of gross lesions had no effect on the overall model. However, it was not clear, how much the results of this study were influenced by differences in water temperature at the various sites, prevalent during the study. Luebke (1997) investigated melano-macrophage centres (MMCs), as indicators for adverse effects of bleached kraft mill effluent and found that MMCs were more prevalent in fish from downstream sites.

Histological effects of sewage treatment plant effluents have been investigated in cage experiments with brown trout (*Salmo trutta*), showing a higher prevalence of necrosis, apoptosis, decreased numbers of mucous cells, decreased epidermal thickness, invasion of leucocytes and extension of melanocytes into the epidermis in the effluent exposed group compared to fish held in tap water (Burkhardt-Holm et al., 1997). Schmidt and coworkers (1999) moreover studied chronic effects of diluted wastewater on rainbow trout (*O. mykiss*). Macroscopically and histologically, only minor changes in gills, skin, and kidney of exposed animals were found compared to fish kept in tap water. Degenerative and inflammatory reactions in the liver of exposed animals were the most prominent findings.

Design of experiments

In order to gain a complete understanding of the immune status of a test fish, many immune parameters should be investigated that are representative of different components (innate and adaptive, as well as humoral and cellular reactions) of the immune system. Köllner and coworkers (2002) propose an experimental set up, which includes functional assays, such as activation and proliferation of leucocyte populations, macrophage phagocytosis and respiratory burst, secretion of antigen-specific antibodies, specific cell-mediated cytotoxicity, as well as challenge models with bacterial and viral pathogens. Investigating an activated immune system, as is the case in a challenge model, reflects the fact, that impacts of pollution of surface water on immune reactions are likely to impair resistance against opportunistic pathogens, leading to higher disease prevalence in fish, which in turn can lead to a decline in fish populations. With pollutants possibly leading to immunosuppression, effects of pollution on immune reactions within an activated immune system should be of higher relevance than impairment of an inactive system. To obtain comparable results, experiments should ideally be conducted during the same season and in the same temperature range, as immune reactions

in fish are strongly influenced by temperature (Le Morvan et al., 1998; Le Morvan-Rocher et al., 1995; Koellner et al., 2000).

In the thesis at hand, effects of sewage treatment water on the immune system and endocrine parameters of rainbow trout (*O. mykiss*) were investigated (chapter II and III). Moreover, the effects of two known immunomodulating substances, namely cortisol and rifampicin, on immune parameters in rainbow trout were examined to get an idea of the nature of measurable immunomodulations (chapter IV and V). In order to gain a clear image of the immune status of the test organisms a wide range of immune methods was used, covering the innate (unspecific), humoral and cellular branches of the immune system, as well as adaptive (specific), cellular and humoral immune reactions. Parameters investigated included blood cell differentials, macrophage activity, reflected in phagocytotic activity and production of reactive oxygen species (oxidative burst), serum lysozyme activity, lymphocyte proliferation and production of specific antibodies against the fish specific bacteria *A. salmonicida*, as well as distribution of white blood cell populations in spleen. “Infection” of fish with *A. salmonicida* also made possible the investigation of effects of pollution on an activated immune system.

Effects of acute exposure to treated sewage effluent on immune function of rainbow trout (*Oncorhynchus mykiss*)

The presence of low concentrations of anthropogenic substances, including pharmaceuticals, health care products and industrial chemicals in sewage treatment effluents and their respective receiving surface waters has raised concerns with regard to their potential adverse effects on the aquatic environment. Concerns have specifically been raised about potential immunomodulating activity of pollutants in wastewater effluents. In this study, immune reactions of rainbow trout (*O. mykiss*) have been examined, after exposure to 10, 30 and 70 % (v/v) sewage treatment effluent for 4 weeks. Immune parameters measured include blood cell differentials, serum lysozyme activity, lymphocyte proliferation, macrophage oxidative burst and phagocytotic activity, as well as *A. salmonicida* specific antibodies in serum. Additional to immune parameters, liver EROD activity was determined. The acute exposure of trout to the high effluent concentrations (30 and 70 %) decreased lymphocyte counts and led to a higher prevalence of degrading erythrocytes in peripheral blood samples. Other immune reactions, measured in this study, were not affected by exposure to effluent. In addition, an induction of microsomal enzymes (mixed-function oxygenase, MFO) in trout liver samples, evident through higher EROD activity, was observed.

Introduction

In recent years, sewage treatment plant effluents have been shown to be polluted with a wide range of anthropogenic substances, including pharmaceutical residues, substances contained in health care products and industrial chemicals (Ternes, 1998; Stumpf et al., 1999; Steger-Hartmann et al., 1999; Steger-Hartmann et al., 1997; Kummerer et al., 1998; Kummerer, 2000; Kummerer, 2001; Daughton & Ternes, 1999). The potential of some of the substances found in sewage, as well as in our surface waters to disrupt endocrine mechanisms in wildlife has raised concerns about possible effects of such contamination on animal and human health, as well as on the stability of our fish populations (Guillette & Guillette, 1996; Jobling et al., 1998). The presence of substances in sewage, which might also affect immune reactions has raised additional concerns. Impairment of immune mechanisms in aquatic organisms due to pollution of our surface waters might lead to reduced resistance against otherwise opportunistic pathogens. Therefore, along with endocrine disruption, effects of contaminants on immune reactions in aquatic organisms should be observed to assess potential immunosuppressing activity (Luebke et al., 1997).

In spite of the growing interest in the field of fish immunotoxicology, knowledge about effects of pollutants on the fish immune system is still sparse to date. Although effects of different substances on selected immune parameters in fish have been investigated (see general introduction, chapter I), no clear pattern for immunomodulating activity has been found. Especially mixed pollution exposure, e.g. in sewage treatment plant (STP) effluent, is difficult to assess and has hardly been investigated so far. Only few publications focus on effects of municipal STP effluent on the immune system of fish (Price et al., 1997; Kakuta, 1997). The field study by Price and coworkers has shown reduced lymphocyte proliferation and lower serum lysozyme activity in carp (*C. carpio*) exposed to river water in south-east England for 47 days compared to a clean water site. Reduced erythrocyte, granulocyte and lymphocyte numbers in cardiac blood, as well as lower blood cell phagocytic activity have been observed in goldfish (*C. auratus*), exposed to treated sewage in a laboratory experiment (Kakuta, 1997).

The study at hand has investigated acute effects of high concentrations of municipal STP effluent on the immune system of rainbow trout (*O. mykiss*) in a controlled lab situation to give a first insight into possible immunomodulating activity in this fish species. Injection of fish with an inactivated form of the fish specific pathogen *A. salmonicida salmonicida* (A.s.s.) should moreover simulate an activated immune system and consequently enable the investigation of effects of effluent on reactivity against a threatening disease. The use of inactivated A.s.s enables the investigation of immune reactions against the bacterial surface molecules, while death of the exposed animals, as could be expected in a challenge with a living pathogen, is avoided. Additional to the immune parameters, liver EROD activity was assessed as an indicator of alterations in microsomal enzyme activity (MFO).

Material and Methods

Experimental set up

To assess acute effects of high concentrations of sewage treatment water on the immune system, one year old rainbow trout (*O. mykiss*), which were purchased from Ngongataha hatchery (Ngongataha, New Zealand), were exposed to either de-chlorinated tap water or 10, 30 or 70 % STP effluent for 27 days. In order to enable assessment of immune competence within an activated immune system, trout were either injected i.p. with formaldehyde inactivated *Aeromonas salmonicida salmonicida*, strain MT 423 (reference strain, National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, Scotland), using a

concentration of 3 x 10^8 cells per 100 g body weight or with phosphate balanced salt solution (PBS) as a control for the injection, prior to the start of the experiment. Trout were held in aquaria throughout the experiment and water was exchanged daily by static renewal of 50 % of the water. Water parameters like conductivity, pH, temperature and dissolved oxygen were measured daily.

Antigen preparation of Aeromonas salmonicida salmonicida (A.s.s.)

A.s.s. strain MT 423, stored in cryotubes (Microbank^{TM}, PRO-LAB Diagnostics, UK), was used for experiments. The bacteria were incubated from cryoconserve in 5 mL sojapepton-casein-pepton-bouillon (CASO-bouillon; SIFIN, Germany) at 22°C for 98 h. After that, the cultures were checked for purity by microscope (gram stain, morphology) and subcultured on CASO-agar plates (SIFIN, Germany). 250 µL of A.s.s.-CASO-bouillon-culture was replanted to a CASO-agar plate and incubated at 22°C for 98 h. The A.s.s. colonies were harvested from 9 plates, resuspended and pooled in 150 mL sterile 0.9 % NaCl and homogenised using a magnetic stirrer. This A.s.s. bacteria pool was inactivated by adding 1.5 mL formaldehyde solution (35 %, DAB, Merck, Germany) and stirred for 60 min at room temperature. After centrifugation (10 min, 4300 rpm, 15°C) in a Hettich Universal 30 F centrifuge, the pellets were resuspended in sterile 0.9 % NaCl and washed twice to remove the formaldehyde. The cell suspension was adjusted to an optical density of 1.0 by adding sterile 0.9 % NaCl (λ=520 nm; Photometer PF-10; Machery-Nagel, Dueren, Germany), which corresponds to 1 x 10^{10} colony forming units (CFU)/mL. The formalin inactivated bacteria were stored in 1 mL aliquots at -20°C.

Sampling

After exposure to effluent for 27 days, fish were sampled on two subsequent days, whereby 5 A.s.s.-injected and 5 PBS-injected fish from every treatment group were used per day. Fish were anesthetised, weighed and their length determined. Peripheral blood was taken from the caudal vein and used for blood smears, to prepare lymphocytes used for a lymphocyte proliferation assay and to get serum samples for the determination of lysozyme activity and A.s.s. specific antibodies. Liver and spleen were dissected and weighed. Part of the head kidney was kept on ice in Leibovitz´s L-15 medium (Invitrogen, Auckland, New Zealand), containing 10 units (U) heparin sodium salt/mL (Sigma, St. Louis, USA) and 100 U/mL penicillin/streptomycin (Pen/Strep; Invitrogen, Auckland, New Zealand), until it was used to

prepare macrophages for phagocytosis and oxidative burst assay. Parts of the liver were kept for subsequent determination of 7-ethoxyresorufin (EROD) activity.

Differential white blood cell counts

Blood smears were covered with May Gruenwald solution (Applichem, Darmstadt, Germany) and incubated for 3 min, the staining solution on the slides was diluted with an equal amount of MQ-H_2O and incubated for another minute, followed by discharging of the staining solution. Giemsa solution (Applichem, Darmstadt, Germany) was diluted 1:20 in phosphate buffer, according to Soerensen (stock solution A (0.067 M KH_2PO_4) and stock solution B (0.067 M Na_2HPO_4 x $2H_2O$) mixed to a ratio of 1:1.23 and filtered through a paper filter). The slides were then covered with the prepared Giemsa solution and incubated for 15 min. Slides were washed thoroughly with MQ-H_2O and air dried. Pictures of the blood smears were later taken with a SV Micro Sound Vision colour camera (Sound Vision Inc, Boston, USA) on a microscope (Zeiss Axiolab) using Axio Vision Version 2.0.5. (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Per slide, a total amount of about 1500 cells were counted on the computer. The different blood cell populations were expressed in percentage of total cells counted.

Preparation of macrophages

The preparation of macrophages from the head kidney was performed according to Secombes (1990) using a Percoll gradient (Sigma, St. Louis, USA). Percoll was diluted with 10 x Hanks balanced salt solution (HBSS; Invitrogen, Auckland, New Zealand) (end concentration: 1 x HBSS) and MQ-H_2O to concentrations of 51 % or 34 %. In a 15 mL Falcon-tube 5 mL 34 % Percoll was layered carefully on 5 mL 51 % Percoll. Tissue was pushed through 100 µm nylon mesh using rubber policemen and rinsed with L-15 medium. The resulting cell suspension was carefully layered on a prepared Percoll-gradient and centrifuged at 400 x g for 25 min at 4°C. The white band at the gradient interface was collected and washed in L-15 medium. After centrifugation at 188 x g for 5 min (4°C) the cell pellet was resuspended in approximately 3 mL of L-15 medium. The cells were counted in a haemocytometer with trypan blue staining to determine viability. Cells were adjusted to a concentration of 1 x 10^6 cells/mL and seeded into 96-well black fluorometer plates (BMG Labtechnologies, Offenburg, Germany) at a density of 2.86 x 10^5 cells/cm^2. After incubation at 18°C for 90 min to allow adhesion, the cells were used for phagocytosis and oxidative burst assays.

Head kidney macrophage phagocytosis

After attachment of cells, media was removed by inverting the plate and drying it carefully on a paper towel. A volume of 100 µL of a 250 µg/mL fluorescein-labeled *E. coli* suspension (K-12 strain, Molecular Probes, Eugene, USA) was added to each well including eight blank wells, which did not contain macrophages. After incubation at 18°C for 2 h, the bacteria were removed by inverting the plate, followed by the addition of 100 µL trypan blue solution (0.025 %). The trypan solution was removed after 1 min of incubation and fluorescence was measured in a fluorescence plate reader (POLARstar Galaxy, BMG Labtechnologies, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters.

Head kidney macrophage oxidative burst

After macrophage attachment, media was removed by plate inversion and either 200 ng/mL PMA (Sigma, St. Louis, USA) in HBSS or HBSS alone was pipetted onto the cells. Measurement of the oxidative burst reaction was started 5 min later by the addition of 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, USA) at a concentration of 10 µg/mL. The time course of H_2O_2-production was measured in a fluorescence plate reader (485 nm excitation and 520 nm emission filters) by detecting the oxidation of H_2DCFDA to 2´,7´-dichlorofluorescein (DCF) over a time period of 15 min. The slope was obtained using the linear portion of the reaction curve. Results were calculated as pmol 2´,7´-dichlorofluorescein (DCF; Acros, Schwerte, Germany) produced per well and min.

Lymphocyte proliferation

The preparation of lymphocytes from rainbow trout peripheral blood follows the description of Karrow et al. (1999). Briefly, peripheral blood was taken from the caudal vein, transferred into a 15 mL tube and centrifuged at 200 x g for 4 min (4°C). The leucocyte buffy coat was collected and diluted to 7 mL with HBSS (5.36 mM KCl; 0.34 mM NaH_2PO_4; 0.44 mM KH_2PO_4; 145 mM NaCl; 10 mM Hepes in MQ-H_2O; pH 7.4) containing 10 U heparin sodium salt/mL (Sigma, St. Louis, USA). The cell suspension was layered on top of 3 mL of Ficoll Histopaque-1077 (Amersham Pharmacia Biotech, Auckland, New Zealand) and centrifuged at 400 x g for 30 min (9°C). Leucocytes at the interface were collected, washed three times and resuspended in L-15 medium (Invitrogen, Auckland, New Zealand). The cells were counted in a haemocytometer with the help of trypan blue dye exclusion, adjusted in L-15 medium, (containing 10 % fetal calf serum (FCS) and 100 U/mL Pen/Strep, both from Invitrogen,

Auckland, New Zealand) to a concentration of 0.5 x 10^6 cells/mL and seeded in 24-well cell culture-plates (1 mL/well) (Invitrogen, Auckland, New Zealand). After keeping the cells at 18°C for 3 h to enable attachment to the cell culture plate, the cells were stimulated with either 100 µg LPS/well (LPS from *E. coli* serotype 0111:B4, Sigma, USA) or 20 µg Concanavalin A/well (Con A; from *Canavalia ensiformis* Type VI, Sigma, USA). After stimulation of lymphocyte proliferation with mitogens the cells were kept in culture at 18°C for 72 h. After addition of 1µCi thymidine-methyl-^{3}H per well (Amersham Pharmacia Biotech, New Zealand), cells were incubated for another 18 h. Finally, the cells were vacuum-filtered on GF/C filter paper (Whatman, England). The filters were put into scintillation vials and 5 mL of scintillation cocktail (2L toluene, 1L Triton X-100, 18 mM 2,5-diphenyloxazole (PPO), 0.55 mM 1,4-bis[2-5-phenyloxazolyl]benzene (POPOP)) was added. Cell proliferation was measured in a Packard BioScience liquid scintillation analyzer Tri-Carb 2100 TR as decays per min (dpm).

Lysozyme activity in trout plasma

The method for measuring serum lysozyme follows the description of Ellis (1990) (turbimetric assay). It is based on the lysis of the gram-positive bacterium *Micrococcus lysodeikticus* by serum lysozyme. The lysis of the bacterium can be detected in a spectrophotometer at 530 nm as a decrease in optical density of the *M. lysodeikticus* solution. 950 µL *M. lysodeikticus* solution (Sigma, St. Louis, USA) (0.5 mg/mL in 0.05M sodium phosphate buffer (pH 6.2)) were pipetted in a cuvette and measured, followed by the addition of 50 µL of serum or plasma. Absorption was measured after 0.5, 1, 2, 3, 4 and 4.5 min, and lysozyme activity was expressed as decrease of optical density (OD) per min.

Aeromonas salmonicida specific antibody ELISA

For the ELISA, 96-well plates (Invitrogen, Auckland, New Zealand) were coated with formaldehyde inactivated *A. salmonicida* antigen (strain MT 423; 20 µg/mL in PBS), and plates were incubated overnight at 4°C. The plates were washed once with PBS and blocked with PBS containing 1 % ovalbumin (Applichem, Darmstadt, Germany) for 1 h at 20°C. Plates were washed, and serum samples (diluted 1:100 in washing buffer: PBS containing 0.1 % Tween 20) were pipetted onto the plates in triplicate. After incubation for 1 h at room temperature, plates were washed 3 times and incubated with a monoclonal mouse anti-trout IgM (4C10) for 1 h at 20°C. After washing, plates were incubated with goat-anti-mouse

IgG/IgM-POD conjugate (Pierce, Bonn, Germany), for 1 h at 20°C. The plates were then washed 3 times and incubated with tetramethylbenzidine (TMB) (Sigma, St. Louis, USA). The colour reaction was stopped by the addition of 0.5 M H_2SO_4 and absorption was measured at 450 nm in a plate reader (POLARstar Galaxy, BMG Labtechnologies, Offenburg, Germany).

Liver EROD activity

Hepatic MFO activity was estimated in post-mitochondrial supernatant (PMS) as 7-ethoxyresorufin-*O*-deethylase (EROD) activity, using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. (1999). Liver extracts were homogenized in a cryopreservative buffer (0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, and 20 % glycerol, pH 7.4) and spun at 9000 x g to obtain the PMS. The EROD reaction mixture contained 0.1 M Hepes buffer pH 7.8 (Sigma, St. Louis, MO, USA), 5.0 mM Mg^{++}, 0.5 mM NADPH (Applichem, Darmstadt, Germany), 1.5 µM 7-ethoxyresorufin (Sigma, St. Louis, MO, USA), and 0.5 mg/mL of PMS protein. The EROD activity was determined kinetically in 96-well plates using one reading every minute for 10 min on a fluorescence plate reader. Resorufin was determined using 544 nm excitation and 590 nm emission filters. Protein content was estimated from fluorescamine (Sigma, St. Louis, MO, USA) fluorescence (390 nm excitation, 460 nm emission filters) against bovine serum albumin (Sigma, St. Louis, MO, USA). EROD activity was calculated as pmol resorufin produced per min and mg protein, with the help of a resorufin standard curve.

Statistics

In every treatment group A.s.s.-injected and sham-injected fish were tested for significant differences using students´ t-test and samples were pooled, where no difference was found in order to provide more representative test groups. Data for all immune parameters were analysed using one-way analysis of variance (ANOVA) with Dunnett´s post test. EROD data did not conform to the assumptions of parametric analysis and were instead compared using a non-parametric Kruskal-Wallis one-way analysis of variance with Bonferroni adjustment for multiple comparisons. Statistical testing was performed using SAS JMP 4.0.4 (SAS Institute Inc., Heidelberg, Germany) and GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software. The level of confidence for all analyses was α=0.05.

Results

In the present study, the only immune parameter affected by exposure to STP effluent were differential blood cell counts. Trout exposed to 30 and 70 % effluent showed a tendency towards lower leucocyte counts in peripheral blood (fig. 1a).

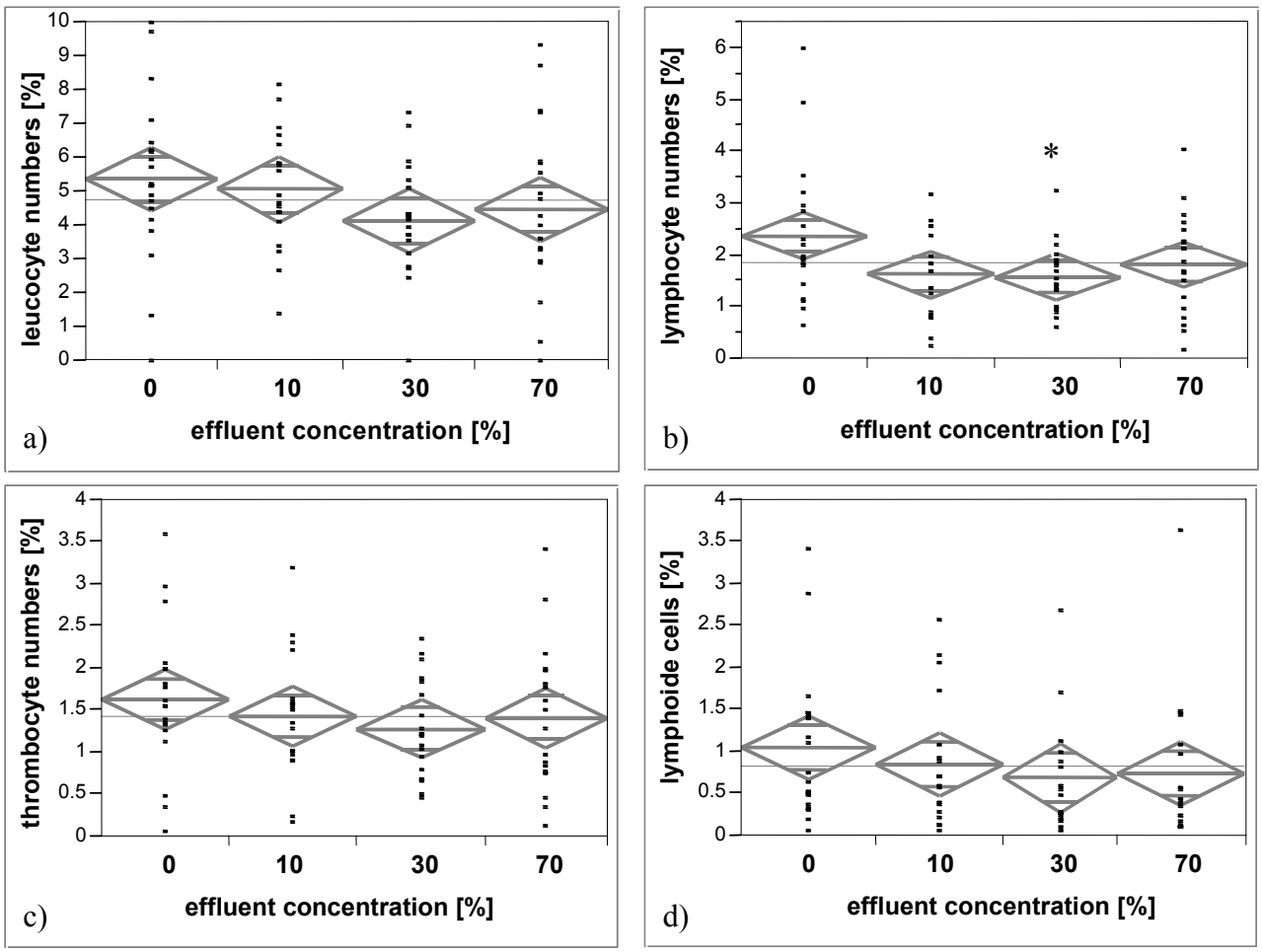


Fig. 1.: Peripheral blood leucocyte numbers in percent of total blood cells counted. A slight decrease in leucocyte numbers after exposure to effluent was observed (a). Lymphocyte numbers were significantly decreased after exposure to 30 % effluent (b). Thrombocyte (c) and lymphoid cell numbers (d) were slightly decreased after exposure to 30 and 70 % effluent. Mean diamonds illustrate sample means and 95 % confidence intervals, whereby the middle line represents the group mean and the vertical span shows the 95 % confidence interval. The horizontal size of the means diamonds represents the sample size. Data from A.s.s.-injected and sham-injected fish were pooled. Data was tested using one-way ANOVA with Dunnett´s post test. Sample size was $n \geq 19$. * $p \leq 0.05$.

Lymphocyte numbers in peripheral blood were lower in all groups exposed to effluent compared to fish held in de-chlorinated tap water, however, only the decrease in lymphocyte numbers in the 30 % effluent group was statistically significant compared to control fish (fig. 1b). Lower leucocyte numbers were mainly due to a decrease in lymphocytes, but thrombocytes and lymphoid cells also showed a tendency towards lower numbers (fig. 1c, d). The percentage of granulocytes in peripheral blood however slightly increased after exposure to effluent (data not shown). All groups exposed to effluent moreover showed a higher prevalence of degrading erythrocytes, with a marked increase in degrading erythrocytes after exposure to 30 and 70 % effluent (fig. 2). However, this effect was only statistically significant for the group exposed to 70 % effluent.

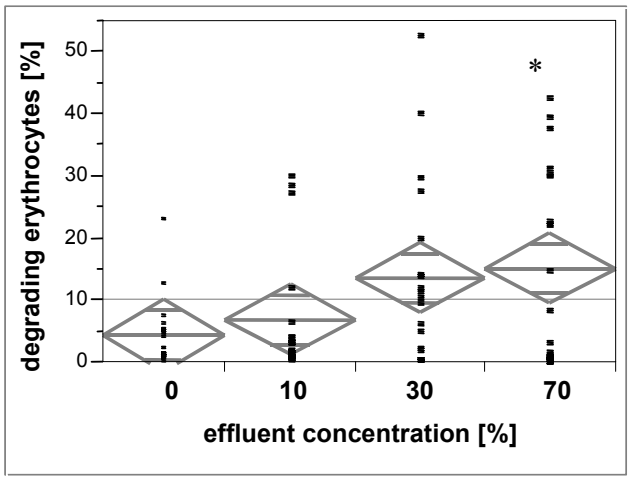


Fig. 2: Numbers of degrading erythrocytes in peripheral blood, given in percent of total blood cells counted. Data from A.s.s.-injected and sham-injected fish were pooled. Sample size was $n \geq 19$. Data was tested using one-way ANOVA with Dunnett´s post test. * $p \leq 0.05$.

Injection of trout with the inactivated fish pathogen *A. salmonicida* did not result in a consistent change in blood cell counts throughout the exposure groups. In most cases blood cell counts were not affected by the injection. However, in the control (tap water) group A.s.s.-injected fish showed significantly lower lymphocyte numbers in peripheral blood compared to sham-injected fish (fig. 3).

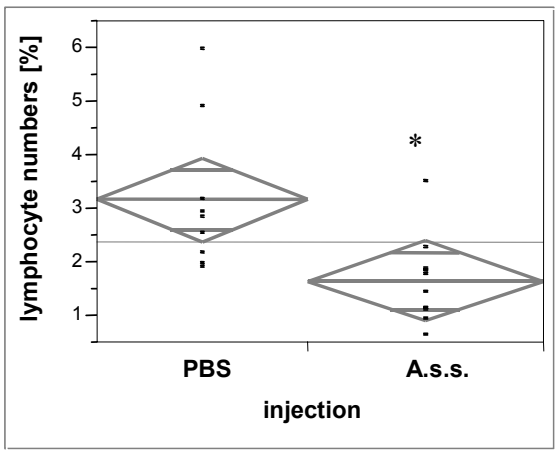


Fig. 3: Peripheral blood lymphocyte numbers in A.s.s.-injected control fish (kept in de-chlorinated tap water) were significantly lower than in sham-injected control fish. Data was tested using student´s t-test. $n \geq 9$; * $p \leq 0.05$.

Lymphocyte proliferation, macrophage activity (phagocytosis and oxidative burst), serum lysozyme activity and production of antibodies against *A. salmonicida* were not affected by acute exposure of rainbow trout to STP effluent. Injection of trout with the inactivated bacteria *A. salmonicida* did not influence any of those immune parameters compared to sham-injected fish.

Liver EROD activity showed a dose-dependent increase after exposure to 10 and 30 % effluent, however, only EROD activity in trout exposed to 30 % STP effluent was significantly higher compared to trout held in de-chlorinated tap water (fig. 4). Exposure to 70 % effluent resulted in only slightly higher liver EROD activity compared to control fish. Liver EROD activity was also not influenced by injection with inactivated *A. salmonicida*.

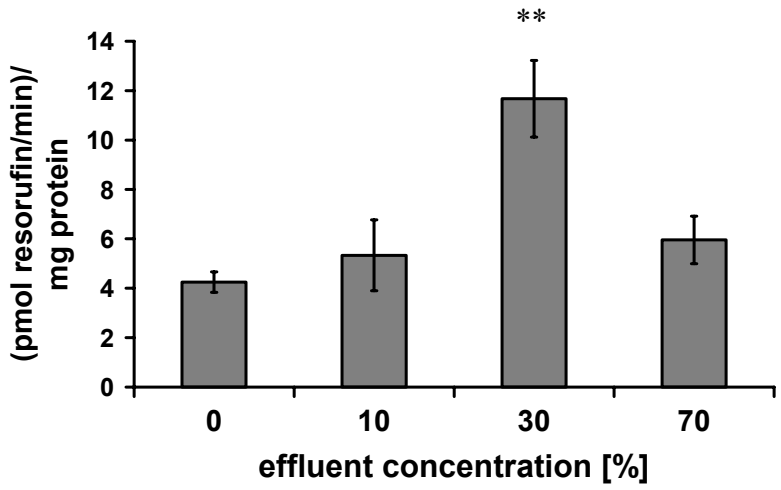


Fig. 4: Liver EROD activity, given as resorufin produced per min and mg protein. Shown are median values with standard error of the means (SEM). Data from A.s.s.-injected and sham-injected fish were pooled. Data was tested using Kruskal-Wallis non-parametric one-way ANOVA. $n \geq 16$; ** $p \leq 0.01$.

Discussion

Exposure of rainbow trout to high concentrations of municipal sewage treatment plant effluent in a controlled lab situation over 4 weeks, did not elicit any effects on lymphocyte proliferation, macrophage activity (phagocytosis and oxidative burst), serum lysozyme activity and production of antibodies against *A. salmonicida*. The only immune parameter, which was shown to be affected by exposure to STP effluent, were peripheral blood cell counts. Trout exposed to effluent showed a tendency towards lower leucocyte counts in peripheral blood. Lower leucocyte numbers were mainly due to a decrease in lymphocytes. A significant decrease in cell numbers could only be shown for lymphocytes in trout exposed to 30 % effluent, however the lack of a significant result in the 70 % group may be due to difficulties in analysing the blood smears. An unusual high prevalence of degrading erythrocytes in the fish exposed to 70 % effluent (and the resulting poor quality of the smears) impeded the identification of all cell types, including lymphocytes, possibly resulting in an over-estimation of lymphocyte counts in these individuals. Effects of exposure to sewage treatment water on blood cell differentials in salmonid fish have been observed previously (Hitzfeld et al., 2000; Prietz et al., 2000). Brown trout (*S. trutta forma fario*) exposed to water samples from different sewage treatment cleaning steps displayed significantly lower leucocyte counts in peripheral blood compared to tap water controls. In line with our results, the decreased leucocyte numbers found in this study were mainly due to lower lymphocyte counts, but thrombocyte numbers were also decreased. Lower numbers of granulocytes and lymphocytes have also been observed in goldfish (*C. auratus*) exposed to 10 and 20 % treated sewage in a laboratory scale experiment (Kakuta, 1997). A decrease in thrombocyte numbers has moreover been shown in dab (*L. limanda*) exposed to sewage sludge in seawater. However, in this study, total leucocyte numbers were not lower in exposed fish compared to controls, which might be due to higher neutrophil numbers (Secombes et al., 1991). Moreover, dab exposed to 16 % diesel oil-based drilling mud were shown to have significantly lower leucocyte numbers in peripheral blood (Tahir et al., 1993).

As mentioned above, trout exposed to high concentrations of STP effluent showed a markedly higher prevalence of degrading erythrocytes in their peripheral blood. Effects of wastewater effluent on erythrocytes in fish have been shown before. Male fathead minnows (*Pimephales promelas*) kept in a constructed wetland system showed decreased haematocrit values after exposure to high effluent concentrations at the inflow site, when compared to control fish or fish further downstream (Hemming et al., 2001). Kakuta (1997) also reported lower

erythrocyte numbers in peripheral blood of goldfish, exposed to treated sewage in a laboratory experiment. Lower haematocrit values are indicative of either degradation of peripheral blood erythrocytes or an active increase of plasma fluid. While measurement of haematocrit does not reveal the nature of the effect on erythrocytes, evaluation of blood smears, in the study at hand, showed sewage treatment effluent to cause degradation of erythrocytes. A possible cause for such an effect can be substances in sewage effluent, which affect ion transport in gills, possibly leading to a disturbed osmotic balance and consequently to swelling and bursting of erythrocytes.

Lymphocyte proliferation, serum lysozyme activity, head kidney macrophage activity and serum level of *A. salmonicida* specific antibodies were not influenced by acute exposure to sewage treatment effluent in the study at hand. In contrast to these results, Price et al. (1997) showed effects of sewage treatment plant effluent on lymphocyte proliferation and serum lysozyme activity. In their study, carp (*C. carpio*) exposed to effluent-receiving river water in south-east England displayed a significant reduction in proliferative responses of T- and B-lymphocytes and in serum lysozyme activity in comparison to a control (high water quality) site. Kakuta (1997) moreover observed a decrease of phagocytotic activity in blood cells of goldfish exposed to treated sewage. The lack of effects on macrophage activity, lymphocyte proliferation and serum lysozyme activity in our study, could be due to differences in the reactivity of the fish species used in the different studies, as well as varying composition of the investigated effluents. As it is difficult to achieve a detailed characterisation of effluent samples, which identifies all compounds possibly leading to adverse effects on immune parameters in fish, results of one study with effluent cannot be rated as indicative for the effects of other effluents. Moreover, detailed characterisations of effects of different substances or whole substance classes on the fish immune system are still lacking. Therefore, with the present knowledge in fish immunology, it is difficult to attribute certain changes in immune parameters to certain types of environmental pollution.

The assessment of effects on immune reactivity is further complicated by the dependence of certain immune parameters on the point of time chosen for their determination. As specific antibody production in trout is a very slow process, assessment of effects on this parameter, can be influenced by a relatively early sampling point of time. Production of *A. salmonicida* specific antibodies in rainbow trout has been shown to only start after 18 days, depending on the amount of bacteria injected, while considerable levels are observed as late as 30 days after challenge (Kollner et al., 2002). Serum antibody levels in the majority of the trout investigated after 28 days in the acute exposure study, were still very low, which indicates

that production of antibodies had only just started. Possible differences in serum antibody level between exposure groups, however, could most likely be observed only when considerable antibody levels are present.

Induction of liver EROD activity is widely used as an indicator for pollution and subsequent activation of detoxification mechanisms (cytochrome P450 1A1) in the vertebrate liver (Cousinou et al., 2000; Bucheli & Fent, 1995) and has mainly been linked to exposure to PAHs and PCBs (Rice & Schlenk, 1995; Stegeman & Hahn, 1994). In the present study, EROD activity in trout liver was observed to increase after exposure to sewage treatment effluent. In accordance with our results, increase in EROD activity has also been shown in roach (*Rutilus rutilus*) and carp (*C. carpio*) exposed to bleached kraft mill effluent and sewage treatment effluent, respectively (Aaltonen et al., 2000a; Sole et al., 2002). The lower level of EROD activity in fish exposed to the highest effluent concentration found in our study, is likely due to inhibition of P450 activity by higher concentrations of toxic substances in liver tissue. Such an inhibiting effect on EROD activity in fish has been demonstrated after application of high concentrations of PCBs *in vitro* and *in vivo* (Hahn et al., 1993; Gooch et al., 1989; Monosson & Stegeman, 1991), as well as in field studies with fish caught at contaminated sites (Elskus et al., 1989).

In general, investigations on effects of mixed substance pollution, like in sewage treatment plant effluents, on the immune system of fish are still sparse to date. Interpretation of results is moreover difficult, due to a lack of knowledge about substance-effect relations in fish immune system. More detailed characterisations of the fish immune system and mechanisms of immunotoxicity in fish are warranted. Establishment of patterns of action for different substance classes would moreover facilitate interpretation of (adverse) effects on immune parameters measured in aquatic organisms. Further studies on mixed pollution effects on immune parameters are necessary to allow statements about immunotoxic effects of polluted surface waters, such as rivers receiving sewage effluent, on the immune competence of exposed fish.

Effects of chronic exposure to treated sewage effluent on reproductive-endocrine and immune function of rainbow trout (*Oncorhynchus mykiss*)

Pollution of surface waters with man-made substances, including pharmaceuticals and pesticides, may lead to adverse effects on aquatic organisms. Potential hazard to fish populations could occur due to alterations of the reproductive system, as well as adverse effects on the immune system, the latter possibly leading to a decreased resistance against fish pathogens. This study focused on the effect of chronic exposure to sewage treatment plant (STP) effluent on the immune system of rainbow trout (*O. mykiss*). Trout were first exposed to 1.5 and 15 % final treated sewage effluent for 26 weeks. Plasma samples, for determination of sex steroid hormone levels and lysozyme activity, were then obtained and half of the fish were injected intra-peritoneally (i.p.) with inactivated *A. salmonicida salmonicida* (A.s.s.) to simulate an infection, while the other half was injected with PBS as control for the immune challenge. Exposure to effluent was continued for another 6 weeks, resulting in an overall exposure time of 32 weeks. Upon termination of the experiment, fish were investigated for effects on head kidney macrophage phagocytosis and oxidative burst, differential blood cell counts, serum lysozyme activity and A.s.s.-specific antibody production. Serum lysozyme activity was decreased in immature (male and female fish pooled) and mature female trout, but not in mature males, after 26 weeks of exposure to effluent. While, after 32 weeks, no effect on macrophage activity, blood cell counts and serum lysozyme activity could be found, A.s.s.-specific serum antibody level were decreased in effluent-exposed mature female trout.

In addition to immune parameters, general physiological and endocrine-reproductive parameters, as well as liver MFO activity was determined. Exposure to 15 % effluent was observed to increase liver and gonad weight in mature male and female fish, whereby the higher gonad weight in females was reflected in an increase in fecundity. Female trout, exposed to 15 % STP effluent moreover displayed a significant increase in plasma 17β-estradiol levels, while male fish showed lower 11-ketotestosterone levels. Liver MFO activity was shown to increase in mature male trout after exposure to 15 % effluent.

Introduction

The presence of a broad array of compounds in surface waters, largely from human waste, has been well documented. Municipal waste has been shown to contain pharmaceutical residues, substances found in household products, such as various derivatives of alkylphenols, as well as natural and synthetic hormones (Ternes, 1998; Halling-Sorensen et al., 1998; Daughton & Ternes, 1999; Jones et al., 2001; Lee et al., 1998; Bennie, 1999; Desbrow et al., 1998). Lately, the effects of such pollutants on endocrine regulation mechanisms and immune reactions in aquatic animals have gained a great deal of attention within the field of environmental toxicology. Several of the pollutants found in our surface waters have been demonstrated to display hormonal or immunomodulating activity (Desbrow et al., 1998; Ashfield et al., 1998; Metcalfe et al., 2000; Payne & Fancey, 1989; Faisal & Huggett, 1993; Lunden & Bylund, 2002; Jobling & Sumpter, 1993). The potential of such substances to disrupt endocrine regulation mechanisms or immune reactions in aquatic animals, can lead to impairment of reproductive performance and immune competence respectively and consequently impede the growth and survival of our fish populations.

The effects of contaminants on the immune system of fish have been demonstrated with a wide variety of chemicals. Some of the chemical classes shown to alter certain immune parameters include polycyclic aromatic hydrocarbons (PAHs) (Payne & Fancey, 1989; Faisal & Huggett, 1993), pesticides (Dunier & Siwicki, 1993), metals (Anderson et al., 1989; Zelikoff et al., 1995), antibiotics (Lundén & Bylund, 2000) and organochlorines (Smith et al., 1999). The natural stress hormone, cortisol, and the reproductive hormone estradiol are also well known immunosuppressors (Verburg-van Kemenade et al., 1999; Wang & Belosevic, 1995).

Changes to immune physiology of fishes in response to complex effluents, such as municipal sewage, have not been examined to the same extent as reproductive effects. In the only study examining exposure to wastewater in the field, caged carp (*C. carpio*) were exposed to river water receiving sewage treatment effluent (Price et al., 1997). Exposed fish displayed a significant reduction in proliferative responses of T- and B-lymphocytes, as well as a decrease in serum lysozyme activity in comparison to fish from a reference site. In a laboratory experiment, exposure of goldfish (*C. auratus*) to treated sewage resulted in a decrease in erythrocyte, granulocyte and lymphocyte numbers in cardiac blood, as well as lower blood cell phagocytotic activity (Kakuta, 1997).

The study of immune effects in aquatic biota, after exposure to complex effluent, has in part been inhibited by the extremely complex nature of the immune system. This is further complicated by the close interactions of immune, stress and endocrine-reproductive functions, and with the logistical and technical difficulties with measuring immunological performance. As a result of the multiple levels of immune protection, a variety of methods from both the innate and humoral immune mechanisms should be used in order to cover a wide range of essential immune reactions. Furthermore, many studies using chemical exposure have examined immune function in an unchallenged state. The relevance of these measurements to the level of disease protection is unknown. Comparison of immune function with and without a pathogen challenge may provide a better indication of the integrity of the immune system (Kollner et al., 2002).

Impairment of endocrine regulation mechanisms in aquatic animals has been demonstrated in numerous studies within the last few years (Jobling et al., 1996, 1998; Crain et al., 1997). Indicators used to detect effects of hormonally active substances in sewage effluent on fish, include induction of plasma vitellogenin (vtg) (Folmar et al., 1996; Harries et al., 1997), alterations in steroid hormone levels (Folmar et al., 1996, 2001) and the occurrence of intersex (Allen et al., 1999; Jobling et al., 1998). Additional to the assessment of endocrine disrupting activity in wastewater, measurement of MFO activity is widely used to determine influences of aquatic pollutants on detoxification mechanisms.

The aim of the present study was to determine if exposure to a sewage effluent from a modern wastewater treatment plant affects immune integrity of rainbow trout (*O. mykiss*). Effects on endocrine-reproductive parameters and MFO activity were investigated to complement possible observations of immunotoxicological effects. In order to provide relevance and eliminate confounding environmental factors such as energy intake, long-term exposure in a mesocosm facility was chosen as the method of exposure. Two year old rainbow trout were exposed to reference water, 1.5 % (v/v) and 15 % (v/v) secondary-treated effluent for 32 weeks, corresponding to the period of reproductive development. Six weeks prior to the termination of the experiment, trout were injected with an inactivated fish pathogen (*A. salmonicida*) to initiate an immunological stimulation. Physiological and biochemical measures of immune, reproductive and general fish condition and growth were measured upon completion of the experiment.

Material and Methods

Fish

For the experiment two year old rainbow trout (*O. mykiss*), which were hatched at Forest Research Institute, were used. Parent fish were purchased from Ngongataha hatchery, Ngongataha, New Zealand. Fish were held in 12,000 L tanks (exposure tanks 1-6, see fig. 5), each containing 50 individuals, and were fed daily with commercial aquaculture feed pellets (Reliance stock food, Dunedin, New Zealand) at a ration of 0.7 % of the wet body weight and the amount was increased at 25 g per month as per the known growth trajectory with fish of this size. All trout were tagged with individually numbered T-bar type tags (HallPrint Pty Ltd, Holden Hill, SA, Australia), weighed and measured at the start of the experiment. Rainbow trout from the strain used in the experiment at hand are known to mature at age 2-3. Thus, maturity at the end of the experiment was assumed for the majority of fish, enabling the assessment of reproductive / endocrine parameters and differentiation of immune reactivity between mature male and female fish.

Sewage treatment plant effluent

Final treated effluent was obtained from a sewage treatment plant (STP) located in Rotorua, New Zealand (fig. 5). This STP employs a pre-treatment step with stop screens and a grit trap, a primary treatment step with sedimentation and secondary activated sludge treatment (Bardenpho Reactor). Subsequently, after going through a plant effluent balance pond, the final treated effluent is pumped to wastewater effluent land treatment holding ponds from where it is sprayed into the forest. Effluent holding tanks at the Forest Research exposure facility were refilled with final treated effluent, collected from the final effluent holding pond with a tanker truck, every second day.

Experimental set up

Trout exposure facility

In the trout exposure experiment activated carbon de-chlorinated tap water was used as the diluent and as the reference treatment (aquifer source) (fig. 5). Water flow was controlled from line pressure using stainless steel globe valves and spring-operated flowmeters. Effluent flow was controlled using a head tank with an overflow to maintain constant pressure, in combination with a PVC orifice calibrated for the nominal flow.

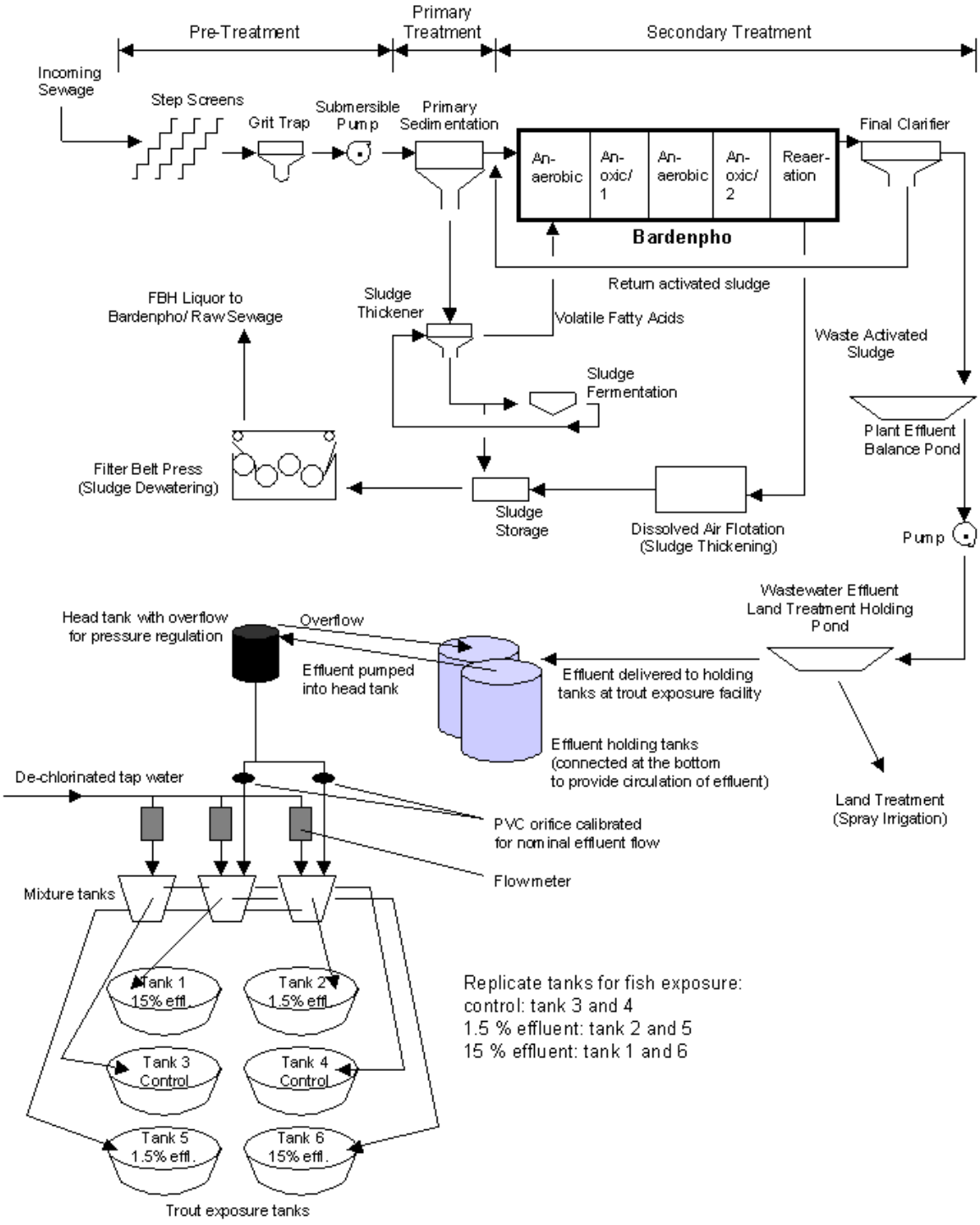


Fig. 5: Schematic overview of wastewater treatment in the Rotorua municipal sewage treatment plant and set up of the trout exposure facility at Forest Research.

Diluent flows were adjusted daily when necessary, and the effluent control orifices were cleaned daily to prevent reduction of flow by bio-fouling. Two replicate fish tanks were used for each treatment (control: tank 3 and 4; 1.5 % effluent: tank 2 and 5; 15 % effluent: tank 1 and 6; see fig. 5). Trout exposure tanks were provided with a constant water flow of 20 L/min, which resulted in a 95 % replacement time of approximately 30 h.

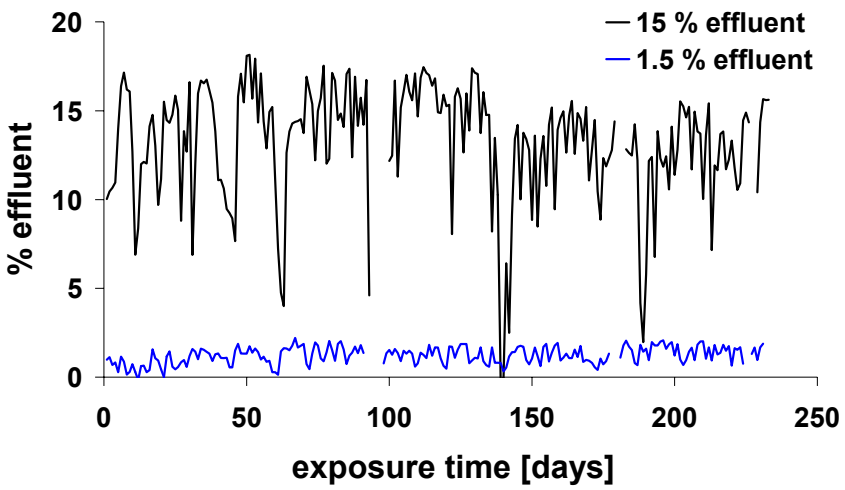
Water parameters

Dissolved oxygen, pH and conductivity (Radiometer Pacific, Auckland, New Zealand) in the fish exposure tanks and in undiluted effluent were measured daily. Additional aeration was provided in the effluent holding tanks and the trout exposure tanks and dissolved oxygen was maintained above 90 % saturation for the duration of the experiment**.** The average pH-values in the exposure tanks were 7.21 ± 0.25 and 7.22 ± 0.25 in the 15% effluent tanks, 7.15 ± 0.28 and 7.20 ± 0.28 in the 1.5% effluent tanks and 7.13 ± 0.29 and 7.13 ± 0.28 in the control tanks. Conductivity in each tank, diluent conductivity, and effluent conductivity was used to calculate the actual effluent concentration in the fish ponds on a daily basis. Temperature was measured hourly using Onset Tidbit temperature loggers (Onset Computer Corp, Bourne, MA, USA). Measured temperature and effluent concentrations over the duration of the experiment are shown in figure 6.

Exposure

Trout were exposed to a nominal concentration of either 1.5 (v/v) or 15 % (v/v) effluent. Control fish were kept in de-chlorinated tap water. The exposure was started on September 22, 2001 and was terminated between May 6 and 14, 2002. After exposure for 26 weeks, trout were anesthetised with ethyl-3-aminobenzoate methanosulfonate (MS222; Fluka, Switzerland) and 1 mL of blood was taken by syringe from the caudal vein. Fish were then either injected with formaldehyde inactivated *A. salmonicida salmonicida* (A.s.s.), strain MT 423 using a concentration of 1 x 10^8 cells per 100 g body weight, or with PBS as a control for the injection. Fish were exposed for a further 6 weeks until they were sampled.

a)



b)

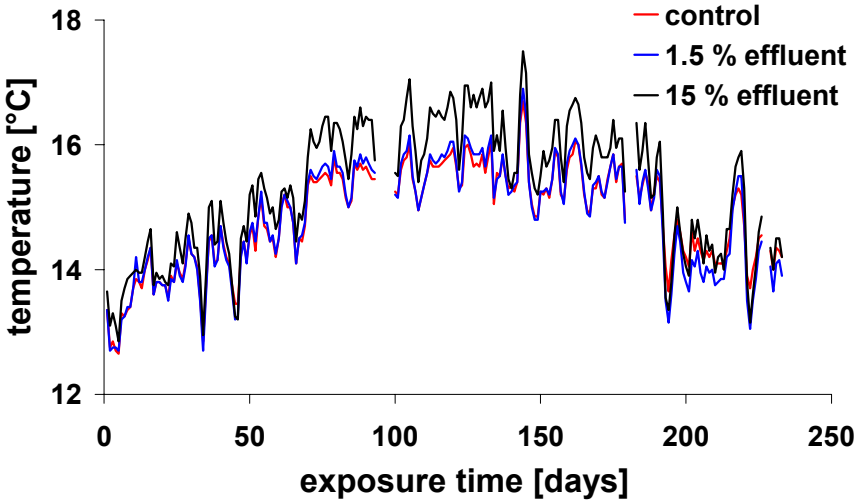


Fig. 6: Graph a) shows effluent concentration in the exposure tanks, given as average of replicate tanks. Percentage of effluent was calculated with the help of conductivity measurements. Graph b) shows temperature in the trout exposure tanks, given as average of replicate tanks.

Antigen preparation of Aeromonas salmonicida salmonicida (A.s.s.)

A.s.s. strain MT 423 (reference strain, NCIMB, Aberdeen, Scotland), stored in cryotubes (Microbank^{TM}, PRO-LAB Diagnostics, UK), was used for experiments. The bacteria were incubated from cryoconserve in 5 mL sojapepton-casein-pepton-bouillon (CASO-bouillon;

SIFIN, Germany) at 22°C for 98 h. Subsequently, cultures were checked for purity by microscope (gram stain, morphology) and subcultured on CASO-agar plates (SIFIN, Germany). 250 µL of A.s.s.-CASO-bouillon-culture was replanted to a CASO-agar plate and incubated at 22°C for 98 h. The A.s.s. colonies were harvested from 9 plates, resuspended and pooled in 150 mL sterile 0.9 % NaCl and homogenised using a magnetic stirrer. This A.s.s. bacteria pool was inactivated by adding 1.5 mL formaldehyde solution (35 %, DAB, Merck, Germany) and stirred for 60 min at room temperature. After centrifugation (10 min, 4300 rpm, 15°C) in a Hettich Universal 30 F centrifuge, the pellets were resuspended in sterile 0.9 % NaCl and washed twice to remove the formaldehyde. The cell suspension was adjusted to an optical density of 1.0 by adding sterile 0.9 % NaCl (λ=520 nm; Photometer PF-10; Machery-Nagel, Dueren, Germany), which corresponds to 1 x 10^{10} CFU/mL. The formalin inactivated bacteria were stored in 1 mL aliquots at -20°C.

Sampling

Female trout were sampled first over two subsequent days by taking out 3 A.s.s.-injected and 3 PBS-injected fish from each tank per day, which resulted in a total of 24 female trout sampled per treatment. Male fish were sampled 7 days later following the same sampling scheme. Blood was removed by caudal puncture and a blood smear was prepared immediately. Blood for serum samples was collected in untreated vacutainers. Blood for plasma was collected in heparinised vacutainers. Blood samples were kept on ice and later spun at 1000 x g (10 min, 4°C) to obtain plasma or serum. Plasma or serum samples were frozen at –80 ºC pending analysis. Body weight, length, liver weight, spleen weight and gonad weight were recorded. Thymus and parts of head kidney, spleen and liver were snap-frozen in liquid nitrogen and stored at -80°C for histology. A 3-5 g sample of ovary for fecundity analysis was weighed and then frozen until egg counts could be made. Head kidney for macrophage preparation was collected and kept on ice in Leibovitz´s L-15-medium (Invitrogen, Auckland, New Zealand) containing 53 mU heparin sodium salt/mL (Sigma, St. Louis, USA; dissolved in HBSS and sterile-filtered) and a mixture of Pen/Strep (Invitrogen, Auckland, New Zealand). A 1 g sample of liver was frozen in liquid nitrogen for the determination of 7-ethoxyresorufin-*O*-deethylase (EROD) activity.

Remaining trout, including immature fish, were sacrificed one week later, to assess gender, state of sexual development, body weight, length, as well as spleen and liver weight.

Condition factor and organ weights

Fulton's condition factor was calculated as body weight ÷ length^3 *100, gonado-somatic index (GSI) as gonad weight ÷ (body weight - gonad weight)*100, liver-somatic index (LSI) as liver weight ÷ (body weight - liver weight)*100, and spleen somatic index (SSI) as spleen weight ÷ (body weight - spleen weight)*100. Only maturing trout were used in the calculation of GSI. For comparison purposes, fecundity was calculated as number of eggs per kg of wet body weight.

Differential blood cell counts

Blood smears were covered with May Gruenwald solution (Applichem, Darmstadt, Germany) and incubated for 3 min. The staining solution on the slides was then diluted with an equal amount of MQ-H_2O and incubated for 1 min, followed by discharging of the staining solution. Giemsa solution (Applichem, Darmstadt, Germany) was diluted 1:20 in phosphate buffer, according to Soerensen (stock solution A (67 mM KH_2PO_4) and stock solution B (67 mM Na_2HPO_4 x $2H_2O$) mixed to a ratio of 1 to 1.23 and filtered through a paper filter). The slides were then covered with the prepared Giemsa solution and incubated for 15 min. Slides were washed thoroughly with MQ-H_2O and air dried. Pictures of the blood smears were later taken with a SV Micro Sound Vision colour camera (Sound Vision Inc, Boston, USA) on a microscope (Zeiss Axiolab) using Axio Vision Version 2.0.5. (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). A total amount of approximately 1500 cells per slide was counted on the computer. The different blood cell populations were expressed in percent of total cells counted.

Preparation of macrophages

The preparation of macrophages from the head kidney was performed according to Secombes (1990) using a Percoll gradient (Sigma, St. Louis, USA). Percoll was diluted with 10 x HBSS (Invitrogen, Auckland, New Zealand) (end concentration: 1 x HBSS) and MQ-H_2O to a concentration of 51 % and 34 % respectively. In a 15 mL Falcon-tube 5 mL of 34 % Percoll was layered carefully on 5 mL of 51 % Percoll. Tissue was pushed through 100 µm nylon mesh using a rubber policeman and rinsed with L-15 medium. The resulting cell suspension was carefully layered on a prepared Percoll-gradient and centrifuged at 400 x g for 25 min at 4°C. The white band at the gradient interface was collected and washed in L-15 medium. After centrifugation at 188 x g for 5 min (4°C) the cell pellet was resuspended in

approximately 3 mL of L-15 medium. The cells were counted in a haemocytometer with trypan blue staining to determine viability. Cells were adjusted to a concentration of 1 x 10^6 cells/mL and seeded into 96-well black fluorometer plates (BMG Labtechnologies, Offenburg, Germany) at a density of 1 x 10^5 cells per well (2.86 x 10^5 cells/cm^2). After incubation at 18°C for 90 min to allow attachment, cells were used for phagocytosis and oxidative burst assays.

Head kidney macrophage phagocytosis

After attachment of the cells, medium was removed by inverting the plate and careful drying on a paper towel. A volume of 100 µL of a 250 µg/mL fluorescein-labeled *E. coli* (K-12 strain, Molecular Probes, Eugene, USA) suspension was added to each well including eight blank wells, which did not contain macrophages. After incubation at 18°C for 2 h the particles were removed by inverting the plate, followed by the addition of 100 µL trypan blue solution (0.025 %). The trypan blue solution was removed after 1 min of incubation and fluorescence was measured in a fluorescence plate reader (POLARstar Galaxy, BMG Labtechnologies, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters.

Head kidney macrophage oxidative burst

After macrophage attachment, medium was removed by plate inversion and either 200 ng/mL PMA (Sigma, St. Louis, USA) in HBSS or HBSS alone was pipetted onto the cells. Measurement of the oxidative burst reaction was started 5 min later by the addition of H_2DCFDA (Molecular Probes, Eugene, USA) at a concentration of 10 µg/mL. The time course of H_2O_2-production was measured in a fluorescence plate reader (485 nm excitation and 520 nm emission filters) by detecting the oxidation of H_2DCFDA to DCF over a time period of 15 min. The slope was obtained using the linear portion of the reaction curve, usually 4 to 10 min. Results were calculated as pmol DCF produced per well and min, using a DCF (Acros, Schwerte, Germany) standard curve.

Serum lysozyme activity

The method for measuring serum lysozyme follows the description of Ellis (1990) (turbimetric assay). It is based on the lysis of the gram-positive bacterium *M. lysodeikticus* by serum lysozyme. Lysis of the bacteria can be detected in a spectrophotometer at 530 nm as a decrease in optical density (OD) of the *M. lysodeikticus* solution. 950 µL *M. lysodeikticus* solution (Sigma, St. Louis, USA) (0.5 mg/mL in 0.05 M sodium phosphate buffer, pH 6.2) were pipetted into a cuvette and measured, followed by the addition of 50 µL of serum or plasma. Absorption was measured after 0.5, 1, 2, 3, 4 and 4.5 min and lysozyme activity was expressed as decrease of OD per min.

Aeromonas salmonicida specific antibody ELISA

For the ELISA, 96-well plates (Invitrogen, Auckland, New Zealand) were coated with formaldehyde inactivated *A. salmonicida* antigen (20 µg/mL in PBS; A.s.s., strain MT 423), and plates were incubated overnight at 4°C. The plates were washed once with PBS and blocked with PBS containing 1% ovalbumin (Applichem, Darmstadt, Germany) for 1 h at 20°C. Plates were washed once with PBS, and serum samples (diluted 1:4000 in washing buffer: PBS containing 0.1% Tween 20) were pipetted onto the plates in triplicate. After incubation for 1 h at room temperature, plates were washed 3 times and incubated with a monoclonal mouse anti-trout IgM (4C10) for 1 h at 20°C. After washing, plates were incubated with goat-anti-mouse IgG/IgM-POD conjugate (Pierce, Bonn, Germany), for 1 h at 20°C. The plates were then washed 3 times and incubated with TMB (Sigma, St. Louis, USA). The colour reaction was stopped by the addition of 1 M H_2SO_4 and absorption was measured at 450 nm in a SLT plate reader 340 ATTC (SLT Labinstruments, Groedig, Austria). As no standards were available for IgM determination, results are given in optical density (OD) measured. To enable comparison without a standard curve all samples were measured in parallel in a single ELISA run. To test the reliability of the test system the ELISA was repeated twice.

Liver EROD activity

Hepatic MFO activity was assessed in post-mitochondrial supernatant (PMS) as EROD activity using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al. (1999). Liver extracts were homogenised in a cryopreservative buffer (0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, and 20 % glycerol, pH 7.4) and spun at 9000 x g to obtain PMS. The EROD reaction mixture contained 0.1 M Hepes buffer pH 7.8 (Sigma, St. Louis, MO, USA), 5.0 mM Mg^{++}, 0.5 mM NADPH (Applichem, Darmstadt, Germany), 1.5 µM 7-ethoxyresorufin (Sigma, St. Louis, MO, USA), and 0.5 mg/mL of PMS protein. The EROD activity was determined kinetically in 96-well plates using one reading every minute for 10 min on a fluorescence plate reader. Resorufin was determined using 544 nm excitation and 590 nm emission filters. Protein content was estimated from fluorescamine (Sigma, St. Louis, USA) fluorescence (390 nm excitation, 460 nm emission filters) against bovine serum albumin (Sigma, St. Louis, USA).

Plasma steroid and serum vitellogenin levels

Steroid hormones were measured by standard radioimmunoassay procedures according to McMaster et al. (1992). Plasma samples were thawed and steroid hormones were extracted with diethyl ether. The steroids testosterone, estradiol, and 11-ketotestosterone (11-KT) were obtained from Sigma (St. Louis, MO, USA). Testosterone and estradiol antibodies were obtained from ICN (Costa Mesa, CA, USA) and 11-KT antibody from Helix Biotech (Vancouver, BC, Canada). Tritiated testosterone and estradiol were obtained from Amersham Life Science (Little Chalfont, Buckhamshire, England) and tritiated 11-KT was purchased from the U.S. Geological Service (Dr. Tim Gross, Florida, USA). The plasma extract from females was analysed for estradiol and testosterone while that from males was analysed for 11-ketotestosterone and testosterone. Plasma vitellogenin (vtg) was measured with a specific rainbow trout vitellogenin EIA kit (Biosense Laboratories, Bergen, Norway).

Statistics

All results were tested for statistical differences between A.s.s.- and sham-injected fish, using student´s t-test. Where no difference due to injection was found, results from both groups were pooled to obtain more representative test groups. Condition factor, liver size, gonad size and fecundity data were analysed using analysis of covariance (ANCOVA) on base-10 logarithmically transformed variables, with body size (length or weight) as the covariate. It

should be noted that although statistical comparisons using ANCOVA were completed on body weight, liver weight, spleen weight and gonad weight and also total fecundity, data are presented as somatic indices for ease of comparison. Growth data and steroid data were compared using analysis of variance (ANOVA) after log-transformation of data where significant heteroscedasticity or departures from normality were observed. Initially, the variability of replicate tanks was tested as a variable in all ANOVAs. Replicate tanks did not add significant variability for any of the endpoints with the exception of growth. Thus, for most endpoints with the exception of growth, individuals from replicate tanks were pooled for all further analyses. Where significant growth variability occurred due to exposure tank, this variable was retained as a factor in the ANOVA when testing treatment-related effects. Dunnett's test was used for post-hoc comparisons of treatment groups to the reference group. EROD and vitellogenin data did not conform to the assumptions of parametric analysis after log transformations and were instead compared using a non-parametric Kruskal-Wallis one-way analysis of variance with Bonferroni adjustment for multiple comparisons. All statistical testing was completed using the SYSTAT® software package (Wilkinson, 1990). The level of confidence used in determining statistical differences for all analyses was α=0.05.

Results

Organ weights and condition factor

Length, body weight and organ weights for liver, spleen and gonad or testis respectively were measured for all mature fish in the fishponds. Condition factors and relative liver somatic index, relative spleen somatic index as well as relative gonad somatic index were calculated for males and females separately. As A.s.s.-injection did not influence any of these parameters, results for A.s.s.- and sham-injected fish were pooled. After exposure to 15 % effluent for 32 weeks, mature trout of both sexes showed significantly higher liver weights as well as significantly higher gonad weights compared to control fish (fig. 7 a-d).

Higher gonad weight in female fish was due to maturation of a higher number of eggs per body weight (higher fecundity), compared to control fish (fig. 7e). There was no significant effect of exposure to 1.5 % effluent on those parameters and exposure to either concentration of effluent did not have an effect on condition factor and spleen weight (data not shown).

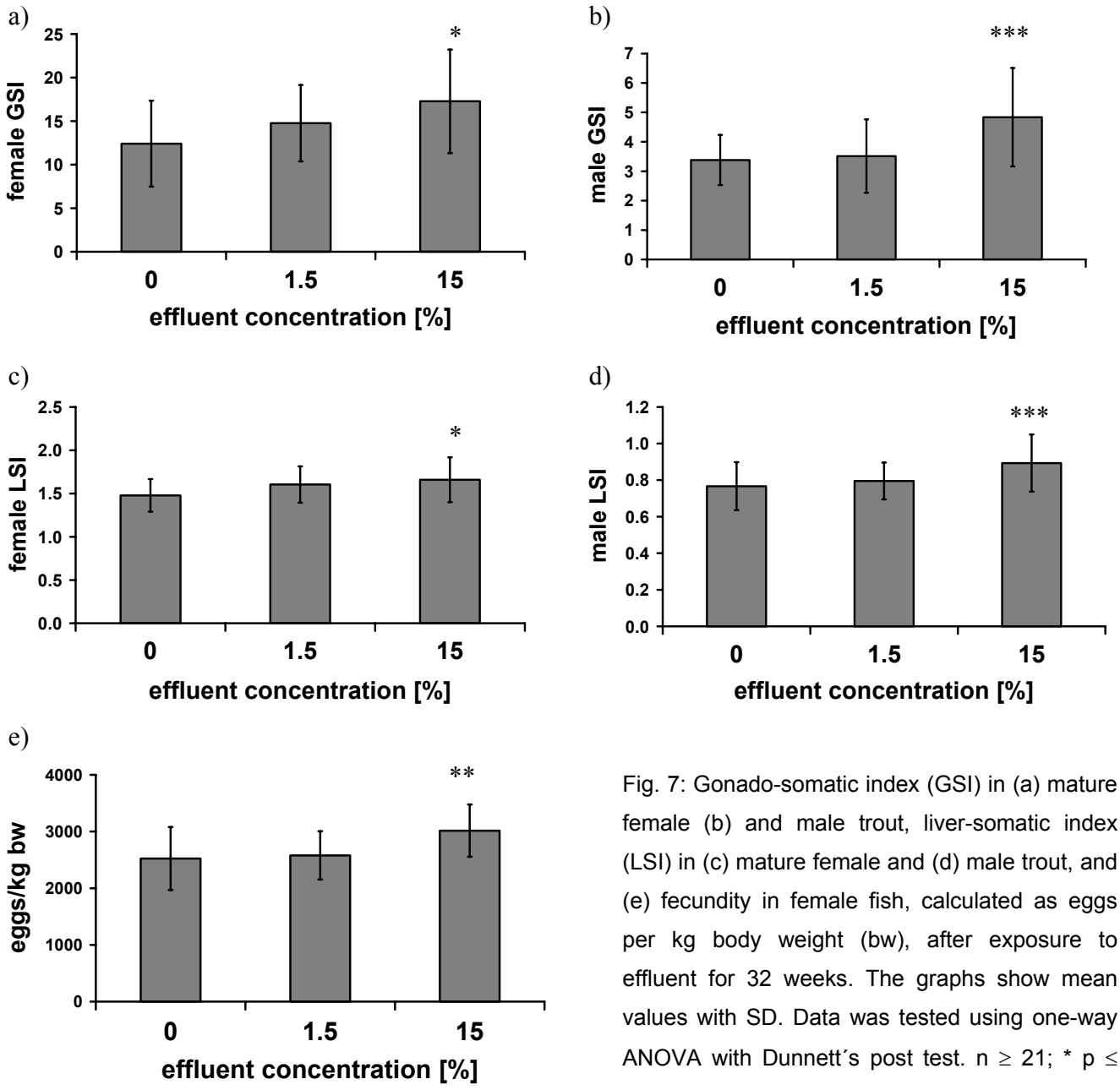


Fig. 7: Gonado-somatic index (GSI) in (a) mature female (b) and male trout, liver-somatic index (LSI) in (c) mature female and (d) male trout, and (e) fecundity in female fish, calculated as eggs per kg body weight (bw), after exposure to effluent for 32 weeks. The graphs show mean values with SD. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 21$; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Plasma steroid level

Exposure of rainbow trout to 1.5 % and 15 % sewage treatment effluent for 26 weeks led to significantly higher plasma estradiol levels in female fish compared to control fish (fig. 8a). After exposure for 32 weeks, plasma estradiol levels were still significantly elevated in female trout exposed to 15 % effluent (fig 8a). Plasma testosterone levels were only significantly raised in female fish after exposure to 1.5 % effluent for 26 weeks (fig. 8b).

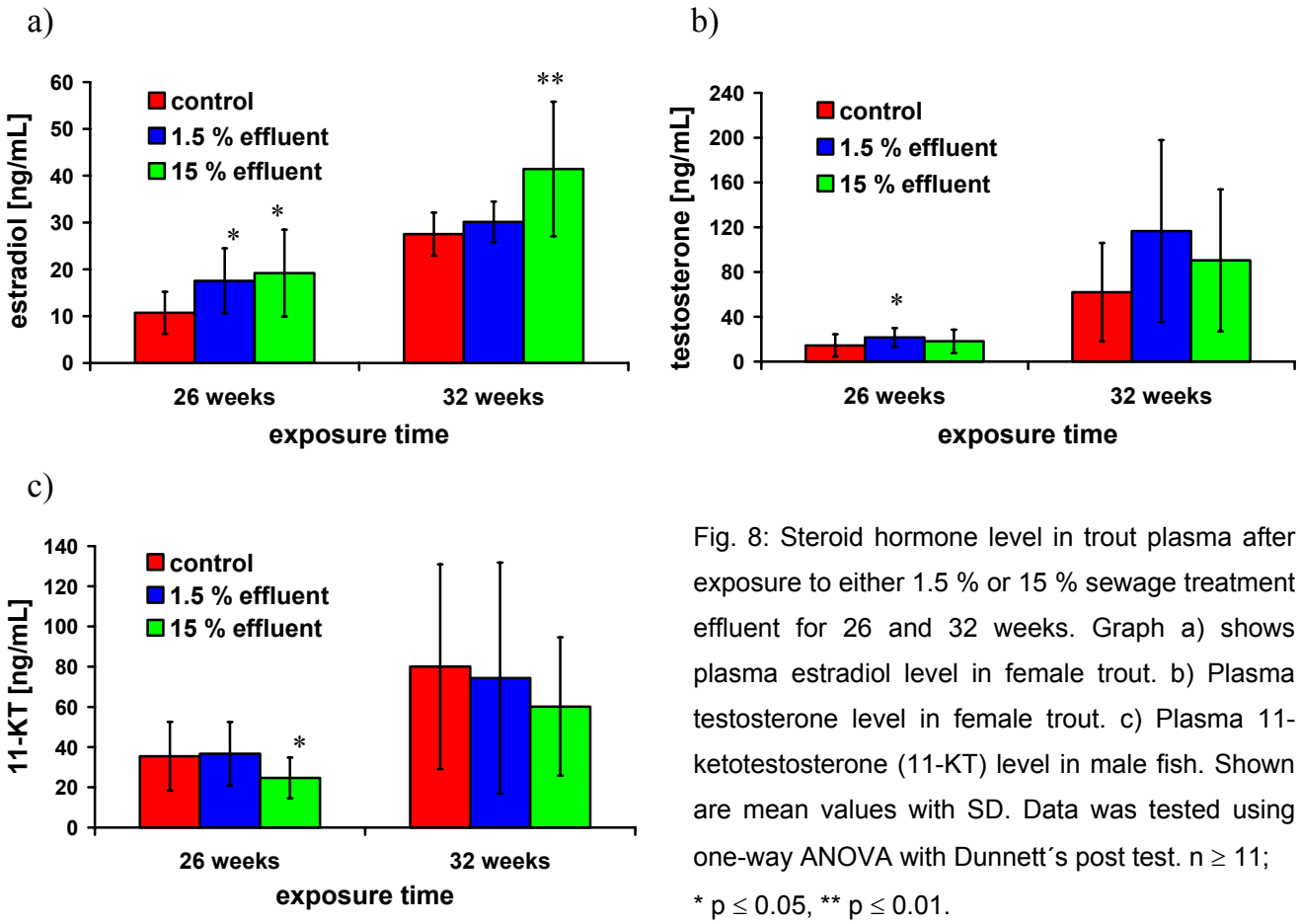


Fig. 8: Steroid hormone level in trout plasma after exposure to either 1.5 % or 15 % sewage treatment effluent for 26 and 32 weeks. Graph a) shows plasma estradiol level in female trout. b) Plasma testosterone level in female trout. c) Plasma 11-ketotestosterone (11-KT) level in male fish. Shown are mean values with SD. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 11$; * $p \leq 0.05$, ** $p \leq 0.01$.

In male fish exposure to effluent did not change plasma testosterone levels significantly (data not shown), but led to a decrease in 11-KT (fig. 8c). However, this decrease in 11-KT was only statistically significant for the exposure to 15 % effluent after 26 weeks. As A.s.s.-injection did not influence steroid levels data from A.s.s.- and sham-injected fish were pooled, in order to obtain more representative test groups.

Serum vitellogenin level

Exposure to sewage effluent did not significantly influence the production of vtg in male trout. However, within the effluent exposed groups, more mature males displayed detectable vtg levels in serum compared to control fish (data not shown). In the group exposed to 15 % effluent a small number of male trout showed strikingly high serum vtg levels. In general, a high variability in the induction of vtg synthesis between individuals was observed.

Liver EROD activity

Exposure to 15 % sewage treatment effluent over 32 weeks resulted in a significant increase in liver EROD activity in mature male fish compared to control fish (fig. 9). Exposure to 1.5 % effluent did not change liver EROD activity. In mature female fish, no effect of sewage treatment effluent on liver EROD activity could be shown (data not shown). As there was no significant difference to be found between A.s.s.-injected and sham-injected fish, data from these groups were pooled, in order to give a more representative control group.

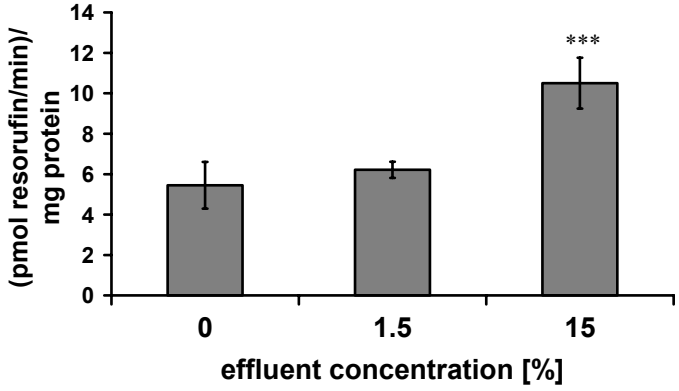


Fig. 9: Liver EROD activity in mature male trout after exposure to STP effluent for 32 weeks. Shown are median values with SEM. Data was tested with Kruskall-Wallis non-parametric one-way ANOVA. $n \geq 17$; *** $p \leq 0.001$.

Differential blood cell counts

Leucocyte numbers in peripheral blood were not influenced by exposure to effluent or injection with *A. salmonicida* (data not shown).

Macrophage oxidative burst and phagocytosis

Exposure to 1.5 % or 15 % STP effluent for 32 weeks did not have an effect on head kidney macrophages oxidative burst (data not shown) or phagocytosis (data not shown). Moreover, injection with inactivated *A. salmonicida* did not influence both parameters.

Serum lysozyme activity

Serum lysozyme activity was measured after 26 weeks of exposure and at the end of the experiment after 32 weeks. After 26 weeks, serum samples from fish exposed to 1.5 % and 15 % effluent did not show altered lysozyme activity compared to samples from control fish if data from all fish was analysed together. If the data was analysed separately according to sex and maturity, as identified with the sampling at the end of the experiment, immature fish (males and females pooled) displayed a significant decrease in serum lysozyme activity after exposure to 1.5 % or 15 % effluent (fig. 10a).

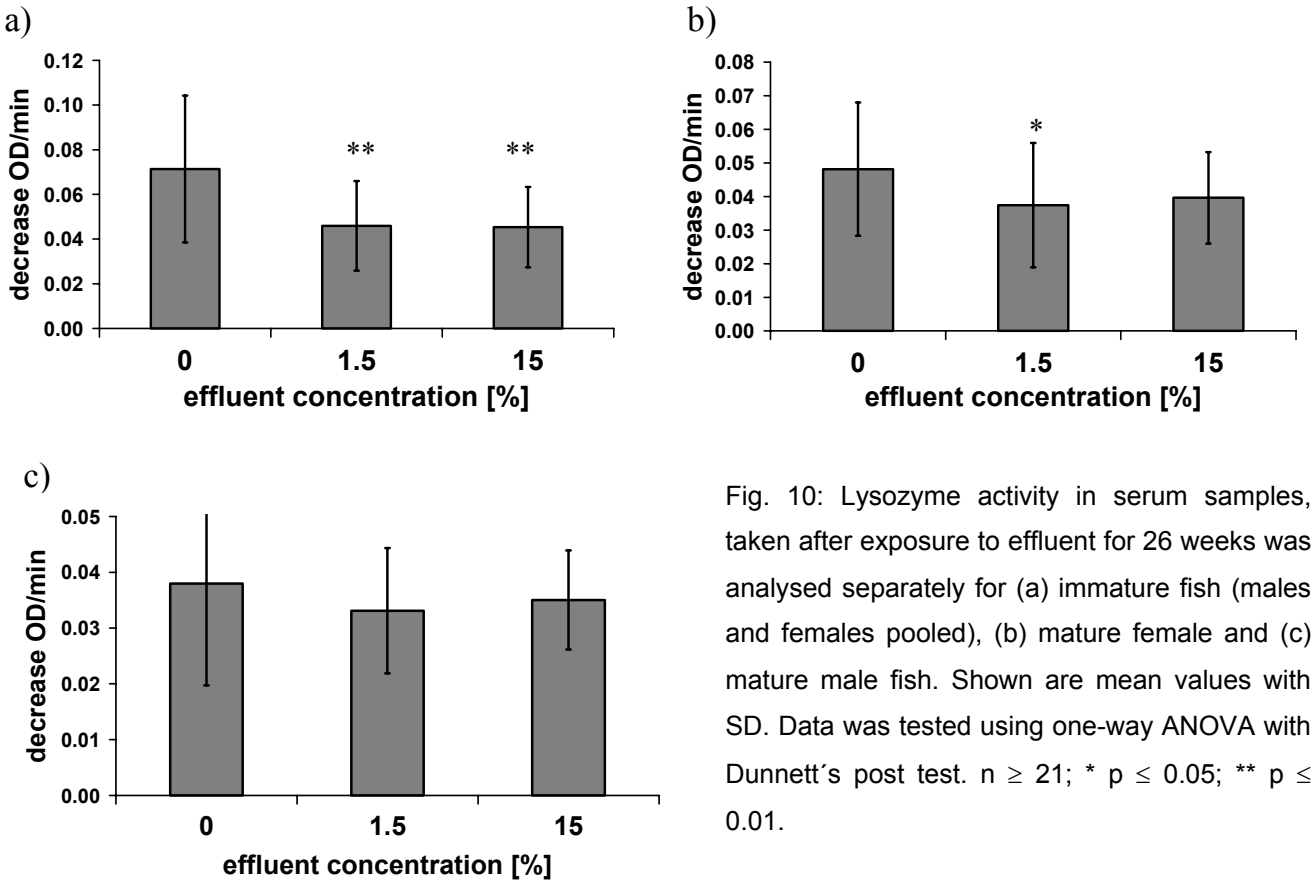


Fig. 10: Lysozyme activity in serum samples, taken after exposure to effluent for 26 weeks was analysed separately for (a) immature fish (males and females pooled), (b) mature female and (c) mature male fish. Shown are mean values with SD. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 21$; * $p \leq 0.05$; ** $p \leq 0.01$.

In mature female trout, exposure to 1.5 % and 15 % effluent resulted in decreased lysozyme activity compared to control female fish, however, this was only statistically significant in the group exposed to 1.5 % effluent (fig. 10b). In mature male fish, exposure to effluent had no significant effect on serum lysozyme activity (fig. 10c). Data from A.s.s.- and sham-injected trout was pooled, as no differences between these groups was found.

Upon termination of the experiment, after 32 weeks, only data for mature male trout was analysed, as the majority of serum samples from female fish caused precipitation in the *M. lysodeikticus* solution used for the lysozyme assay. Exposure to 1.5 % or 15 % STP effluent for 32 weeks did not have an effect on serum lysozyme activity (data not shown). However, in control fish exposed to de-chlorinated tap water, i.p.-injection with the inactivated trout pathogen *A. salmonicida* resulted in a significant increase in serum lysozyme activity compared to PBS-injected fish (fig. 11). In the fish exposed to effluent, no difference in lysozyme activity between pathogen-injected and sham-injected trout could be found.

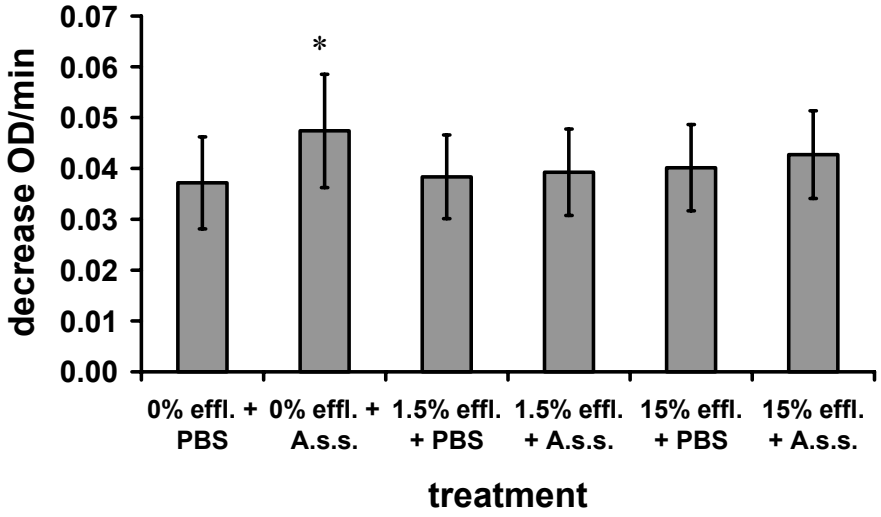


Fig. 11: Lysozyme activity in male trout after exposure to 1.5 % and 15 % effluent (effl.) for 32 weeks, compared to control fish kept in de-chlorinated tap water. The graphs show mean values with SD. Difference between A.s.s. and PBS injection was tested with students t-test; n = 12 for 0 % effl. + PBS and 0 % effl. + A.s.s.; $n \geq 7$ for the effluent-exposed groups; * $p \leq 0.05$.

Aeromonas salmonicida specific antibody ELISA

As no standards were available for IgM determination, results are given in optical density (OD) measured in the *A. salmonicida* specific anti-IgM-ELISA at a wavelength of 450 nm. To enable comparison without a standard curve, all samples were measured in parallel in a single ELISA run. To test the reliability of the test system, the ELISA was repeated twice and no difference was detected in the results. In mature female trout, exposure to 1.5 % or 15 % STP effluent resulted in lower serum levels of specific antibodies, produced against inactivated *A. salmonicida*, however, the decrease in serum antibody levels was only statistically significant in the group exposed to 1.5 % effluent (fig. 8).

Mature male trout did not display different levels of specific antibodies after exposure to effluent compared to tap water exposed fish (data not shown).

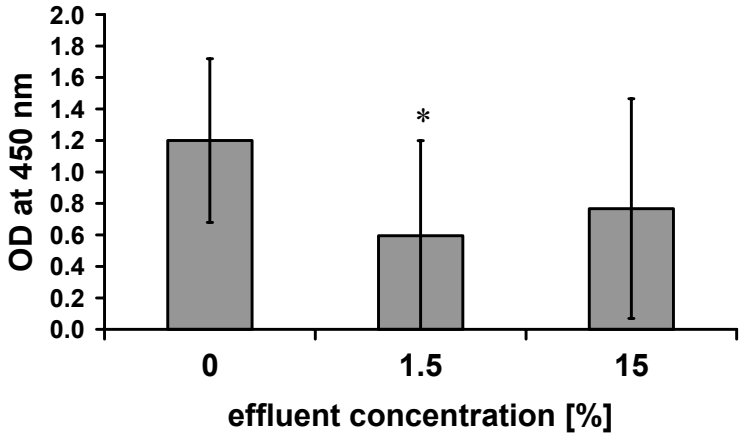


Fig. 8: *A. salmonicida*-specific antibody level in serum samples from female trout, after exposure to effluent for 32 weeks. Values are obtained in parallel in a single ELISA. As no standards for the determination of IgM were available, results for the different exposure groups can only be expressed as OD relative to each other. Data was tested using one-way ANOVA with Dunnett´s post-test. Shown are mean values with SD.
n=12 for the groups exposed to 0 % and 1.5 % effluent; n=8 for the 15 % effluent group; * $p<0.05$

Discussion

Chronic exposure of rainbow trout to sewage effluent from the municipal sewage treatment plant in Rotorua, New Zealand, resulted in alterations of general physiological and endocrine-reproductive endpoints and influenced reactivity of selective components of the trout immune system.

Reproductive alterations included a stimulation of gonad growth in both males and females exposed to 15 % effluent. In females, the higher gonad weight was reflected in a significantly higher fecundity as estimated by an index of egg count per body weight. During mid-vitellogenesis (after 26 weeks of exposure), the female trout displayed significantly augmented plasma 17β-estradiol levels after exposure to both 1.5 % or 15 % effluent. Closer to ovarian follicle maturation (after 32 weeks of exposure), estradiol levels in plasma were still significantly higher at the 15 % effluent exposure concentration. These findings are in line with studies by Folmar et al. (2001), who have also reported elevated plasma estradiol levels in female walleye (*Stizostedion vitreum*) downstream of a STP effluent outfall. As in our experiments, Folmar and coworkers did also not find significantly changed plasma

testosterone levels in female fish after effluent exposure. In the study at hand, exposure to 15 % effluent also led to significantly higher testis weight in mature male trout. Plasma testosterone levels in these male fish were not significantly changed, while plasma 11-KT levels were significantly lower in the group exposed to 15 % effluent, after 26 weeks of exposure. At the end of the experiment (after 32 weeks) there was still a trend in 11-KT to be reduced in fish exposed to 15 % effluent. An increase in serum vitellogenin (vtg) levels, due to exposure to effluent was only observed in few individual males, compared to control fish. In general, estrogenic activity has been shown for several sewage treatment plant effluents in various countries. However, there are often contradictory results for single parameters, such as gonad weight. In a study with sexually maturing male fish, Harries et al. (Harries et al., 1999) found that exposure to effluent from a United Kingdom sewage treatment plant led to significantly increased plasma vtg level, but did not affect testis weight in those fish, while in a previous study, they had shown that exposure to effluent led to smaller gonad indices (Harries et al., 1997). Hemming et al. (2001) also found that exposure to sewage effluent led to lower GSI and higher vtg levels in male fathead minnow (*P. promelas*), however, effluent concentrations used in this study were quite high, with up to 100 %. Several studies on endocrine effects also showed that fish exposed to sewage effluent displayed enlarged livers, which was linked to the production of vtg (Harries et al., 1997; Hemming et al., 2001; Porter & Janz, 2003).

Our study also showed an increase in liver somatic index in male and female trout after exposure to 15 % effluent. Liver size can be impacted by many factors, particularly energy intake increases. However, the exposed fish were on controlled rations and physiological data does not show increases in energy storage (condition factor when excluding organ weights). As it is difficult to attribute this change to increased energy storage in the liver, this observation may be due to increased biosynthesis in the liver, due in part to reproductive functions as well as contaminant metabolism, such as EROD activity, which was induced in male trout exposed to 15 % effluent.

In general, it can be said that effluent from the Rotorua municipal sewage treatment plant showed endocrine activity at a concentration of 15 %. The stimulation of both males and females suggests that a similar mechanism must be present in both sexes. It is possible that very low amounts of estrogens present in the effluent stimulated the initiation of gonadal development at a critical point in reproductive development. Previous studies with rainbow trout exposed to pulp and paper mill effluent have shown that they are most sensitive to alterations in gonad growth when exposed prior to gonad growth being initiated (van den

Heuvel & Ellis, 2002). The signal to start gonad growth in both males and females is thought to be estrogen dependent, explaining these effects in both sexes. Also, the recruitment of ovarian follicles in females occurs due to hormonal signals at a very specific period in development, which further supports an endocrine mechanism, rather than one of differing energy utilisation. The limited induction of vtg would suggest that estrogens are present in the effluent, though specific compounds were not measured. However, vtg appears to be expressed on a very individual specific basis, suggesting a significant level in genetic polymorphism in those elements responsible for transduction of this signal.

A wide range of immunological endpoints were investigated in mature trout after exposure to STP effluent in an attempt to examine the competence of the many varied components of the immune system. Though many functions of the immune system were unimpacted, plasma lysozyme and antibody production in response to an immune challenge were decreased in effluent-exposed trout.

Injection of fish with the fish specific pathogen *A. salmonicida* did not affect any of the examined parameters, except for serum lysozyme activity. In mature male trout, i.p. injection with the inactivated pathogen resulted in a significantly enhanced lysozyme activity, compared to sham-injected fish. This result is in agreement with the findings of Moyner et al. (Moyner et al., 1993) who showed that infection of Atlantic salmon (*S. salar*) with *A. salmonicida* leads to increased serum lysozyme levels. In the groups exposed to effluent, A.s.s.-injected trout did not display altered lysozyme activity compared to the PBS-injected fish suggesting a possible influence of the effluent on the ability to express this bacteriolytic enzyme. This hypothesis would support the findings that various types of stressors can suppress components of the innate immune system of fish (Yin et al., 1995; Tahir et al., 1993; Möck & Peters, 1990). Similar effects of effluent exposure on serum lysozyme activity have been shown by Price et al. (1997). In this study carp (*C. carpio*), which were held in an effluent intake river, displayed lower serum lysozyme activity. In agreement with these findings, we could show a decrease of serum lysozyme activity for immature fish (males and females pooled) and to some extend in mature female trout after 26 weeks of exposure. In male fish, lysozyme was not affected by exposure to STP effluent. The fact that a clear, significant decrease in serum lysozyme activity after 26 weeks of exposure could only be shown in immature fish, but not in males and females at the onset of maturation, suggests that lysozyme production or activity is also influenced by sexual development (Hutchinson & Manning, 1996; Fletcher et al., 1977).

The observation that serum samples from mature female fish (after 32 weeks of exposure) caused precipitation of the *M. lysodeikticus* solution, used for the lysozyme assay, could not be explained so far. It is, however, possible that upon maturation, female fish have higher amounts of immunologically active proteins in blood, which are transported to the ovarian follicles. Substances like lectin, C-reactive protein (CRP) and lysozyme are present in fish eggs as a defence against infection (Yousif et al., 1991, 1994a, 1994b; Balakhnin & Dudka, 1990; Balakhnin et al., 1990; Fletcher et al., 1977; Nunomura, 1991). There are possibly types of proteins among those which can cause precipitation in a bacterial solution. For example, coho salmon (*O. kisutch*) egg lectin has been shown to agglutinate *A. salmonicida* (Yousif et al., 1994b).

The ability of mature female trout to produce specific antibodies to an injected antigen was decreased after exposure to 1.5 % and 15 % STP effluent compared to control fish, however, the decrease in serum antibody levels in the group exposed to 15 % effluent was not statistically significant. Possibly, the lack of a significant result is due to a smaller sample size in this group, which in contrast to the control and 1.5 % group (n=12) only consisted of 8 individuals. As we could not observe lower peripheral blood lymphocyte numbers in blood smears after exposure to effluent, a decrease in A.s.s.-specific serum antibody levels would have to be explained by a decrease in stimulation of B-lymphocytes to mature into antibody-producing plasma cells or a lower productivity of existing plasma cells. This hypothesis however, cannot be proven, as we did not have the ability to apply a corresponding method to show lower plasma cell activity.

Marked adverse effects on the immune system of fish are usually related to known point sources of identifiable toxicants in the waters investigated. Significant changes in immune parameters like macrophage activity have e.g. be linked with PAH or metal contamination (Kelly-Reay & Weeks, 1994; Weeks & Warinner, 1986; Bols et al., 2001) and oil-contaminated sediments (Tahir et al., 1993). Effects on the immune system have also be shown in dab (*L. limanda*) exposed to sewage sludge (Secombes et al., 1991, 1992, 1995). Except for the studies conducted by Price et al. (Price et al., 1997) and Kakuta (1997), in which exposure to STP effluent led to decreased serum lysozyme activity and reduced lymphocyte proliferation in carp, and lower blood erythrocyte, granulocyte and lymphocyte numbers, as well as decreased blood cell phagocytotic activity in goldfish respectively, adverse effects of sewage treatment plant effluent on the immune system of fish have not been shown so far.

In the present study, effluent from a STP with state-of-the-art treatment was observed to cause changes in both reproductive and immunological parameters, suggesting a potential hazard for fish exposed to such effluents. Like in most vertebrates, close interactions between endocrine and immune functions are to be expected in fish. However, in our study, only indirect evidence for interaction between the two systems could be shown, reflected in differences in immune reactivity between gender and at different stages of sexual development (immature vs. mature). A direct association between the adverse effect on endocrine and immune parameters observed in this study could not be found. As in trout both, the endocrine and immune system have been found to be affected by exposure to sewage effluent, monitoring of aquatic pollution should include the assessment of endocrine-reproductive and immune parameters. While, in recent years, the applicability of endocrine parameters for the assessment of aquatic pollution has been widely investigated and discussed, immunotoxicology of fish is still undeveloped due to the lag in the discovery of basic immunology in fishes and the immense complexity of the immune system. The experimental design, needed to enable the assessment of immune parameters, is complicated by various characteristics of the immune system. As immune reactions are temporally very dynamic it might be necessary to concentrate more on time courses of the investigated reactions, rather than use an endpoint determination, which is a snapshot in time that misses most of the events occurring (Köllner, 2003, personal communication). An example of this can be seen in this study: the duration of post-challenge exposure was optimised in order to measure antibody production, which is slow in trout. However, this overlooked rapid responses, such as oxidative burst, that are well known to respond to *A. salmonicida* within very short periods of time. Few toxicological experiments using immune endpoints, utilise an immune challenge. As adverse effects of pollutants on immune reactions become most relevant with the threat of opportunistic diseases, investigations of an activated system are of higher relevance than assessment of immune parameters within a resting, unchallenged system. A challenge situation should therefore be included in all laboratory experiments, studying effects of pollution on the fish immune system (Kollner et al., 2002). A better understanding of the fish immune system is essential for the interpretation of results, suggesting effects of environmental contamination on immune parameters in fish. Identification of mechanisms and patterns of action of pollutants in the aquatic environment would facilitate estimation of adverse effects on fish health. While the study at hand provides clear evidence for adverse effects of sewage effluents on the fish immune system, further studies are necessary to

provide a better assessment of possible immunotoxicological effects of STP effluents on immune competence in fish.

Influence of chronic exposure to treated sewage effluent on the distribution of white blood cell populations in rainbow trout (*Oncorhynchus mykiss*) spleen

Impediment of immune reactions in aquatic animals has been supposed as a possible consequence of low-level contamination of our surface waters with anthropogenic substances. Intake of man-made substances into surface waters mainly happens through discharge of, more or less completely treated wastewater into rivers, lakes and oceans. The study at hand has investigated the prevalence and distribution of different leucocyte populations in spleen samples of rainbow trout (*O. mykiss*), chronically exposed to sewage treatment plant effluent. After exposure for 26 weeks, trout were injected intra-peritoneally with inactivated *A. salmonicida salmonicida* (A.s.s.) to simulate an infection, or with PBS as a control for the immune challenge. Exposure to effluent was continued for another 6 weeks, resulting in an overall exposure time of 32 weeks. Histochemical analysis of spleen cryosections was conducted using monoclonal antibodies, specific for different leucocyte populations in rainbow trout, namely B-lymphocytes, granulocytes, monocytes and thrombocytes. Moreover, the expression of MHC class I and II on spleen cells could be assessed. Exposure of trout to effluent resulted in a marked decrease of thrombocyte numbers, increase of monocytes, altered distribution of B-cells and higher immunoglobulin surface expression, as well as activation of MHC class II expression. The most prominent finding of the study at hand, however, was the occurrence of intraplasmatic deposits or inclusions with strong autofluorescence in spleen sections from effluent exposed trout. Results for leucocyte prevalence and distribution are discussed in context with effects of A.s.s.-injection on these cell populations.

Introduction

In the last few years attention of scientists in the field of environmental toxicology has shifted from the observation of strong, direct toxicity to the identification of more subtle effects of pollution. While not leading to massive fish kills or other clearly visible effects, a variety of substances, including pharmaceuticals, industrial chemicals and compounds contained in many household products, have recently been shown to affect endocrine as well as immunological mechanisms in vertebrates at relatively low concentrations (Daughton & Ternes, 1999). Contamination of our surface waters with a cocktail of substances, at however low concentrations, has ever since been suspected to alter e.g. reproductive performance, possibly leading to a reduction in fish populations (Jobling et al., 1996; Larsson et al., 1999;

Matthiessen & Sumpter, 1998; Robinson et al., 2003). The main cause of such a mixture pollution is the intake of municipal, agricultural and industrial wastewater into our surface waters (Daughton & Ternes, 1999; Desbrow et al., 1998; Hirsch et al., 1999; Ternes, 1998).

Histological investigations within the field of fish toxicology have classically focused on general pathology (externally visible disease, skin structure and lesions, liver lesions, necrosis and apoptosis, as well as inflammatory reactions) and tumour incidence (Vethaak et al., 1992; Burkhardt-Holm et al., 1997; Schmidt et al., 1999; Wahli et al., 2002). Only in the last few years have environmental toxicologists started to consider effects of aquatic pollution on immune competence of fish. Being a commonly present feature in osteichthyes, melanomacrophage centres (MMCs) in liver, spleen and head kidney, have been suggested and already used as indicators of adverse effects of aquatic pollution on the fish immune system (Wolke et al., 1985; Bucke et al., 1992; Payne & Fancey, 1989; Luebke et al., 1997; Kranz & Gercken, 1987). Although, it is generally accepted, that MMCs play a role in immunological mechanism in fish (along with other putative functions), their functions have not been clearly elucidated yet (Wolke, 1992). Therefore, the meaning of changes in MMCs for immune reactions in fish remains questionable. Moreover, clear cause-effect relationships could not be established, where changes in MMCs were observed.

With specific antibodies against fish immune cells becoming more available, it is now moreover possible to track leucocyte populations in peripheral blood, as well as in organ samples. In the field of immunotoxicology, occurrence and distribution of different white blood cell populations, e.g. in haematopoietic tissues is of major relevance. Alterations in prevalence and activity of different types of leucocytes points to changes in a variety of immune reactions, as immunological activity is almost exclusively based on leucocyte integrity.

In the study at hand, specific antibodies against rainbow trout leucocytes have been used to investigate effects of sewage treatment effluent on occurrence and distribution of white blood cells in the spleen. Therefore, rainbow trout (*O. mykiss*) have been exposed to realistic concentrations (calculated with a dilution factor, known for German intake rivers) of STP effluent over a period of 32 weeks (chronic exposure). Subsequently, B-lymphocytes, monocytes, granulocytes and thrombocytes, as well as the cell surface molecules MHC class I and MHC class II have been detected in spleen sections.

Material and Methods

Fish

For the experiment two year old rainbow trout (*O. mykiss*), which were hatched at Forest Research Institute were used. Parent fish were purchased from Ngongataha hatchery, Ngongataha, New Zealand. Fish were held in 12,000 L tanks (exposure tanks 1-6, see fig. 5, chapter III), each containing 50 individuals and were fed daily with commercial aquaculture feed pellets (Reliance stock food, Dunedin, New Zealand) at a ration of 0.7 % of the wet body weight and the amount was increased at 25 g per month as per the known growth trajectory with fish of this size. All trout were tagged with individually numbered T-bar type tags (HallPrint Pty Ltd, Holden Hill, SA, Australia), weighed, and measured at the start of the experiment.

Sewage treatment plant effluent

Final treated effluent was obtained from a sewage treatment plant (STP) located in Rotorua, New Zealand (fig. 5, chapter III). This STP employs a pre-treatment step with stop screens and a grit trap, a primary treatment step with sedimentation and secondary activated sludge treatment (Bardenpho Reactor). Subsequently, after going through a plant effluent balance pond the final treated effluent is pumped to wastewater effluent land treatment holding ponds from where it is sprayed into the forest. Effluent holding tanks were refilled with final treated effluent, collected from the final effluent holding pond with a tanker truck, every second day.

Experimental set up

Trout exposure facility

In the trout exposure experiment activated carbon de-chlorinated tap water was used as the diluent and as the reference treatment (aquifer source) (fig. 5, chapter III). Water flow was controlled from line pressure using stainless steel globe valves and spring-operated flowmeters. Effluent flow was controlled using a head tank to maintain constant pressure in combination with a PVC orifice calibrated for the nominal flow. Diluent flows were adjusted daily when necessary, and the effluent control orifices were cleaned daily to prevent reduction of flow by bio-fouling. Two replicate tanks were used for each treatment. Trout exposure tanks were provided with a constant water flow of 20 L/min, which resulted in a 95 % replacement time of approximately 30 h.

Water parameters

Dissolved oxygen, pH and conductivity (Radiometer Pacific, Auckland, New Zealand) in the fish exposure tanks and in undiluted effluent were measured daily. Additional aeration was provided in the effluent holding ponds and the trout exposure tanks and dissolved oxygen was maintained above 90 % saturation for the duration of the experiment**.** The average pH-values in the exposure tanks were 7.21 ± 0.25 and 7.22 ± 0.25 in the 15 % effluent tanks, 7.15 ± 0.28 and 7.20 ± 0.28 in the 1.5% effluent tanks and 7.13 ± 0.29 and 7.13 ± 0.28 in the control tanks. Conductivity in each tank, diluent conductivity, and effluent conductivity was used to calculate the actual effluent concentration in the fish ponds on a daily basis. Temperature was measured hourly using Onset Tidbit temperature loggers (Onset Computer Corp, Bourne, MA, USA). Measured temperature and effluent concentrations over the duration of the experiment are shown in figure 6, chapter III.

Exposure

Trout were exposed to a nominal concentration of either 1.5 (v/v) or 15 % (v/v) effluent. Control fish were kept in de-chlorinated tap water. The exposure was started on September 22, 2001 and was terminated between May 6 and 14, 2002. After exposure for 26 weeks, trout were anesthetised with ethyl-3-aminobenzoate methanosulfonate (MS222) (Fluka, Switzerland) and 1 ml of blood was taken by syringe from the caudal vein. Fish were then either injected with formaldehyde inactivated *A. salmonicida*, strain MT 423 (reference strain, NCIMB, Aberdeen; Scotland) using a concentration of 1 x 10^8 cells per 100 g body weight, or with PBS as a control for the injection. Fish were exposed for a further 6 weeks until they were sampled.

Antigen preparation of Aeromonas salmonicida salmonicida (A.s.s.)

A.s.s. strain MT 423, stored in cryotubes (Microbank^{TM}, PRO-LAB Diagnostics, UK), was used for experiments. The bacteria were incubated from cryoconserve in 5 ml sojapepton-casein-pepton-bouillon (CASO-bouillon; SIFIN, Germany) at 22°C for 98 h. After that the cultures were checked for purity by microscope (gram stain, morphology) and subcultured on CASO-agar plates (SIFIN, Germany). 250 µl of A.s.s.-CASO-bouillon-culture was replanted to a CASO-agar plate and incubated at 22°C for 98 h. The A.s.s. colonies were harvested from 9 plates, resuspended and pooled in 150 ml sterile 0.9 % NaCl and homogenised using a magnetic stirrer. This A.s.s. bacteria pool was inactivated by adding 1.5 ml formaldehyde

solution (35 %, DAB, Merck, Germany) and stirred for 60 min at room temperature. After centrifugation (10 min, 4300 rpm, 15°C) in a Hettich Universal 30 F centrifuge, the pellets were resuspended in sterile 0.9 % NaCl and washed twice to remove the formaldehyde. The cell suspension was adjusted to an optical density of 1.0 by adding sterile 0.9 % NaCl (λ=520 nm; Photometer PF-10; Machery-Nagel, Düren, Germany), which corresponds to 1 x 10^{10} CFU/ml. The formalin inactivated bacteria were stored in 1 ml aliquots at -20°C.

Sampling

Female trout were sampled first over two subsequent days by taking out 3 A.s.s.-injected and 3 PBS-injected fish from each tank per day. Male fish were sampled 7 days later following the same sampling scheme. Small parts of spleen were dissected and snap-frozen in liquid nitrogen and stored at -80°C for histology.

Immuno-histology

Spleen samples were cut on a cryostat microtome (Leica CM3050, Leica, Germany). Approximately 8 µm thick organ sections were placed on poly-L-lysine (0.1 % w/v in water; Sigma, Steinheim, Germany) coated glass slides. After fixation in 100 % acetone for 10 min at 4°C, the sections were air-dried. Dry sections were incubated with primary antibodies (list of antibodies used, see table 4) for 1 h at room temperature. The slides were washed twice in Iscove´s Modified Dulbecco´s medium (Invitrogen, Karlsruhe, Germany) and subsequently incubated with secondary, fluorescence labelled antibodies (see table 4) for 1 h. After washing twice in medium, slides were mounted in PBS, containing 10 % glycerine and 2.5 % 1,4 diazobicyclooctan (Dabco, Sigma, Steinheim, Germany), covered with cover slips and examined for specific fluorescence using a LSM 510 confocal laser scanning microscope (Carl Zeiss, Hallbergmoos, Germany). The sections were scanned using a 40 x oil immersion objective. The 488 nm line of an argon laser was used for Alexa 488 and fluorescein-isothiocyanat (FITC) excitation and the 543 nm line of a helium/neon laser for R-Phycoerythrin (R-PE) and indocarbocyanin (CY3) exitation. The fluorescence emission was recorded using a main beam splitter at 488/543/633 nm in combination with a second beam splitter at 505-550 nm and an emission filter from 560-615 nm. The obtained scans were analysed using the LSM 510/2.1 software package (Carl Zeiss, Hallbergmoos, Germany).

Table 4: Primary and secondary antibodies used for immuno-histochemical staining

Cell type or surface marker	Primary antibody	Secondary antibody
Granulocytes	Monoclonal antibody (mAb) Q4E (Kuroda et al., 2000)	Goat-anti-mouse IgG_3, FITC-conjugate (Medac, Hamburg, Germany)
Monocytes	mAb 45 (Köllner et al., 2001)	Goat-anti-mouse IgM (μ), R-PE-conjugate (Caltag, Hamburg, Germany)
B-cells	mAb N2 (Fischer & Köllner, 1994)	Goat-anti-mouse IgG-Alexa Fluor 488 (MoBiTec, Goettingen, Germany)
Thrombocytes	mAb 42 (Köllner et al., submitted for publication)	Goat-anti-mouse-$IgG_1(\gamma)$, FITC-conjugate (Caltag, Hamburg, Germany)
MHC I	mAb H9 (Dijkstra et al., 2003)	Goat-anti-mouse-IgG, Cy3-conjugate (Dianova, Hamburg, Germany)
MHC II	Oslo antiserum (Koppang et al., 2003)	a-rabbit-FITC (Sigma, Steinheim, Germany)

Results

Spleen

Spleen tissue sections from fish exposed to 15 % effluent appeared more disintegrated, compared to tissue samples from control fish. Moreover, a high prevalence of an intense orange/red and patchily distributed autofluorescence was observed after exposure to 15 % effluent (effl.) (fig. 9). As shown in double staining using mab 45 (anti-trout monocytes) and mab Q4E (anti-trout granulocytes), this autofluorescence seems to be concentrated in trout monocytes.

Monocytes and granulocytes

The patches with strong autofluorescence observed in trout, were shown to coincide with the specific fluorescence staining of monocytes using mab 45 (fig. 10). Furthermore, the injection of trout with *A. salmonicida* together with the exposure to 15 % effluent induced an increased infiltration of monocytes into spleen tissue. However, the distribution of monocytes within spleen tissue was not observed to be altered by exposure to STP effluent.

Granulocytes were found to distribute evenly within the sections (fig. 10). Cell numbers in spleen from PBS injected fish exposed to 15 % effluent did not seem lower than in control fish, however granulocytes displayed diverse intensity of staining, with some cells showing

lower surface expression of the marker stained by mab Q4E. In contrast, all granulocytes in spleen sections from control fish showed an equally strong staining and cell aggregates were observed in some distance to MMCs.

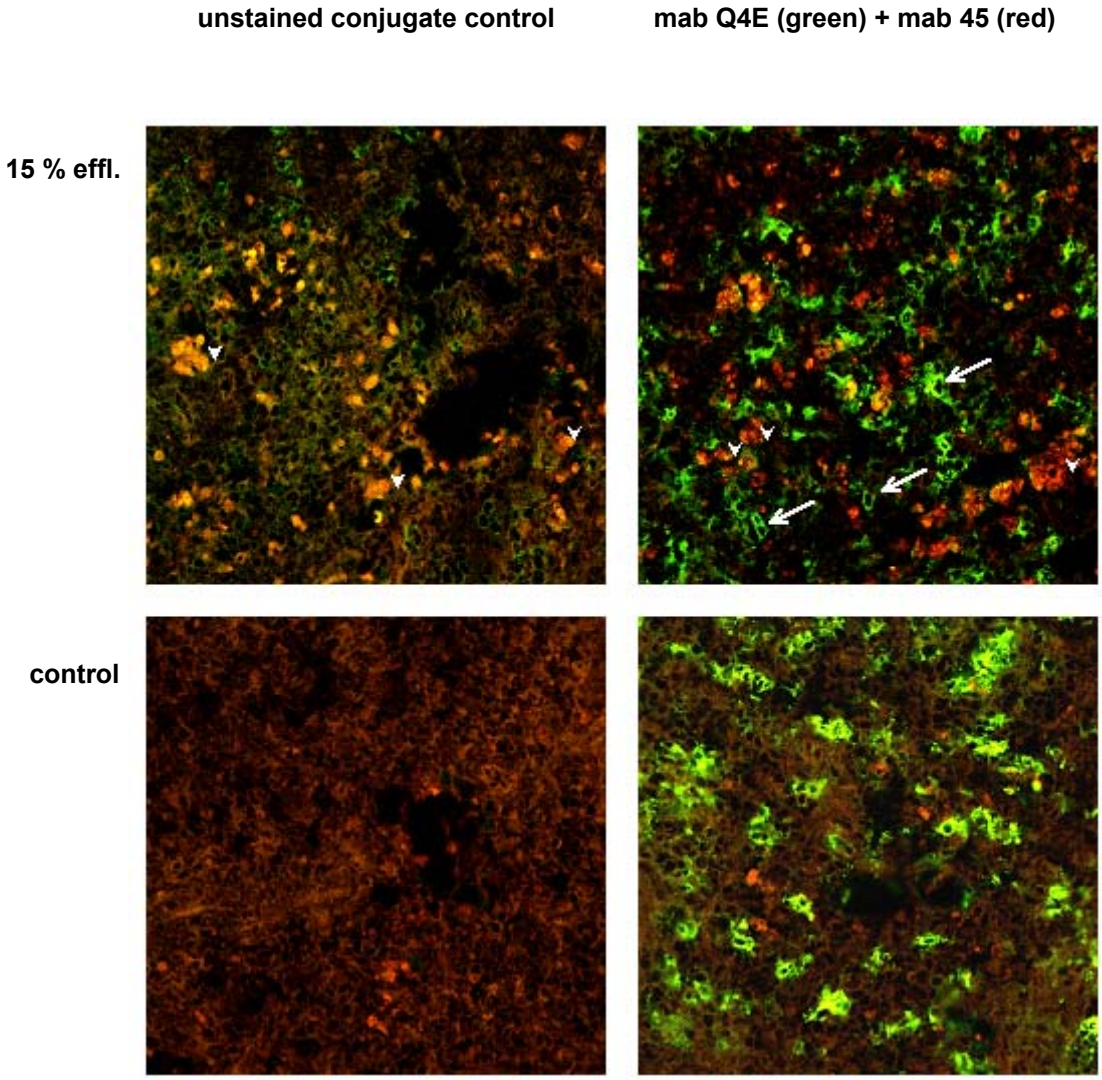


Fig. 9: Laser scanning microscopy of spleen cryosections from trout exposed to 15 % effluent (effl.) (above) and control fish (below). Note the strong orange autofluorescence of large leucocytes in the 15 % effluent group compared to the control group. These cells were labelled with mab 45 (red fluorescence; arrowheads), whereas granulocytes labelled with mab Q4E (green fluorescense, arrows) showed no intracytoplasmatic fluorescence.

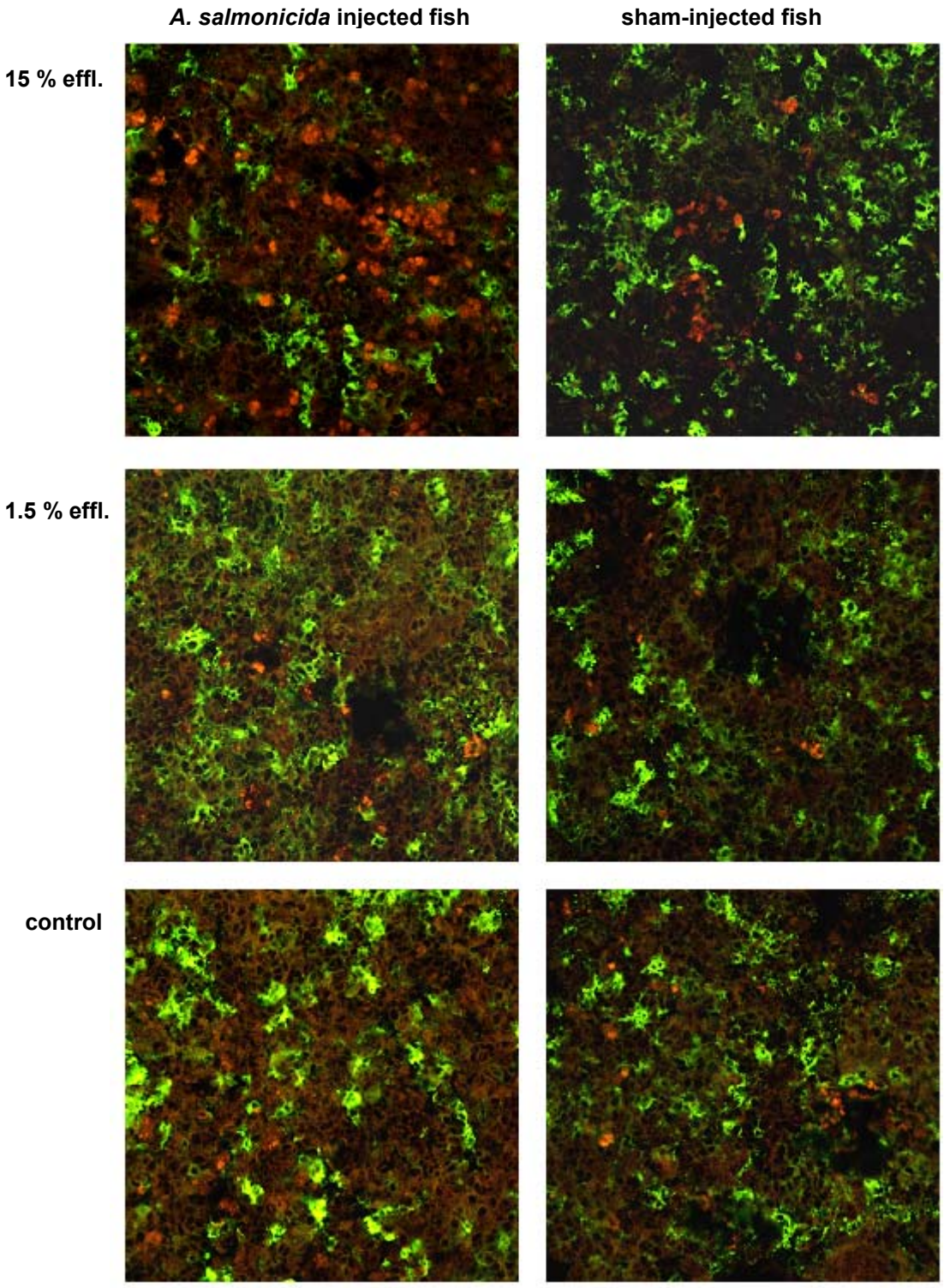


Fig. 10: Laser scanning microscopy of spleen cryosections from control fish and trout exposed to 1.5 or 15 % effl., labelled with mab 45 (anti-trout monocytes, red fluorescence) and mab Q4E (anti-trout granulocytes, green fluorescence). The exposure to 15 % effl. induced a strong increase in the number of monocytes (stained by mab 45, red membrane fluoroscence), showing intracellular autofluorescence. In the *A. salmonicida* injected fish the number of labelled monocytes was further increased compared to PBS-injected fish. Granulocytes seemed to be influenced by exposure to 15 % effl. as shown in changed distribution and slightly decreased fluorescence intensity.

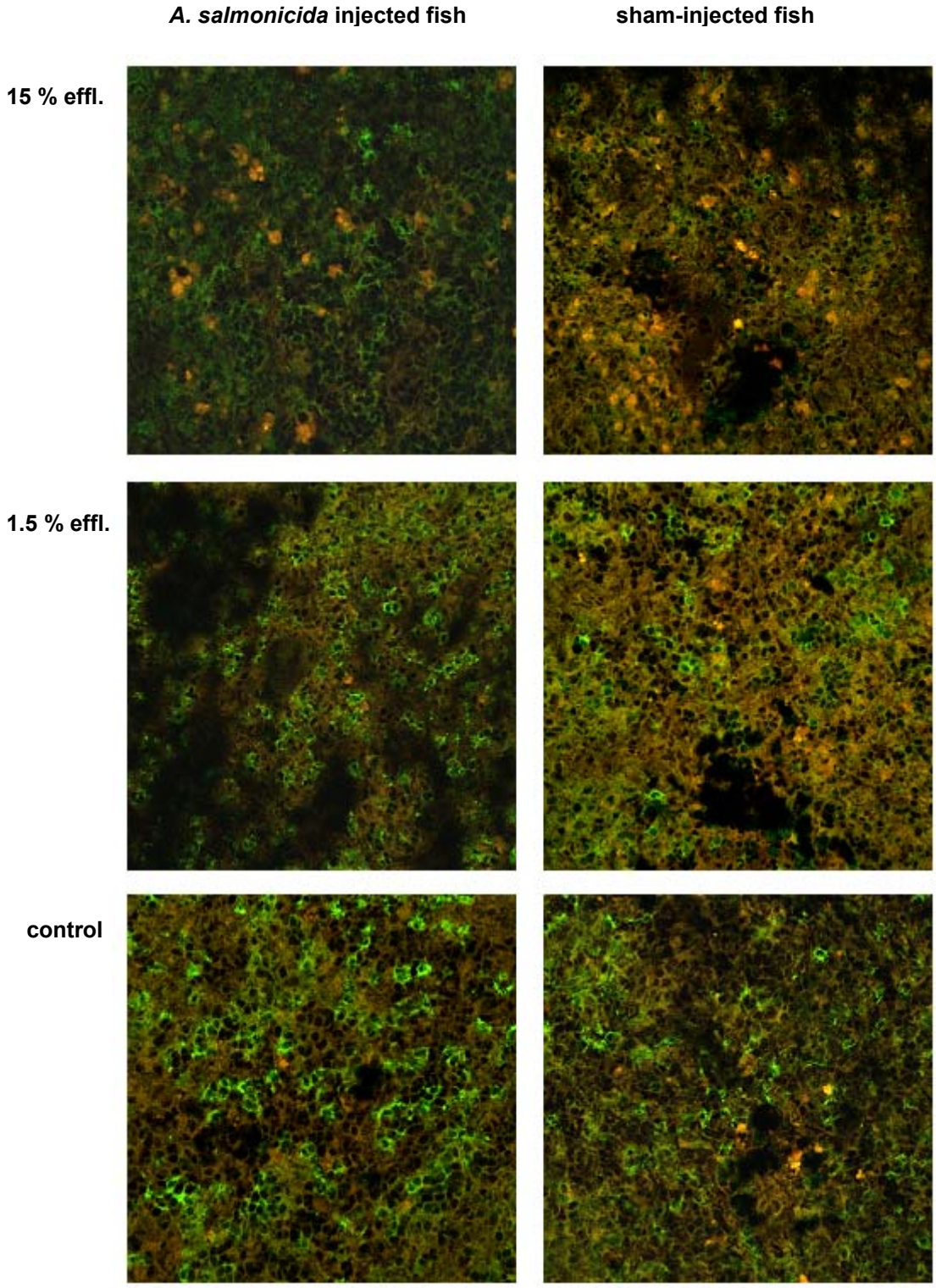


Fig. 11: Laser scanning microscopy of spleen cryosections from control fish and trout exposed to 1.5 or 15 % effl., after labelling with mab 42 (anti trout thrombocytes, green fluorescence).
Note the strong decrease in thrombocyte numbers and lower fluorescence intensity on cell surfaces in 15 % effl. group.

Thrombocytes

Spleen sections from control fish displayed an even distribution of thrombocytes (fig. 11). Staining intensity in these sections seemed to be increased in *A. salmonicida* injected trout compared to sham-injected fish. In tissue samples of trout exposed to 15 % effluent, staining intensity and number of thrombocytes was markedly decreased compared to control fish. In the 15 % effluent group injection of trout with *A. salmonicida* did not have any influence on thrombocyte numbers in spleen tissue, compared to sham-injected fish.

B-lymphocytes

In spleen from control trout and trout exposed to 1.5 % effluent B-lymphocytes showed a homogenous distribution and expression of surface IgM within the tissue. Exposure of trout to 15 % effluent led to an uneven distribution of B-cells in spleen tissue (fig. 12). Larger cells with a high expression of surface IgM assembled around MMCs while in other parts of the sections lower numbers of B-cells were stained, displaying lower fluorescence intensity.

MHC class I and MHC class II

The expression of MHC class I and MHC II in spleen leucocytes seemed to be influenced by both the exposure to 15 % STP effluent and by the injection of trout with *A. salmonicida* particles (fig. 13).

MHC II positive cells were unequally distributed within spleen tissue, with aggregates of MHC II expressing cells, as well as completely unstained areas. Spleen leucocytes in control fish and the 1.5 % effluent group showed a very low expression of MHC class II. The exposure to 15 % STP effluent resulted in a strong increase of MHC class II (green fluorescence) along with the above described occurrence of autofluorescence (yellow / orange cytoplasmatic fluorescence) in larger cells.

In *A. salmonicida* injected fish a stronger expression of MHC class I molecules (orange or orange / red fluorescence) in larger leucocytes surrounding the MMCs (fig. 13, arrows) was found compared to PBS treated control trout. It should be noted that in controls and in the 1.5 % STP effluent group not all MHC class I positive cells also displayed a MHC II specific staining, while in the 15 % effluent group cells showed a specific MHC I / MHC II double staining.

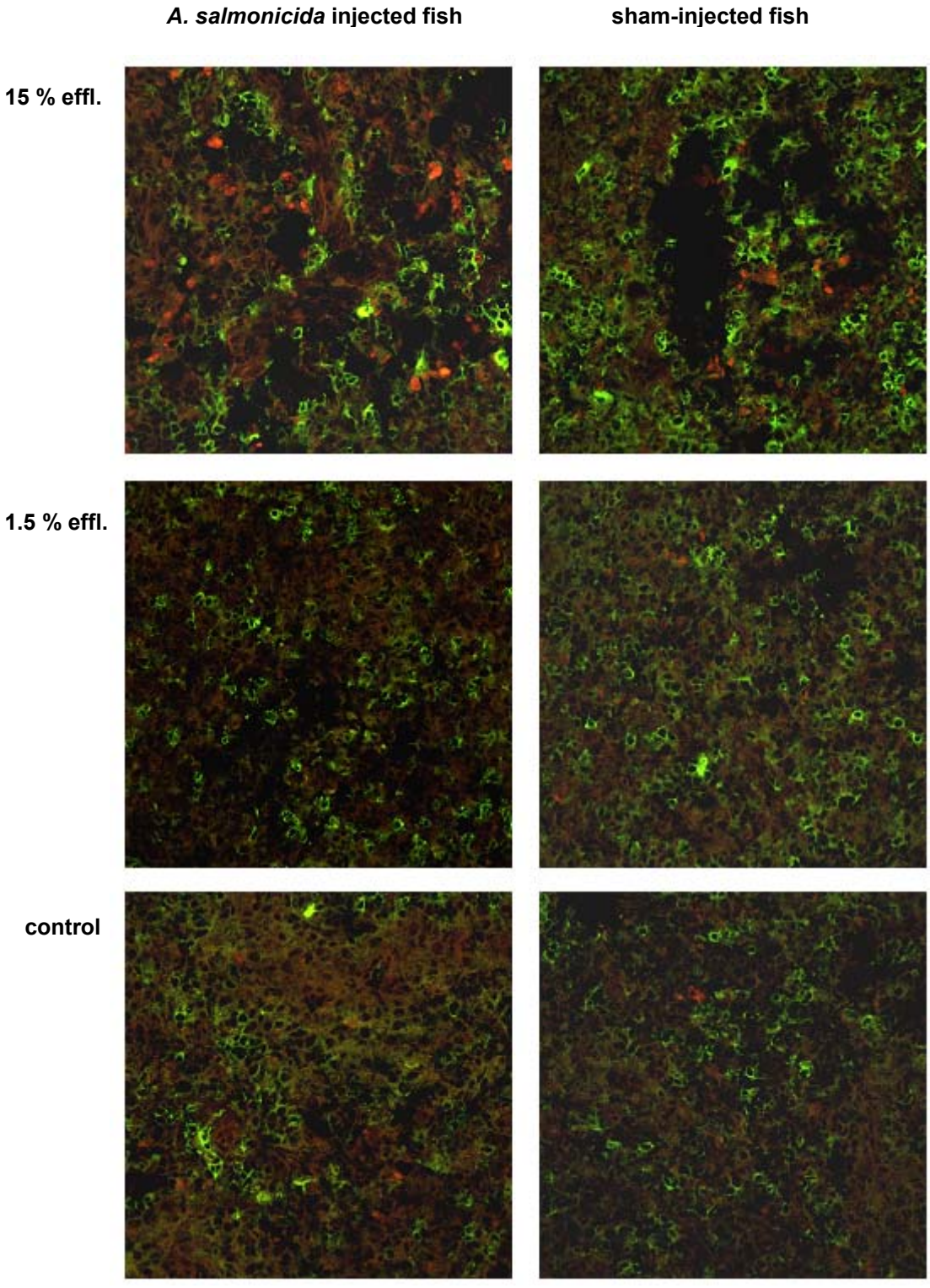


Fig. 12: Laser scanning microscopy of spleen cryosections from control fish and trout exposed to 1.5 or 15 % effl., after labelling with mab N2 (anti trout IgM, green fluorescence).
Note the altered distribution and increased staining intensity in trout exposed to 15 % effl. compared to control fish.

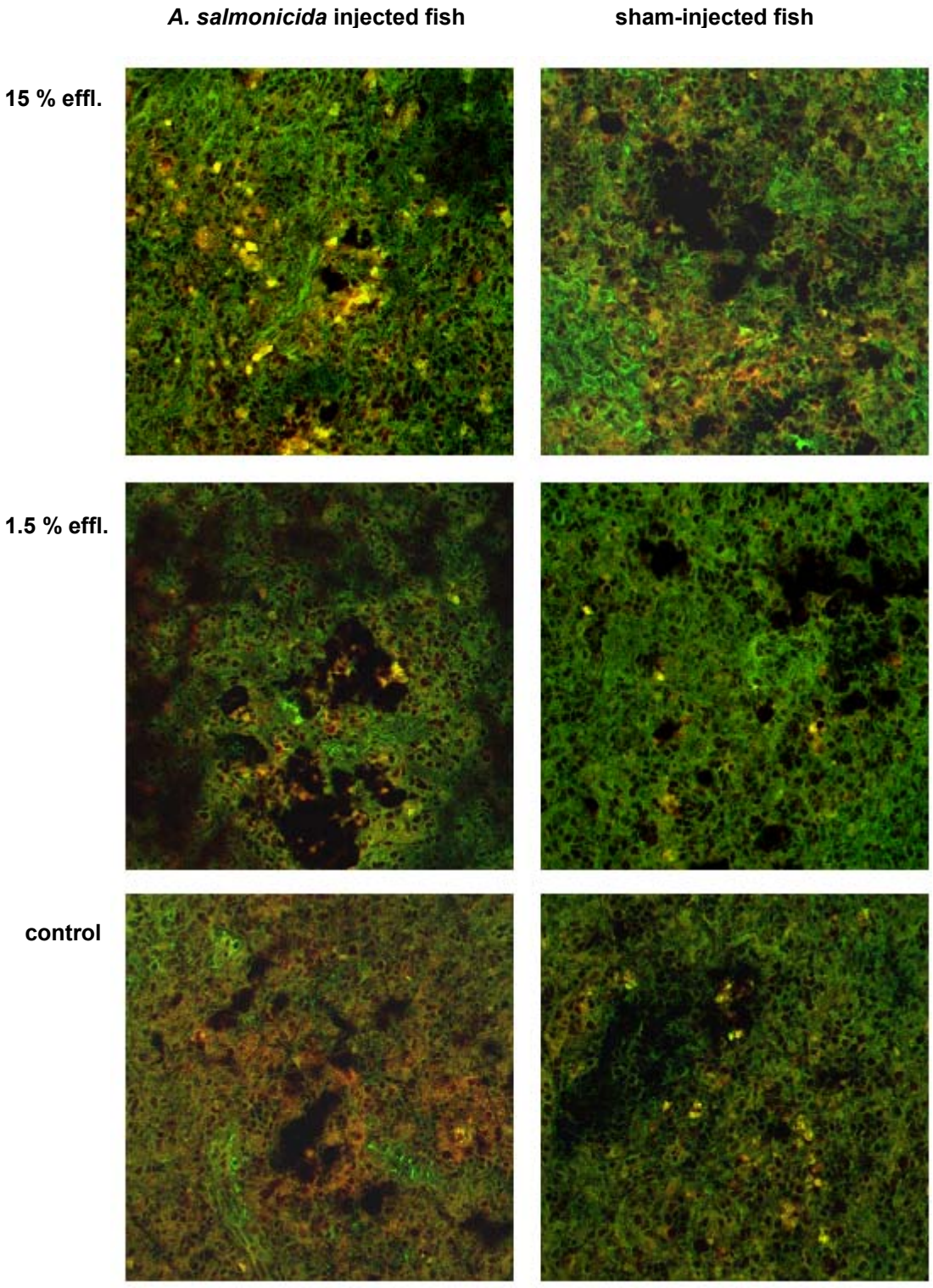


Fig. 13: Laser scanning microscopy of spleen cryosections from control fish and trout exposed to 1.5 or 15 % effl., after labeling with mab H9 (anti trout MHC class I, red fluorescence). and anti salmon MHC II antiserum (green fluorescence).
In the 15 % effl. group, a strong increase in the number of MHC II positive cells was found. In spleen sections from *A. salmonicida* injected trout MHC class I positive cells were found to surround the MMCs.

Discussion

In this study, chronic exposure of rainbow trout to STP effluent has been shown to alter occurrence and distribution of different leucocyte populations in spleen tissue. The predominant finding was a high prevalence of a strong, intracytoplasmatic orange autofluorescence, especially in large leucocytes, in spleen sections from trout exposed to 15 % effluent. These deposits or inclusions have been found to coincide with the specific antibody staining of monocytes and might therefore be thought to constitute depositions in macrophages. Lipofuscin, which can usually be found in MMCs is e.g. known to be autofluorescent (Wolke, 1992). The identity of these deposits or inclusions could not be elucidated or satisfactorily explained. However, they may be considered to reflect an adverse effect of chronic exposure of trout with STP effluent. This impression is further supported by a slightly more disintegrated character of the investigated spleen tissue compared to samples from control fish.

The increase of monocytes / macrophages, detected in spleen samples from trout injected with *A. salmonicida*, is in agreement with results from previous studies (Köllner & Kotterba, 2002). Additional to the effects of *A. salmonicida* injection, it seems that the exposure to 15 % STP effluent had a "co-stimulatory-like" effect on monocytes, indicating the unspecific activation of cells involved in innate immune functions. This finding can be interpreted as a hint to a possible chronic inflammation response to compounds found in STP effluent.

The marked decrease in thrombocyte numbers and lower staining intensity on these cells found in spleen samples from trout exposed with 15 % effluent are further, remarkable results. The main function of thrombocytes, the phylogenetic precursors of platelets in lower vertebrates, is blood clotting (Rowley et al.,1997). Recent findings, however, indicate that trout thrombocytes may also be involved in antigen presentation (Utke et al., 2003). Lower thrombocyte numbers in spleen tissue after exposure to 15 % effluent might be due to an efflux of these cells into blood or other body compartments, possibly reflecting a chemotactive response of thrombocytes towards foreign material in STP effluent. Lower staining intensity might be due to a decreased expression of the CD42 like surface marker recognized by mab 42 (Köllner et al., 2003, personal communication). As it was shown that this molecule is involved in thrombocyte aggregation, a decrease in surface expression could indicate a disturbance of aggregatory funtion after chronic exposure to 15% effluent.

B-lymphocytes in spleen tissue from trout exposed to 15 % effluent were observed to gather close to MMCs, in contrast to B-cells in spleen of control fish, which were evenly distributed in the tissue. Spleen B-cells in the 15 % effluent group moreover displayed a markedly higher intensity of fluorescence staining. The B-cells were detected using an mab against trout IgM (Thuvander et al., 1990). Therefore, not only the distribution of B-lymphocytes can be detected, but also an activation of these cells, leading to an increased expression of surface immunoglobulin (sIgM), reflected in a higher intensity of fluorescence staining on single cells. Gathering of those B-cells, with increased sIgM expression, around macrophage centres might be connected to antigen presentation in these areas of the spleen. This is a further indication of an unspecific activation of the immune system due to exposure to STP effluent.

The enhanced MHC class II specific staining in spleen sections observed after exposure to 15 % effluent is a further indication of effluent induced stimulation of immune cells. It is well known, that activation and proliferation of immune cells after antigenic or mitogenic stimulation results in an increased expression of MHC II molecules on monocytes and activated B and T-lymphocytes (Grusby & Glimcher, 1995; Rohn et al., 1996). The higher MHC II specific staining found in our study might thus also be connected to activation of antigen presenting cells (monocytes, B-cells). However, the functional relevance of such a finding would have to be proved using tests, like phagocytosis assay or mixed leucocyte reaction (MLR), which could not be realized within the scope of this study.

The measurement of *A. salmonicida* specific antibodies in serum of *A. salmonicida* injected trout, revealed lower levels in mature female trout exposed to 1.5 and 15 % effluent, compared to control fish (see chapter III). However, a direct association between putative antigen-presentation and specific antibody levels in serum cannot be drawn, as suppression of antibody production could happen at several other stages, following antigen-recognition (Sharma & Zeeman, 1980). Anyhow, mere antigen presentation does not necessarily result in the production of antibodies by B-cells. Moreover, the nature of the putative antigen, presented in this case is not known. Exposure to effluent might have resulted in incorporation of other antigens, additional to *A. salmonicida* and humoral immune reactions have been found to vary substantially with the antigen applied (Davis et al., 2003; Sharma & Zeeman, 1980).

Although, an effect of chronic exposure to 15 % effluent on occurrence and distribution of leucocytes, as well as a possible deposition of degradation materials in spleen of rainbow trout has been shown, a clear characterization of the (putative adverse) influence cannot be gained in the study at hand. However, our results can be regarded as an indicator for potential

adverse effects of STP effluents on the fish immune system, reflected in an induction of a response, similar to chronic inflammation and a constant unspecific stimulation of different leucocyte populations. Therefore, our findings further support the inclusion of immune parameters into monitoring of aquatic pollution, as has been suggested before by several scientists (Zelikoff et al., 1998, 2000; Wester et al., 1994; Van Muiswinkel, 1992). Analysis of occurrence and distribution of white blood cell populations in haematopoietic tissue, with the help of specific antibodies, might specifically be regarded as a useful method to assess effects of environmental contamination on immune reactions. Adverse effects observed in haematopoietic tissues might reflect or subsequently lead to alterations in several immune reactions.

However, a single test method is not of great help to assess the overall immune competence of an organism. Therefore, investigations on immunotoxicity warrant the assessment of a range of immune parameters. Alterations in leucocyte numbers and expression of surface markers on different leucocyte populations, observed with the help of immuno-histochemistry, should be complemented with functional assays, in order to elucidate the implications of the histological effects. A final characterisation of the overall immune competence has moreover to be based on the investigation of a stimulated immune system and immunosuppression in its last consequence can only be demonstrated with the help of challenge experiments. Further investigations on the effects of sewage treatment water on immune functions in fish are desirable. It will however be indispensable to complete such investigations with studies on mechanisms of immunotoxicity, in order to better understand how immune competence of aquatic organisms can be influenced by different types of pollution.

Effects of water-borne cortisol on the immune system of rainbow trout (*Oncorhynchus mykiss*)

Cortisol has been shown to elicit immunosuppressive activity in teleost fish. Its role as an internal mediator for stress-responses has been investigated in several fish species and its functions and effects have been shown to be comparable to mechanisms in mammals. While in most studies examination of effects of cortisol in fish is linked to stress-related immunosuppression, this study employed external cortisol as a model substance for immunomodulating effects. Acute exposure of rainbow trout (*O. mykiss*) to cortisol via the water for three weeks was followed by investigation of haematocrit and leucocrit, peripheral blood cell differentials, serum lysozyme activity and head kidney macrophage phagocytotic and oxidative burst activity. Beside these immune parameters, general physiological parameters, like condition factor and liver and spleen weight were assessed. In accordance with literature data, trout exposed to cortisol displayed significantly decreased leucocrit values, reflected in lower peripheral blood lymphocyte numbers, assessed with the help of blood cell differentials. Serum lysozyme activity was also shown to be slightly lower in cortisol exposed trout. Head kidney macrophage activity however was not influenced by cortisol. Exposure to cortisol moreover led to a higher spleen weight. Except for macrophage activity, cortisol applied via the water has been shown to cause similar effects as described in the literature for stress-induced internal cortisol and external cortisol applied by injection or feeding.

Introduction

In fish, cortisol has mainly been investigated in context with its internal function as a stress marker. In fish, like in mammals, stress is associated with alteration of immune parameters and internal cortisol has been suspected to be a mediator, leading to immunosuppression. Effects of stress on the internal cortisol level in fish and consequent alterations of immune reactions have been shown by Narnaware and coworkers (1994), whereby injection stress given under light anaesthesia, or a longer noise stress combined with confinement, both enhanced cortisol levels and significantly reduced the level of phagocytic activity of macrophages from the spleen and pronephros of rainbow trout (*O. mykiss*). Application of external cortisol has also been shown to affect immune reactions in fish. A profound lymphopenia and eosinopenia in carp blood after injection with cortisol has been shown by Wojtaszek (2002). Feeding of masu salmon (*O. masou*) with cortisol for 2 weeks was shown

to reduce blood plasma immunoglobulin M (IgM) concentrations (Nagae et al., 1994) and in striped bass (*M. saxatilis*) levels of virus-neutralising antibodies after infection with pancreatic necrosis virus were decreased due to exogenous corticosteroids (Wechsler et al., 1986). Moreover reduced disease resistance was shown in juvenile carp (*C. carpio*) against *I. multifiliis* and in coho salmon (*O. kisutch*) infected with *L. salmonis* after injection or implantation of cortisol, respectively (Houghton & Matthews, 1990; Johnson & Albright, 1992).

In vitro experiments with fish cells showed a dose-dependent inhibition of chemotaxis, phagocytosis and nitric oxide production by cortisol in a long-term goldfish macrophage cell line (Wang & Belosevic, 1995). Carp lymphocytes cultured together with cortisol showed reduced proliferation, which was connected to induction of a high level of apoptosis in those cells (Weyts et al., 1997; Verburg-van Kemenade et al., 1999). Reduced LPS mitogenesis due to cortisol was moreover shown in Atlantic salmon (*S. salar*) (Espelid et al., 1996).

Routes of exposure of fish to external cortisol are usually implantation, injection or feeding. In the experiment at hand, rainbow trout (*O. mykiss*) were exposed to water-borne cortisol in a flow-through system, supplying a constant replacement of water and test substance. Therefore this experiment includes the uptake of cortisol from the water by the test organism. The experiment was designed to characterise a possible manifestation of immunomodulation in rainbow trout, employing a model substance, with known immunosuppressive activity. Immune parameters chosen to investigate effects of water-borne cortisol after exposure for three weeks were haematocrit and leucocrit, blood cell differentials, phagocytosis and oxidative burst in head kidney macrophages and serum lysozyme activity. Beside the immune parameters, physiological parameters, like condition factor, liver-somatic index and spleen-somatic index were assessed.

Material and Methods

Experimental set up

Rainbow trout (*O. mykiss*) were purchased from the hatchery Hugo Strobel (Hausen am Andelsbach, Germany) and kept in 360 L aquaria, at groups of 12 fish, for the duration of the experiment. After an acclimatisation period of 21 days, rainbow trout were exposed to water-borne hydrocortisone (11β,17α 21-trihydroxypregn-4-ene-3,20-dione; Sigma, Steinheim, Germany) in a flow-through system for 21 days (fig. 14). Hydrocortisone (cortisol) was dissolved in dimethylsulfoxid (DMSO, for synthesis, Merck, Darmstadt, Germany) and

diluted with water from Lake Constance to gain stock solutions of 0.91 g/L (2.51 mM) and 0.091 g/L (0.251 mM), both in 49.3 % DMSO. Control groups were either exposed to DMSO (stock solution: 49.3 % DMSO) as a solvent control or lake water only.

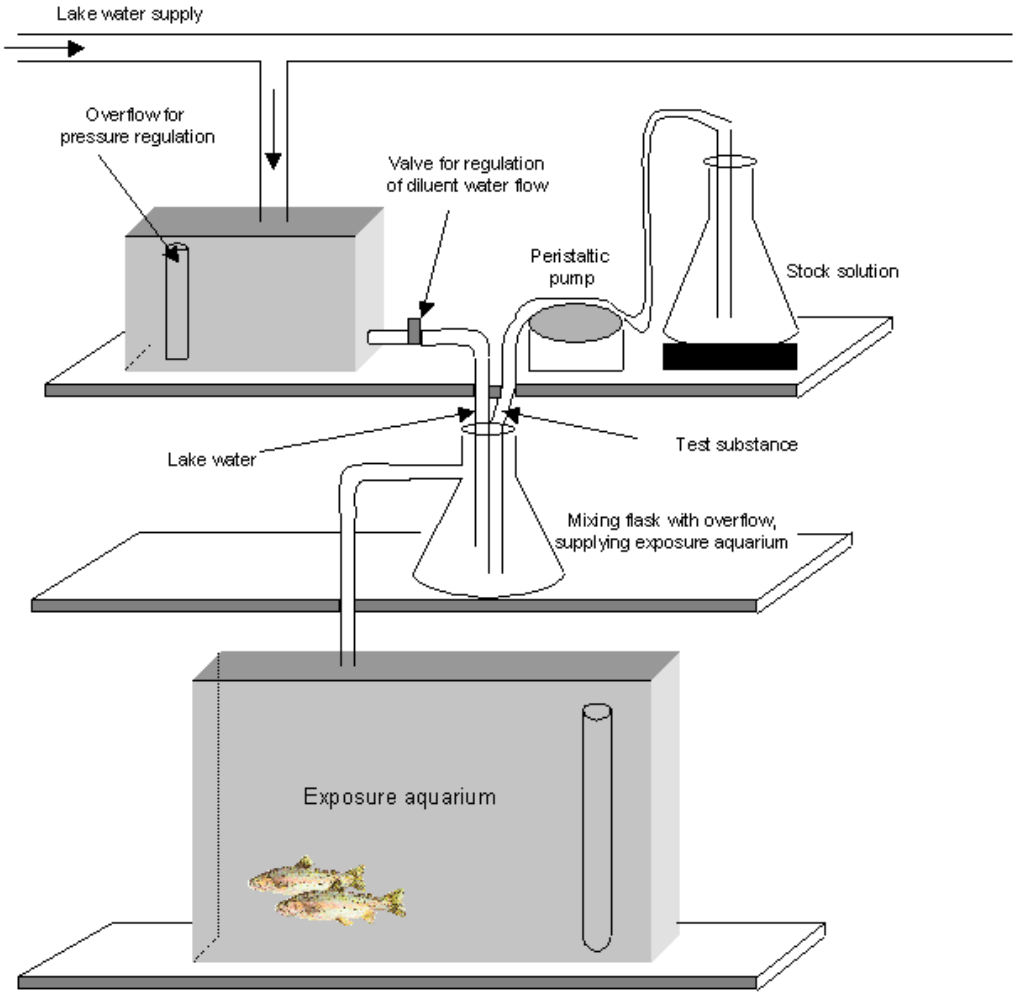


Fig. 14: Exposure of trout to water-borne cortisol in a flow-through system with constant replacement of water and test substance.

Stock solutions were kept in 5 L conical flasks under constant stirring and pumped into mixing flasks at a rate of 2 mL/min with the help of peristaltic pumps. Lake water flow into the mixing flasks was 530 mL/min. Therefore, stock solutions were diluted to final exposure concentrations of 3.43 mg/L (9.47 µM) and 0.343 mg/L (0.947 µM) cortisol in 0.186 % DMSO. According to a flow of 32 L/h from the mixing flasks into the aquaria, replacement time for a complete exchange of water in the aquaria was 48 h. To monitor cortisol

concentrations in the aquaria, daily water samples were frozen at -20°C and later analysed with HPLC.

High Pressure Liquid Chromatography (HPLC) analysis of water samples

For HPLC analysis water samples were filtered through folded filters (595 ½, S&S folded filter, Schleicher&Schuell, Dassel, Germany), to get rid of particles. Hydrocortisone standards were dissolved in 0.2 % DMSO in MQ-H_2O at concentrations of 0.1, 0.5, 1, 5 and 10 mg/L. Liquid phases were 100 % methanol (MeOH) + 0.1 % tri-fluor acetic acid (TFA) and MQ-H_2O + 0.1 % TFA. Separation of standards and samples was performed on a C18 column (Grom-Sil 12 ODS 4 HE, 5µm, Grom, Herrenberg, Germany; HPLC: Beckman System Gold, 125 Solvent Module, 597e Autosampler, Beckman, Muenchen, Germany) using the following elution method:

0-3 min: 30 % MeOH

3-20 min: gradient from 30 % MeOH to 90 % MeOH

20-25 min: 90 % MeOH

25-30 min: gradient from 90 % to 30 % MeOH

Cortisol peaks were detected with a diode array detector (SPD-M10A VP, Shimadzu, Muenchen, Germany) at a wavelength of 254 nm. A correlation coefficient between area under the curve and cortisol concentration was determined for the cortisol standards.

Sampling of trout

Rainbow trout were sampled on two subsequent days, using 6 trout per aquarium per day. Trout were sacrificed and blood was taken from the caudal vein with a syringe. Blood smears were prepared, followed by determination of haematocrit / leucocrit values. Remaining blood was kept overnight at 4°C, centrifuged at 1000 x g, for 15 min, at 4°C and serum was frozen at -80°C, pending further analysis. Fish were weighed and their length determined before further dissection. Liver and spleen were removed and also weighed. Head kidney was kept on ice, in Leibovitz´s L-15 medium (Invitrogen, Karlsruhe, Germany), containing 2 % fetal calf serum (FCS, PAA Laboratories, Coelbe, Germany), 100 U Pen/Strep per mL (Invitrogen, Karlsruhe, Germany) and 53 mU heparin sodium salt/mL (Sigma, Steinheim, Germany) until used for preparation of macrophages.

Growth data

Fulton's condition factor was calculated as body weight ÷ length^3 *100, liver-somatic index (LSI) as liver weight ÷ (body weight - liver weight)*100, and spleen somatic index (SSI) as spleen weight ÷ (body weight - spleen weight)*100.

Peripheral blood parameters (haematocrit, leucocrit and blood cell differentials)

To determine haematocrit and leucocrit values, natrium-heparin treated micro haematocrit capillaries (Roth, Karlsruhe, Germany) were filled with fresh peripheral blood, closed with haematocrit sealing compound (Brand, Wertheim, Germany) and centrifuged at 12000 x g, for 5 min in a Biofuge haemo with a special haematocrit rotor (Heraeus / Kendro Laboratory Products, Hanau, Germany). Haematocrit and leucocrit were measured in percent filling of the capillary with the help of a haematocrit harp (Heraeus / Kendro Laboratory Products, Hanau, Germany).

Blood smears were covered with May Gruenwald solution (modified eosin methylene blue solution in methanol, Merck, Darmstadt, Germany) and incubated for 3 min. Staining solution on the slides was diluted with an equal amount of MQ-H_2O and incubated for another minute, followed by discharging of the staining solution. Giemsa solution (azure eosin methylene blue in methanol, Merck, Darmstadt, Germany) was diluted 1:20 in phosphate buffer, according to Soerensen (stock solution A (67 mM KH_2PO_4) and stock solution B (67 mM Na_2HPO_4 x $2H_2O$) were mixed to a ratio of 1:1.23 and filtered through a paper filter). The slides were then covered with the prepared Giemsa solution and incubated for 15 min. Slides were washed thoroughly with MQ-H_2O and air dried. Pictures of the blood smears were later taken with a SV Micro Sound Vision colour camera (Sound Vision Inc, Boston, USA) on a microscope (Zeiss Axiolab) using Axio Vision Version 2.0.5. (Carl Zeiss Vision GmbH) and per slide a total amount of about 1000 cells were counted on the computer. The different blood cell populations were expressed in percent of total cells counted.

Preparation of head kidney macrophages

The preparation of macrophages from the head kidney was performed according to Secombes (1990) using a Percoll gradient (Amersham Pharmacia, Freiburg, Germany). Percoll was diluted with 10 x HBSS (53.6 mM KCl, 3.4 mM NaH_2PO_4; 4.4 mM KH_2PO_4; 1.45 M NaCl, 100 mM Hepes) (end concentration: 1 x HBSS) and MQ-H_2O to a concentration of 51 % and

34 % respectively. In a 15 mL Falcon-tube 5 mL of 34 % Percoll was layered carefully on 5 mL of 51 % Percoll. Tissue was pushed through 100 µm nylon mesh using rubber policemen and rinsed with L-15 medium. The resulting cell suspension was carefully layered on a prepared Percoll-gradient and centrifuged at 400 x g for 25 min at 4°C. The white band at the gradient interface was collected and washed in L-15 medium. After centrifugation at 188 x g for 5 min (4°C) the cell pellet was resuspended in approximately 3 mL of L-15 medium. The cells were counted in a haemocytometer with trypan blue staining to determine viability. Cells were adjusted to a concentration of 1 x 10^6 cells/mL and seeded into 96-well flat-bottom tissue culture plates (Greiner, Frickenhausen, Germany) at a density of 2.86 x 10^5 cells/cm^2. After incubation at 18°C for 48 h to allow attachment, the cells were used for phagocytosis and oxidative burst assays.

Head kidney macrophage phagocytosis

After attachment of cells, media was removed by inverting the plate and drying carefully on a paper towel. A volume of 100 µL of a 400 µg/mL fluorescein-labeled *E. coli* (K-12 strain, Molecular Probes, Eugene, USA) suspension was added to each well including eight blank wells, which did not contain macrophages. After incubation at 18°C for 2 h, the particles were removed by inverting the plate followed by the addition of 100 µL trypan blue solution (0.025 %). After 1 min of incubation with trypan blue solution, the solution in the wells was removed. Fluorescence was measured in a fluorescence plate reader (FL600 Microplate Fluorescence Reader Bio-Tek Instruments Inc., Vermont, USA) with 485 nm excitation and 520 nm emission filters.

Head kidney macrophage oxidative burst

After macrophage attachment, media was removed by plate inversion and either 500 ng/mL PMA (Sigma, Steinheim, Germany) in HBSS (5.36 mM KCl, 0.34 mM NaH_2PO_4; 0.44 mM KH_2PO_4; 145 mM NaCl, 10 mM Hepes) or HBSS alone was pipetted onto the cells. Measurement of the oxidative burst reaction was started 5 min later by the addition of H_2DCFDA (Sigma, Steinheim, Germany). The time course of H_2O_2-production was measured in a fluorescence plate reader (485 nm excitation and 520 nm emission filters) by detecting the oxidation of H_2DCFDA to DCF over a time period of 15 min. The slope was obtained using the linear portion of the reaction curve, usually 4 to 10 min.

Serum lysozyme activity

The method for measuring serum lysozyme follows the description of Ellis (1990) (turbimetric assay). It is based on the lysis of the gram-positive bacterium *M. lysodeikticus* by serum lysozyme. The lysis of the bacterium can be detected in a spectrophotometer at 530 nm as a decrease in optical density of the *M. lysodeikticus* solution. 950 µL *M. lysodeikticus* solution (Sigma, Steinheim, Germany) (0.5 mg/mL in 0.05 M sodium phosphate buffer (pH 6.2)) were pipetted in a cuvette and measured, followed by the addition of 50 µL of serum or plasma. Absorption was measured after 0.5, 1, 2, 3, 4 and 4.5 min, and lysozyme activity was expressed as decrease of optical density (OD) per min.

Results

HPLC analysis of water samples

Correlation coefficient between area under the curve and cortisol concentration for standards between 0.1 and 10 mg/L was 0.9998. Nominal cortisol concentrations in aquaria were 0.354 mg/L in the lower exposure group (aquarium 2) and 3.54 mg/L in the higher exposure group (aquarium 1), and actual concentrations ranged from 0.18 to 0.477 mg/L and from 1.728 to 4.169 mg/L, respectively (fig. 15).

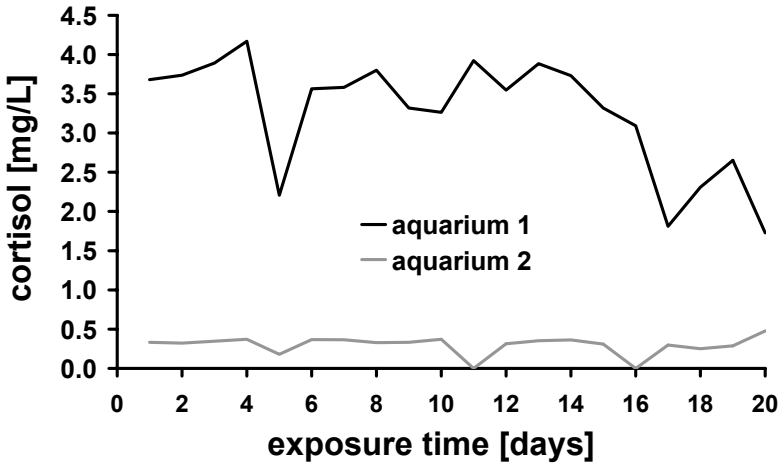


Fig. 15: Cortisol concentration in aquaria, determined by HPLC-analysis. Nominal concentration was 3.54 mg/l for aquarium 1 and 0.354 mg/L for aquarium 2.

Growth data

Condition factor and liver-somatic index were not influenced by exposure to cortisol (data not shown). Spleen-somatic index was significantly elevated after exposure to a nominal cortisol (C) concentration of 3.5 mg/L compared to DMSO exposed fish (fig. 16).

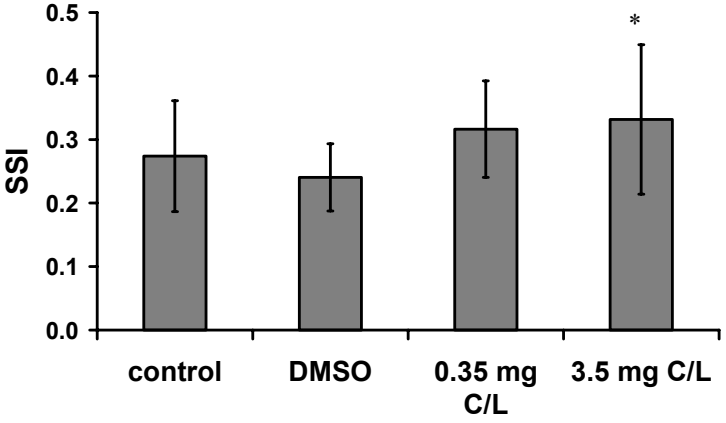


Fig. 16: Spleen-somatic index (SSI) in trout after exposure to nominal cortisol (C) concentrations of 0.35 mg/L and 3.5 mg/L cortisol, or DMSO as solvent control. Shown are mean values with SD. Data was tested with one-way ANOVA with Dunnett´s post test, using DMSO-exposed fish as control. * $p \leq 0.05$, $n \geq 12$

Haematocrit, leucocrit and differential blood cell counts

Exposure of rainbow trout to cortisol for 3 weeks resulted in significantly decreased leucocrit values in peripheral blood, compared to control fish, however, the value was not significantly lower if compared with the DMSO exposed group (fig.17). Haematocrit values were not changed after exposure to cortisol (data not shown).

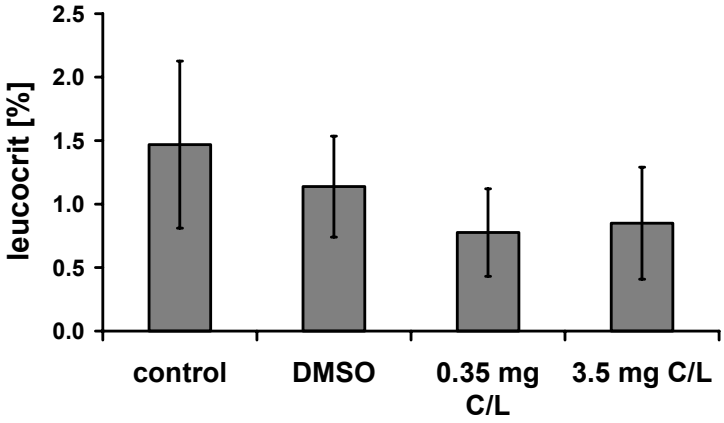


Fig. 17: Leucocrit is given as packed cell volume in percent of blood volume used. Leucocrit values were significantly decreased in trout blood after exposure to 0.35 mg cortisol/L or 3.5 mg cortisol/L compared to control fish. However, the decrease in leucocrit in cortisol exposed fish was not statistically significant if compared to the DMSO group. Shown are mean values with SD. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 12$.

Results for leucocrit values were supported by the analysis of blood smears, also revealing lower peripheral blood leucocyte counts in trout exposed to either concentration of cortisol, compared to control fish (fig. 18a). Lower leucocyte counts were mainly due to a decrease in blood lymphocyte counts (fig. 18b), but thrombocyte numbers were also reduced (fig. 18c). Lymphocyte as well as total leucocyte counts were significantly lower in trout exposed to both concentrations of cortisol, compared to control fish held in lake water, but not compared to DMSO (solvent)-exposed control fish.

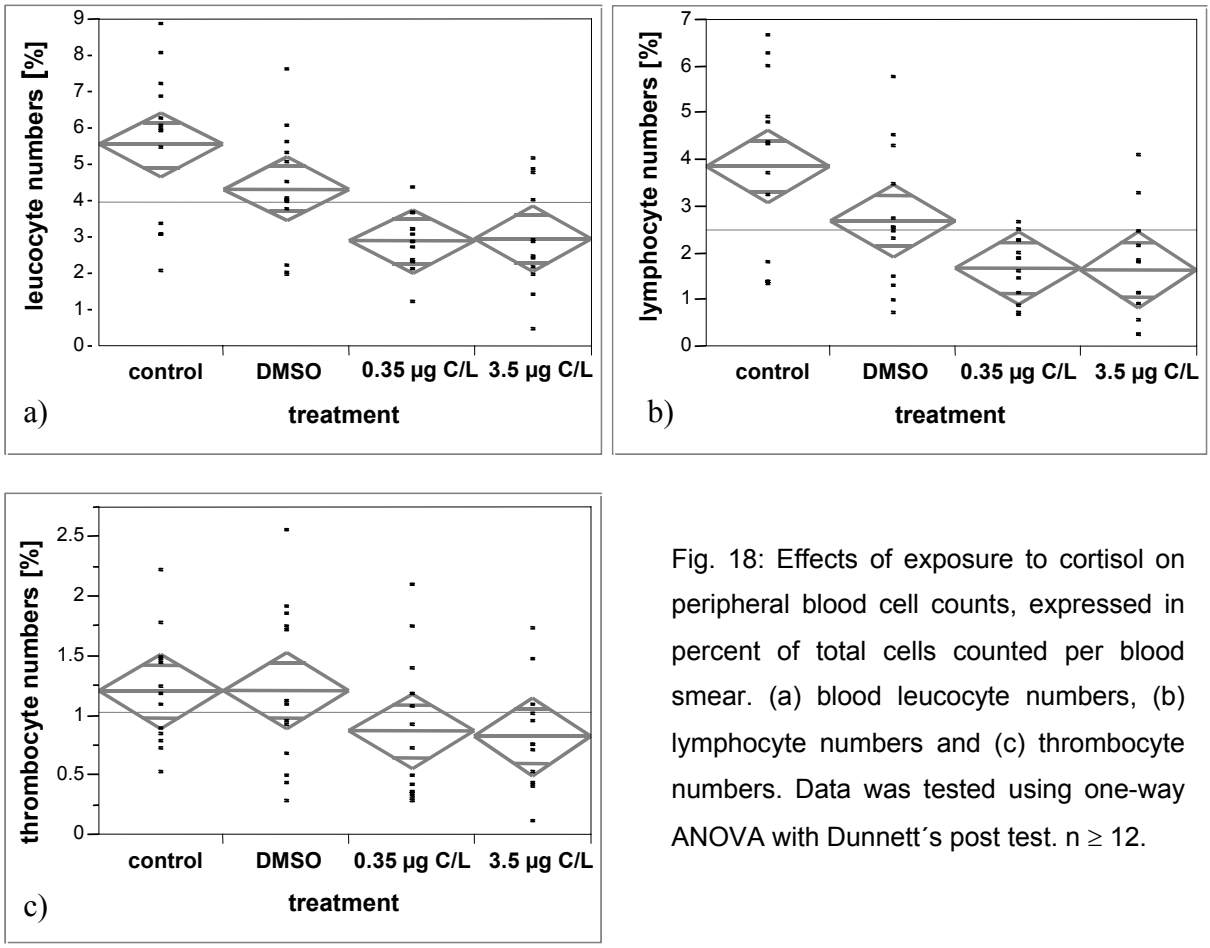


Fig. 18: Effects of exposure to cortisol on peripheral blood cell counts, expressed in percent of total cells counted per blood smear. (a) blood leucocyte numbers, (b) lymphocyte numbers and (c) thrombocyte numbers. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 12$.

Serum lysozyme activity

Exposure of rainbow trout to 3.5 mg cortisol/L resulted in decreased serum lysozyme activity compared to the control group (lake water) and fish exposed to either DMSO or 0.35 mg cortisol/L (fig. 19). However this decrease was not statistically significant.

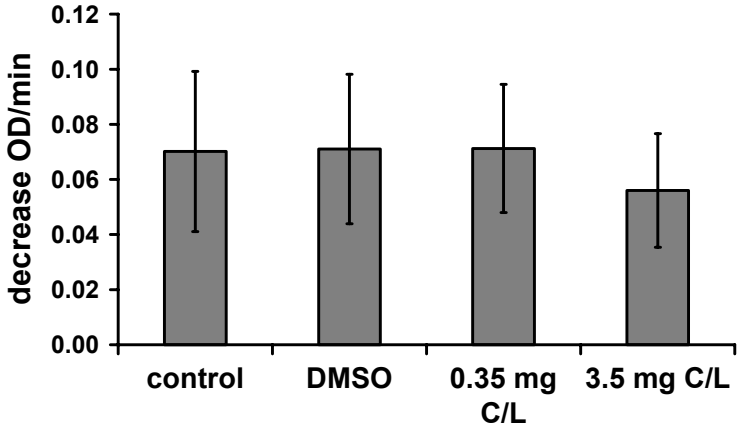


Fig. 19: Serum lysozyme activity in trout after exposure to cortisol, compared to DMSO-exposed fish (solvent control) and fish kept in lake water (control). Shown are mean values with SD. Data was tested using one-way ANOVA with Dunnett´s post test. $n \geq 12$.

Head kidney macrophage phagocytosis and oxidative burst

Phagocytotic and oxidative burst activity of head kidney macrophages were not influenced by exposure of fish to cortisol.

Discussion

Immunomodulating activity of external cortisol as well as internal cortisol, induced by stress has been shown in fish before. In fish, as well as in mammals, cortisol leads to profound leucopenia (Verburg-van Kemenade et al., 1999; Wojtaszek et al., 2002). In accordance with the literature, we found significantly reduced leucocyte levels in rainbow trout peripheral blood, after exposure to cortisol. Lower leucocyte counts were mainly due to a decrease in lymphocyte numbers. Effects of cortisol on lymphocyte activity and viability have been observed before, reflected in suppression of mitogen-stimulated proliferation and induction of apoptosis in this cell type (Verburg-van Kemenade et al., 1999; Weyts et al., 1997). Additional to the effects on leucocyte numbers, cortisol also slightly suppressed serum lysozyme activity in the trout exposed to a nominal concentration of 3.5 mg/L, however, this suppression was not significant. Unlike in other investigations, we could not find an effect of cortisol on macrophage phagocytosis and oxidative burst (Wang & Belosevic, 1995;

Narnaware et al., 1994). However, this might be due to the difference in exposure route, possibly leading to lower internal cortisol concentrations (in head kidney) and / or different distribution in fish exposed via the water.

Beside looking at immune reactions we also assessed common physiological parameters, like condition factor, liver somatic index and spleen index and found a significant increase of spleen weight after exposure to cortisol. The mechanism of action, leading to an increase in spleen weight could not be elucidated in this experiment. As the spleen is a haematopoietic organ, the effect of cortisol on spleen weight might reflect an influence on immune cells. In most cases, an increase in organ weight is due to cell proliferation or activation of enzymatic activity.

To our knowledge, this is the first study to show effects of cortisol on the immune system of fish after exposure via the water. However, the influence of cortisol on rainbow trout immune reactions is not as pronounced as shown in other studies. The failure to demonstrate an effect of cortisol on macrophage activity is most likely due to the exposure route, possibly leading to lower internal cortisol concentrations. As uptake and distribution of cortisol in the organism was not analysed in this study, a clear statement on dose-effect relationships cannot be made. It can however be assumed that cortisol concentrations in trout head kidney were not high enough to induce the inhibiting effect on macrophage phagocytosis and oxidative burst, found in other studies, in which cortisol was applied via injection or implantation, or isolated macrophages were exposed *in vitro* (Wang & Belosevic, 1995; Narnaware et al., 1994).

Although exposure of trout to cortisol via the water does not reflect a real life situation, this study demonstrates the ability of water-borne pollutants to affect the fish immune system. As contamination of our surface waters with a wide variety of substances, including pharmaceuticals, ingredients of health care products and industrial chemicals, becomes more prevalent, immunomodulating activity of such pollutants in aquatic organisms should be assessed. In spite of an increasing interest in the investigation of immune competence within the field of aquatic toxicology, useful exposure-effect relationships for different substance classes could not be established. This might be due to the complexity of the immune system and a general lack of knowledge on functioning and regulation of fish immune reactions. Moreover, the existence of mixed-substance pollution in our surface waters further complicates the assessment of effects on immune competence of aquatic organisms. Therefore, the investigation of model substances with supposed or known immunomodulating activity can only give a superficial idea of possible effects of water contamination with immunological active substances. Further investigations are needed to enable the inclusion of

immune parameters into the assessment of water pollution and its adverse effects on aquatic organisms.

Effects of rifampicin on immune parameters in rainbow trout (*Oncorhynchus mykiss*) exposed via the water

Immunosuppressive activity of the antibiotic rifampicin has been observed in patients undergoing anti-tuberculosis therapy as well as in laboratory animals, including rats, mice and rabbits. Effects of rifampicin on the immune system of fish have not been investigated so far, as exposure of fish to this antibiotic seems unlikely. In this study, rifampicin has been used as a model substance, with known immunosuppressive activity in other vertebrates, to characterise possible immunosuppressive effects in rainbow trout (*O. mykiss*). Trout have been exposed to water-borne rifampicin at nominal concentrations of 4.27 and 0.427 mg/L over a period of 21 days. Immune parameters measured were haematocrit and leucocrit, blood cell differentials, phagocytosis and oxidative burst in head kidney macrophages and serum lysozyme activity. A suppression of leucocrit and serum lysozyme activity was observed in rainbow trout exposed to the higher rifampicin concentration. The significance of this finding is discussed in relation to aquatic contamination and immunotoxicology.

Introduction

Rifampicin is an antibiotic which has been used for the treatment of tuberculosis since the 1970s. In mammals, adverse side-effects of rifampicin on immune reactions have been observed. In humans, several formulations of rifampicin have been shown to inhibit *in vitro* neutrophil chemotaxis in the range of their therapeutic levels (Bersani et al., 1987; Van der Auwera & Husson, 1989). Van der Aurewa & Husson found that the inhibition of random migration could be explained by a significantly increased adherence of the human neutrophils to polysterene after administration of rifampicin. Rifampicin has also been shown to significantly inhibit *in vitro* human monocyte oxidative metabolism in a dose-dependent manner at therapeutic concentrations (Nielsen, 1989). *In vitro* lymphoproliferative response of human lymphocytes stimulated with mitogens and *in vitro* synthesis of polyclonal immunoglobulin in human peripheral blood cells have also been reduced after exposure to rifampicin (Ibrahim et al., 1987). Beside observations of immunosuppressive activity of rifampicin in patients and *in vitro* studies with human immune cells, effects of rifampicin on the immune system have also been investigated in different animal models. Using the split-heart allograft technique, Bellahséne and Forsgren (1980) have demonstrated a delay in graft rejection in mice after daily application of a therapeutic dose of rifampicin. In this study, the number of plaques of haemolysis and the humoral antibodies to sheep erythrocytes were also

reduced by a human therapeutic dose. Serrou et al. (1972) also reported longer survival time of skin allografts in rabbits treated with rifampicin.

Effects of rifampicin in fish have not been investigated so far, as direct contact of fish with this antibiotic is unlikely. In this study, rifampicin has been chosen as a reference substance with supposed immunosuppressive activity to help establish an image of possible immunomodulation in fish after exposure to substances via the water. Test methods used to assess putative immunomodulating activity of rifampicin exposure in rainbow trout were haematocrit and leucocrit, blood cell differentials, phagocytosis and oxidative burst in head kidney macrophage and serum lysozyme activity. Beside these immune parameters, growth factors, including Fulton's condition factor, liver-somatic index (LSI) and spleen somatic index (SSI) were assessed.

Material and Methods

Experimental set up

Rainbow trout (*O. mykiss*) were purchased from the hatchery Hugo Strobel (Hausen am Andelsbach, Germany) and kept in 360 L aquaria, at groups of 12 fish, for the duration of the experiment. After an acclimatisation period of 21 days, rainbow trout were exposed to water-borne rifampicin (kindly provided by Gruenenthal GmbH, Aachen, Germany) in a flow-through system for 21 days (scheme of exposure facility see fig. 14, chapter V). Rifampicin was dissolved in DMSO (for synthesis, Merck, Darmstadt, Germany) and diluted with water from Lake Constance to gain stock solutions of 1.132 g/L (1.38 mM) and 0.1132 g/L (0.138 mM), both in 4.52 % DMSO. Control groups were either exposed to DMSO (stock solution: 4.52 % DMSO) as a solvent control or lake water only. Stock solutions were kept in 5 L conical flasks under constant stirring and were pumped into mixing flasks at a rate of 2 mL/min with the help of peristaltic pumps. Lake water flow into the mixing flasks was 530 mL/min. Therefore, stock solutions were diluted to 4.27 mg/L (5.20 µM) and 0.427 mg/L (0.52 µM) rifampicin in 0.017 % DMSO. According to a flow of 32 L/h from the mixing flasks into the aquaria, replacement time for a complete exchange of water in the aquaria was approximately 48 h.

Sampling of trout

Rainbow trout were sampled on two subsequent days, using 6 trout per aquarium per day. Trout were sacrificed and blood was taken from the caudal vein with a syringe. Blood smears, for latter assessment of blood cell differentials, were prepared, followed by determination of haematocrit / leucocrit. Remaining blood was kept overnight at 4°C, centrifuged at 1000 x g, for 15 min, at 4°C and serum was frozen at –80°C, pending further analysis. Fish were weighed and their length determined before further dissection. Liver and spleen were removed and also weighed. Head kidney was kept on ice in Leibovitz´s L-15 medium (Invitrogen, Karlsruhe, Germany), containing 2 % fetal calf serum (FCS, PAA Laboratories, Coelbe, Germany), 1 U Pen/Strep per mL (Invitrogen, Karlsruhe, Germany) and 53 mU heparin sodium salt/mL (Sigma, Steinheim, Germany) until used for preparation of macrophages.

Growth data

Fulton's condition factor was calculated as body weight ÷ length^3 *100, liver-somatic index (LSI) as liver weight ÷ (body weight - liver weight)*100, and spleen somatic index (SSI) as spleen weight ÷ (body weight - spleen weight)*100.

Peripheral blood parameters (haematocrit, leucocrit and blood cell differentials)

To determine haematocrit and leucocrit values, natrium-heparin treated micro haematocrit capillaries (Roth, Karlsruhe, Germany) were filled with fresh peripheral blood, closed with haematocrit sealing compound (Brand, Wertheim, Germany) and centrifuged at 12000 x g, for 5 min in a Biofuge haemo with a special haematocrit rotor (Heraeus / Kendro Laboratory Products, Hanau, Germany). Haematocrit and leucocrit were measured in percent filling of the capillary with the help of a haematocrit harp (Heraeus / Kendro Laboratory Products, Hanau, Germany).

Blood smears were covered with May Gruenwald solution (modified eosin methylene blue solution in methanol, Merck, Darmstadt, Germany) and incubated for 3 min. Subsequently, the staining solution on the slides was diluted with an equal amount of MQ-H_2O and incubated for another minute, followed by discharging of the staining solution. Giemsa solution (azure eosin methylene blue in methanol, Merck, Darmstadt, Germany) was diluted 1:20 in phosphate buffer, according to Soerensen (stock solution A (67 mM KH_2PO_4) and stock solution B (67 mM Na_2HPO_4 x $2H_2O$) were mixed to a ratio of 1:1.23 and filtered

through a paper filter). The slides were then covered with the prepared Giemsa solution and incubated for 15 min. Slides were washed thoroughly with MQ-H_2O and air dried. Pictures of the blood smears were later taken with a SV Micro Sound Vision colour camera (Sound Vision Inc, Boston, USA) on a microscope (Zeiss Axiolab) using Axio Vision Version 2.0.5. (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) and per slide a total amount of about 1000 cells were counted on the computer. The different blood cell populations were expressed in percent of total cells counted.

Preparation of head kidney macrophages

The preparation of macrophages from the head kidney was performed according to Secombes (1990) using a Percoll gradient (Amersham Pharmacia, Freiburg, Germany). Percoll was diluted with 10 x HBSS (53.6 mM KCl, 3.4 mM NaH_2PO_4; 4.4 mM KH_2PO_4; 1.45 M NaCl, 100 mM Hepes) (end concentration: 1 x HBSS) and MQ-H_2O to a concentration of 51 % and 34 % respectively. In a 15 mL Falcon-tube 5 mL of 34 % Percoll was layered carefully on 5 mL of 51 % Percoll. Head kidney tissue was pushed through 100 µm nylon mesh using a rubber policeman and rinsed with L-15 medium. The resulting cell suspension was carefully layered on a prepared Percoll-gradient and centrifuged at 400 x g for 25 min at 4°C. The white band at the gradient interface was collected and washed in L-15 medium. After centrifugation at 188 x g for 5 min (4°C) the cell pellet was resuspended in approximately 3 mL of L-15 medium. The cells were counted in a haemocytometer with trypan blue staining to determine viability. Cells were adjusted to a concentration of 1 x 10^6 cells/mL and seeded into 96-well flat-bottom tissue culture plates (Greiner, Frickenhausen, Germany) at a density of 2.86 x 10^5 cells/cm^2. After incubation at 18°C for 90 min to allow attachment, the cells were used for phagocytosis and oxidative burst assays.

Head kidney macrophage phagocytosis

After attachment of cells, media was removed by inverting the plate and drying carefully on a paper towel. A volume of 100 µL of a 400 µg/mL fluorescein-labeled *E. coli* (K-12 strain, Molecular Probes, Eugene, USA) suspension was added to each well including eight blank wells, which did not contain macrophages. After incubation at 18°C for 2 h the particles were removed by inverting the plate followed by the addition of 100 µL trypan blue solution (0.025 %). After 1 min of incubation with trypan blue solution, the solution in the wells was removed. Fluorescence was measured in a fluorescence plate reader (FL600 Microplate

Fluorescence Reader, Bio-Tek Instruments Inc., Vermont, USA) with 485 nm excitation and 520 nm emission filters.

Head kidney macrophage oxidative burst

After macrophage attachment, media was removed by plate inversion and either 500 ng/mL PMA (Sigma, Steinheim, Germany) in HBSS (5.36 mM KCl, 0.34 mM NaH_2PO_4; 0.44 mM KH_2PO_4; 145 mM NaCl, 10 mM Hepes) or HBSS alone was pipetted onto the cells. Measurement of the oxidative burst reaction was started five minutes later by the addition of 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma, Steinheim, Germany). The time course of H_2O_2-production was measured in a fluorescence plate reader (485 nm excitation and 520 nm emission filters) by detecting the oxidation of H_2DCFDA to DCF over a time period of 15 min. The slope was obtained using the linear portion of the reaction curve, usually 4 to 10 min.

Serum lysozyme activity

The method for measuring serum lysozyme follows the description of Ellis (1990) (turbimetric assay). It is based on the lysis of the gram-positive bacterium *M. lysodeikticus* by serum lysozyme. The lysis of the bacterium can be detected in a spectrophotometer at 530 nm as a decrease in optical density of the *M. lysodeikticus* solution. 950 µL *M. lysodeikticus* solution (Sigma, Steinheim, Germany) (0.5 mg/mL in 0.05 M sodium phosphate buffer, pH 6.2) were pipetted in a cuvette and measured, followed by the addition of 50 µL of serum or plasma. Absorption was measured after 0.5, 1, 2, 3, 4 and 4.5 min and lysozyme activity was expressed as decrease of optical density per min.

Results

Growth data

Condition factor, liver weight and spleen weight of rainbow trout were not influenced by exposure to rifampicin via the surrounding water (data not shown).

Head kidney macrophage phagocytosis and oxidative burst

Head kidney macrophage phagocytosis and oxidative burst were both not influenced by exposure to rifampicin in the water (data not shown).

Haematocrit, leucocrit and blood cell differentials

Exposure of rainbow trout to a nominal rifampicin (rif) concentration of 4.27 mg/L resulted in lower peripheral blood leucocrit levels. However, this result is only significant, if compared to the lake water control, but not compared to the solvent (DMSO) control (fig. 20).

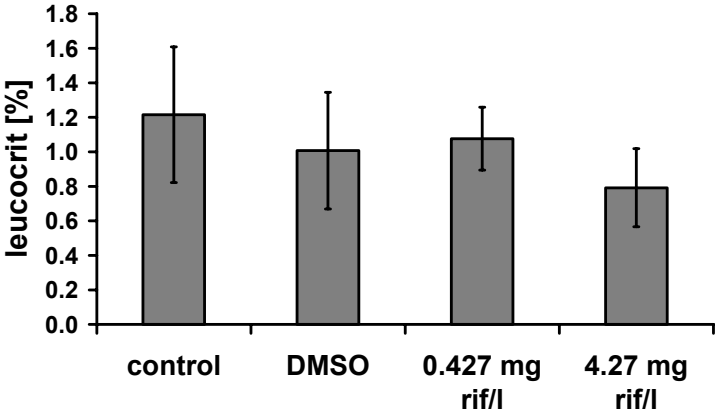


Fig. 20: Leucocrit values, given in percent of blood volume, after exposure to rifampicin (rif). Shown are mean values with SD; n = 12. Data was tested using one-way ANOVA with Dunnett´s post test.

Exposure to either concentration of rifampicin or the solvent control did not significantly change haematocrit values. Peripheral blood cell counts were not influenced by exposure to rifampicin.

Serum lysozyme activity

Exposure of rainbow trout to 4.27 mg rif/L resulted in a significantly decreased serum lysozyme activity compared to control fish, kept in lake water and trout exposed to DMSO (solvent control) (fig. 21).

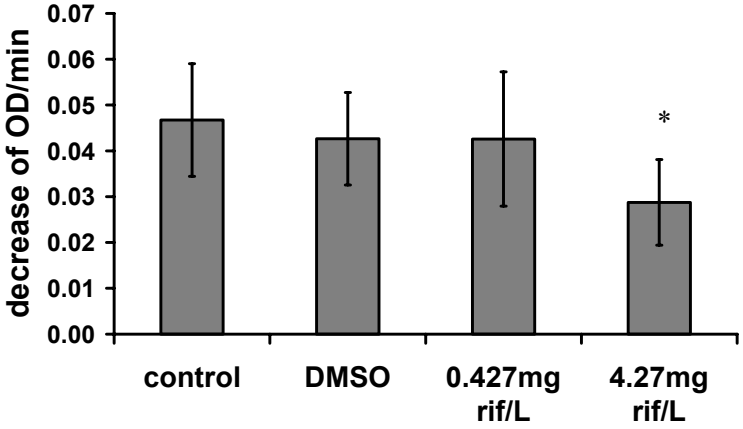


Fig. 21: Effects of rifampicin on lysozyme activity in trout serum, measured as decrease of optical density (OD) of a *M. lysodeikticus* solution. Shown are mean values with SD; $n \geq 11$; * $p \leq 0.05$. Data was tested using one-way ANOVA with Dunnett´s post test.

Discussion

In the study at hand, rifampicin has been shown to elicit immunomodulating activity in rainbow trout after exposure to high concentrations via the water. Serum lysozyme activity was significantly depressed by a nominal concentration of 4.27 mg rifampicin/L. Serum lysozyme is an unspecific immune parameter, which has been shown to react to a variety of substances, environmental pollutants (Karrow et al., 1999; Tahir et al., 1993; Price et al., 1997) and stress (Yin et al., 1995; Ruane et al., 1999; Caruso et al., 2002), as well as internal and environmental factors, like sexual development (Fletcher et al., 1977) and temperature (Hutchinson & Manning, 1996). Therefore, this immune parameter cannot be regarded as specifically indicative of exposure to a certain contaminant or substance class in surface waters. However, serum lysozyme levels can be used as a general indicator for environmental contamination and suppression of this immune parameter by rifampicin shows a possible effect of such a substance on the immune system of aquatic animals, exposed via the water.

Beside serum lysozyme activity, the only other immune parameter found to be affected by rifampicin exposure were blood leucocrit values. However, the decrease in leucocrit in the group exposed to the higher rifampicin concentration was only significant, if compared to control fish kept in lake water, but not if compared to trout exposed to DMSO as a solvent control. Moreover did the analysis of blood smears not confirm the finding of lower leucocrit values. Therefore, this effect could not be characterised in more detail and its implications remain unidentified.

In our fish model, effects of rifampicin on macrophage activity could not be shown. This finding does not correspond to the immunosuppressive effects of rifampicin demonstrated in humans and mammalian animal models. However, differences in immunomodulating activity of rifampicin might not only be due to species differences, but could be caused by the different exposure route, which possibly led to lower substance concentrations or different distribution in the body.

To our knowledge, this study is the first to show an effect of an antibiotic on the fish immune system after exposure via the water. Although uptake and distribution of rifampicin in trout tissue was not investigated, this study suggests a significant uptake of this antibiotic, leading to suppression of an unspecific humoral immune parameter. This finding might be of low relevance for the real life situation, as exposure of fish to such high concentrations of rifampicin in our surface waters seems unlikely. Rifampicin is no longer used to a great extent in chemotherapy nowadays and has not been detected in the aquatic environment. However,

several other antibiotics have been found in sewage effluents and surface water samples, including an erythromycin degradation product, roxithromycin and sulfamethoxazole (Hirsch et al., 1999). Immunosuppressive effects of antibiotics in fish have been demonstrated for oxolinic acid (OA), oxytetracycline (OTC) and florfenicol (FF). These antibiotics inhibited *in vitro* lymphocyte proliferation in a dose-dependent manner, with FF being the most effective antibiotic tested in this study (Lundén & Bylund, 2000). It was also shown that these antibiotics lead to suppressed mitogenic response of the head kidney cells, after oral administration of a therapeutic dose (Lundén & Bylund, 2000).

Although the exposure of trout to such high concentrations of rifampicin used in this study, might not reflect a real life situation, our findings support concerns about putative effects of human pharmaceuticals, including antibiotics, on the immune competence of fish. Further investigations on immunomodulating activity of pharmaceuticals in our surface waters, possibly affecting fish health, are therefore warranted.

General discussion

The health of aquatic environments is not only important for the animals living in our surface waters, but also lays the foundation for human life. Not only are we dependent on clean drinking water, which, in many regions, is gained from surface waters, but aquatic animals moreover constitute an important part of our food. In spite of considerable improvement in water quality in Central Europe since the 1980s, pollution of our surface waters and subsequent adverse effects on aquatic life are an ongoing concern. In recent years, low level contamination of rivers and lakes with a mixture of substances has moved into the centre of attention within aquatic toxicology (Jones et al., 2001; Ternes, 1998; Daughton, 2003). Mostly, these low concentrations of chemicals do not lead to acute toxic effects. However, several pollutants have been found to disrupt sensitive mechanisms in animals, including endocrine regulation, at the low concentrations found in surface waters (Allen et al., 1999; Bennie, 1999; Desbrow et al., 1998). To date, the potential of a broad array of substances to influence hormonal regulation of sexual development and reproduction has been thoroughly investigated. Several aquatic pollutants, including pharmaceuticals, ingredients of household products and industrial chemicals, have been found to display hormone-like activity (Matthiessen & Sumpter, 1998; Spies & Rice, 1988). These substances thus have the potential to disrupt normal development and reproduction, which in turn might endanger the survival of animal populations. Concerns about adverse effects of water pollution on reproduction in aquatic animals has been complemented by the discovery, that impairment of immune mechanisms through exposure to low level contaminants might be equally harmful to aquatic organisms. Several pollutants have since been suggested to affect immune reactions in aquatic animals, with the main focus set on fish (Sharma & Zeeman, 1980; Bols et al., 2001). Low level contamination of water with immunological active substances might lead to decreased immune competence, ultimately resulting in higher disease prevalence in fish populations. Consequently, the inclusion of immune parameters in monitoring aquatic health has been suggested by several scientists, in the field of environmental toxicology (Zelikoff et al., 2000; Sharma & Zeeman, 1980).

The aim of the doctoral thesis at hand was to gain a more detailed insight into immunotoxicology in rainbow trout (*O. mykiss*) and to evaluate different test parameters. In order to achieve this, rainbow trout were exposed to two different substances with known immunosuppressive activity in mammalians, namely hydrocortisone and rifampicin and to municipal sewage treatment effluent, reflecting a complex mixture exposure. Rainbow trout

was chosen as the test organism because of its status as the best investigated fish species in the field of fish immunology, which in turn is partly due to its commercial value.

Effects of rifampicin and cortisol on selected immune parameters in rainbow trout

The investigations with cortisol and rifampicin are of minor relevance in context with environmental pollution, as exposure of aquatic animals to these substances is unlikely. Exposure of trout to cortisol and rifampicin was chosen as a model to examine the possibility of water-borne substances, with known immunosuppressive potential, to influence immune reactions in fish.

Effects of cortisol on fish immune parameters have been thoroughly studied, mainly in connection with its function as a stress mediator (Verburg-van Kemenade et al., 1999; Narnaware et al., 1994; Espelid et al., 1996; Davis et al., 2003). In agreement with other investigations and with its described function, cortisol was shown to reduce leucocyte numbers in peripheral blood in the study at hand, mainly reflected in a decrease in lymphocyte counts. Serum lysozyme levels were also slightly reduced. Cortisol was moreover observed to increase spleen weight, which might also reflect an effect on the immune system, as spleen is an important haematopoietic organ. However, the mechanism leading to spleen enlargement could not be elucidated in this study. In contrast to other studies with cortisol, an effect on macrophage phagocytosis or oxidative burst was not observed (Narnaware et al., 1994). This might be due to the difference in exposure route, possibly leading to lower cortisol concentrations in head kidney, compared to stressing of fish or application via injection, implantation or feeding.

Exposure of trout to water-borne rifampicin was found to decrease serum lysozyme activity. The other immune parameters investigated in this study were not influenced by rifampicin. Due to the lack of investigations in fish, the results of this study can only be compared to observations in mammalian test systems. Human monocytes e.g. showed decreased oxidative burst *in vitro* at therapeutic doses (Nielsen, 1989). In contrast to these findings, exposure of trout to rifampicin via the water did not affect macrophage activity. However, *in vitro* studies in our lab also revealed a suppressive effect of rifampicin on macrophage oxidative burst. It can be assumed, that rifampicin concentrations in head kidney after exposure via the water were not high enough to elicit any effects on macrophage activity.

Effects of sewage treatment effluent on selected immune parameters in rainbow trout

While effects of several chemical pollutants, including metals, pesticides, PAHs and PCBs on the fish immune system have been investigated at some length (see general introduction, chapter I), studies on immunologic effects of mixed substance pollution are scarce to date. Some studies have focused on pulp and paper mill effluents (Ahmad et al., 1998; Aaltonen et al., 1997, 2000a, 2000b; Fatima et al., 2001; Lappivaara & Oikari, 1999) or waters, which were already known to be seriously contaminated with industrial chemicals (Weeks & Warinner, 1986). To our knowledge, only two studies so far have focused on effects of sewage treatment effluent on fish immune reactions (Price et al., 1997; Kakuta, 1997). While the study by Price and coworkers was conducted in the field, the investigations, presented here are, together with the study by Kakuta, the first to employ exposure of fish to defined sewage effluent concentrations in a controlled lab situation.

Exposure of trout to 10, 30 and 70 % effluent over a period of 4 weeks, was conducted to assess acute effects of high sewage concentrations on the immune system. In this experiment, exposure to high concentrations of effluent (30 and 70 %) was shown to affect peripheral blood cell counts, along with liver cytochrome P450 1A (EROD) activity. The other immune parameters measured, including serum lysozyme activity, macrophage oxidative burst, macrophage phagocytotic activity, and serum levels of *A. salmonicida* specific antibodies were not observed to be influenced by acute exposure to sewage effluent. However, as specific antibody production in trout is a very slow process, the lack of an observed effect in this parameter may be due to the sampling point in time. Production of *A. salmonicida* specific antibodies in rainbow trout has been shown to only start after 18 days, depending on the amount of bacteria injected, while considerable levels are observed as late as 30 days after challenge (Kollner et al., 2002). In contradiction to the findings from Kollner and coworkers, serum antibody levels in the majority of the trout, investigated after 28 days in our acute exposure study, were still very low, which might have prevented the assessment of possible differences between the exposure groups. A possible explanation for the low antibody levels, found in our study, might be a lower reactivity of the rainbow trout strain used, causing a further delay in antibody production.

Chronic exposure of rainbow trout (over 32 weeks) to 1.5 and 15 % effluent closely resembles a real life situation and was shown to influence immune reactions along with growth and endocrine-reproductive parameters, as well as mixed-function oxygenase activity. Parameters affected in this experiment include serum lysozyme activity, specific antibody production and

prevalence and distribution of leucocytes in haematopoietic organs, as well as liver EROD activity, liver size, steroid hormone levels, gonad size and fecundity. However, like in the acute experiment with high effluent concentrations, an influence of effluent on macrophage activity could not be demonstrated. In contrast to the acute exposure, effects on peripheral blood cell counts could not be shown in the chronic exposure experiment.

In general, effects of acute exposure with high effluent concentrations and chronic exposure with low, realistic concentrations on selected immune parameters in rainbow trout could be demonstrated. It can be assumed that acute exposure to high concentrations of polluted water over a short time period might affect other immune reactions than chronic exposure with low concentrations. As the immune system is a highly dynamic system, with activation and down-regulation of different reactions at different points in time, assessment of adverse effects on the system at a single point in time, most likely does not cover all reactions and influences, happening during the whole exposure. With the current state of knowledge on mechanisms in fish immunotoxicity, elaborate cause and effect relationships can hardly be defined. Although the exposure studies presented in this thesis make a valuable contribution to the assessment of hazardous environmental pollution, by clearly demonstrating adverse effects of STP effluent on immune reactions in trout, valid cause and effect patterns could not be identified. A clear relation between type of exposure, namely short-term exposure to high effluent concentrations versus chronic exposure to lower concentrations, and effects on different immune reactions cannot be deduced, due to a lack of background knowledge on mechanisms and a more detailed insight into time courses of immune reactions.

Possible (immuno-) toxic substances in STP effluent

Within the scope of the thesis at hand a detailed chemical analysis of the STP effluent used for the exposure experiments could not be conducted. Basic chemical analysis of final effluent at the sewage treatment plant, includes regular monitoring of ammonia nitrogen (NH_4-N). Ammonia is known to be acutely toxic to fish, however, definite toxicity levels for ammonia are difficult to assess, due to the influence of several parameters on toxicity, including temperature and pH, and different susceptibility of fish species (Hrubec et al., 1996). During the long-term exposure study, elevated ammonia concentrations in final effluent caused acute toxicity in trout, resulting in mortality at two occasions. Concerning effects on the immune system, exposure of rainbow trout (*O. mykiss*) to sublethal ammonia concentrations has been shown to reduce survival rates after infection with a virulent *Streptococcus iniae* strain

(Hurvitz et al., 1997). As no correclation between antibody titres and exposure to ammonia and protection could be found, Hurvitz and coworkers concluded that the decrease of protection against *S. iniae* in ammonia exposed trout could be attributed to suppression of the cellular or the non-specific defence mechanisms or to the effect of ammonia on other physiological systems. Adverse effects of ammonia on the immune system have also been demonstrated in carp (*C. carpio*) infected with blood fluke (*Sanguinicola inermis*) (Schuwerack et al., 2001). In this study, integrity of thymus and head kidney tissue was impaired, along with alterations in leucocyte numbers in both organs and effects on proliferation of head kidney lymphocyte. Intermittent high ammonia concentrations in the course of exposure to effluent can thus be expected to affect immune parameters in trout. However, a clear association between exposure of trout to ammonia and adverse effects on immune reactions found in our studies with effluent, namely decrease in serum lysozyme activity, lower periperhal blood leucocyte numbers and lower A.s.s.-specific serum antibody levels, cannot be established.

Heavy metal content of effluent is measured every 6 months at the Rotorua STP, including assessment of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), zinc (Zn) mercury (Hg) and lead (Pb), for which effects on the fish immune system have been observed (reviewed by Zelikoff, 1993). Chronic exposure to chromium and copper has e.g. been shown to greatly reduce serum antibody levels in carp (*C. carpio*) and brown trout (*S. trutta*) immunized with MS2 bacteriophages (O´Neill, 1981a) and to increase susceptibility of rainbow trout to infectious haematopoietic necrosis virus (Hetrick et al., 1979) at concentrations, which could also be found in effluent used in our exposure studies (STP, Rotorua, New Zealand). In this study, suppression of serum antibody levels was also shown after exposure to Ni and Zn, however, the exposure concentrations of these metals were markedly higher than amounts found in the effluent from the Rotorua STP. In most of the studies, in which effects of metals on immune reactions in fish have been shown, exposure concentrations clearly exceeded concentrations measured in the Rotorua STP effluent.

Due to a lack of chemical analysis, specific for known immunotoxic substances and scarcity of data on possible effects of chemical parameters, such as ammonia content, on the fish immune system, alterations in immune reactions, found in the thesis at hand, could not clearly be related to possible causing compounds. More detailed chemical analysis of effluent along with extensive laboratory studies on the immunotoxicity of a broad array of substances would be necessary to elucidate cause and effect patterns.

Test methods

In order to include investigations on immunotoxicity into monitoring of environmental pollution, test methods have to meet several criteria. Beside sensitivity of the chosen immune parameters to different pollutants, applicability and reliability are essential to successful use. In the thesis at hand, several test methods, covering various parts of the immune system, have been applied in experiments with sewage treatment effluent, as an example for mixed pollution, as well as in studies with model substances (single substance exposure) known to elicit immunomodulating effects. Exposure of rainbow trout to sewage treatment effluent has been shown to alter lysozyme activity, peripheral blood leucocyte counts, erythrocyte viability, leucocyte distribution in spleen and specific antibody production.

Beside being sensitive to exposure to sewage treatment effluent, serum lysozyme activity has moreover been demonstrated to react on exposure to rifampicin and cortisol. Along with its sensitivity to different substances and pollutants, the fact that measurement of serum lysozyme activity is an inexpensive, relative fast and reliable, easy-to-handle test method makes it a promising parameter to be included into monitoring of immunotoxicity in aquatic toxicology.

In this thesis, trout peripheral blood cell differentials have been observed to react on exposure to STP effluents, as well as exposure to cortisol. Moreover, in the literature several substances and pollutants are described to influence blood leucocyte counts. Blood cell differentials should therefore be considered as a useful, basic immune parameter in investigations on adverse effects of environmental contamination on the immune competence of aquatic organisms. However, test methods, which are at our disposal are either time consuming (counting of cells) or expensive and demanding (FACS-analysis).

Immuno-histochemical analysis, assessing occurrence and distribution of leucocyte populations in haematopoietic organs, has only been conducted with tissue samples from trout chronically exposed to sewage effluent. Effects on prevalence and distribution of leucocytes in spleen tissue could be demonstrated, along with a high prevalence of deposits or inclusions with strong autofluorescence. Although, the identity of these deposits could not be elucidated, their presence suggests an adverse effect of exposure to STP effluent on haematopoietic tissue. Effects of chronic exposure to effluent on the prevalence of leucocyte populations in spleen tissue could not be associated with an influence on blood cell differentials. However, assessment of blood cell differentials and leucocyte prevalence at a single point in time, as is the case in our studies, might not meet the capabilities of these methods. Assessment of time

courses might be more useful, possibly providing information on the influence of environmental stressors on recruitment and distribution of white blood cells in different tissues in the course of an immune reaction. Besides mere identification of certain leucocyte populations, the use of antibodies specific for surface markers, such as MHC and IgM can give information on the state of activation of the cells detected. Due to its potential to provide a range of information, immuno-histochemistry could therefore constitute a valuable tool for the assessment of environmental pollution.

Head kidney macrophage activity, measured as oxidative burst and phagocytotic activity have, in this study, neither been influenced by exposure to effluent, nor by the model substances cortisol and rifampicin. This observation is partly in contrast to investigations described in the literature. Macrophage activity has been shown to be sensitive to a variety of substances and pollutants and has thus been suggested as a valuable test parameter in fish immunotoxicology (Weeks & Warinner, 1986; Rice et al., 1996; Kelly-Reay & Weeks, 1994). The lack of an effect on macrophage activity in the studies at hand could be due to low substance concentrations at the place of action, in this case head kidney. This explanation is likely for the experiment with cortisol and rifampicin, as exposure via the water might not result in high enough concentrations in the investigated organ, compared to experiments using injection, implantation or oral application as route of exposure. In the case of the experiments with effluent, a correlation between influences on immune parameters and certain pollutants cannot be made, due to a lack of chemical analysis of the wastewater used in the studies at hand. Possibly, the effluent does not contain high enough concentrations of substances, which can affect head kidney macrophage activity. Beside a certain insensitivity of macrophage activity towards certain environmental pollution, supposed by this thesis, other aspects also contradict their employment in environmental monitoring. Preparing of primary macrophage cultures from higher sample numbers (which are inevitable in environmental toxicology) is very time consuming and labour-intensive and therefore also susceptible to mistakes. The failure to demonstrate effects on macrophage activity in this study might therefore also be due to difficulties with the test method.

Specific antibody production is an immune parameter, which, in this thesis, has been shown to react on exposure to STP effluent. Beside its supposed sensitivity to pollution of surface waters, its high relevance is in favour for its application within environmental monitoring. In fish, the significance of this immune parameter is not due to its effectiveness in fighting disease, as, in fish, protection against diseases is believed to rely more on the unspecific, innate part of the immune system. The significance of this test method lies in the fact that the

immune system can be investigated in an activated state, after exposure to a pathogen, rather then studying immune parameters of a resting system. Beside exposure of fish with inactivated bacteria, which is still effective in activating immune reactions, immune challenges with living bacteria should also be considered for laboratory scale investigations. Limitations of employing disease challenges with living as well as inactivated pathogens are due to its time-consuming and handling-intensive nature, especially as fish might have to be exposed by injection, rather than immersion in water. Exposure with living pathogens can moreover exclude assessment of immune parameters if the disease leads to high mortality rates and death ratios are the main end point.

For the assessment of immunotoxicological effects within aquatic toxicology, employment of several immune parameters will always be inevitable, in order to cover the whole immune system and subsequently be able to estimate the resulting over-all immune competence of affected organisms. With the present, limited knowledge on mechanisms in fish immunotoxicology, to date final conclusions on the immune competence of affected organisms might only be possible after the observation of an actual decrease in disease resistance.

Assessment of aquatic pollution

Investigation of adverse effects of anthropogenic pollution on aquatic organisms within the field of environmental toxicology is widely influenced nowadays by the search for suitable biomarkers to facilitate regular monitoring. However, the relevance of biomarkers is controversially discussed. The use of biomarkers often does not necessarily allow conclusions on the overall health status of the investigated organism, let alone on the state of the whole population. The complexity of the immune system emphasises this problem in the field of immunotoxicology. The investigation of a limited number of immune parameters will not allow a detailed assessment of the actual overall immune status of the test organism. Moreover, there is a lack of conclusive data on effects of different substances on certain immune reactions in fish. No clear patterns for effects of certain substance classes on immune parameters investigated have been found so far, but results are often contradictory (see general introduction, chapter I). Therefore, alterations in immune parameters cannot be traced back and clearly related to causing substances, and substance classes have not been found to elicit comparable, characteristic effects on certain immune parameters. More effort has to be put into studying the mechanisms involved in the modification of immunologic processes by

exogenous and even endogenous chemicals, to allow a more effective use of immune parameters as indicators of environmental pollution (Sharma & Zeeman, 1980; Wester et al., 1994). Moreover should the investigation of the immune system of a given organism distinguish between an inactive and an activated immune system (Kollner et al., 2002). Man-made contaminants in surface waters might e.g. cause immunosuppression and impair resistance against opportunistic pathogens, leading to higher disease prevalence. Assessment of adverse effects of pollutants on an unchallenged immune system, however, might not reflect influences of the same pollution on a challenged, active system. As adverse effects on the immune system become relevant during infection, investigations including an immune challenge most likely yield the more relevant information, compared to assessment of immune parameters within a resting system.

The immune system is also highly dynamic on a temporal scale. Measurement of certain immune reactions at only one point in time (usually one final sampling upon termination of an exposure experiment) might not assess all adverse effects happening throughout the course of an exposure to pollutants. In order to better understand influences on the immune system, various immune parameters should be measured at several points in time. Thus, not only modulation in the strength of a reaction can be assessed, but also possible delays in reactivity.

Beside the lack of cause and effect relationships in immunotoxicology, investigations on immune parameters are further complicated by the variety of factors influencing immune reactions (Bly et al., 1997). Aquatic organisms, living in close interaction with the surrounding medium water, are strongly influenced by exogenous factors, like temperature (Le Morvan-Rocher et al., 1995). Immune reactions in fish have been observed to be vastly modulated by temperature, ranging from a highly activated immune system at warm temperatures in summer to an almost inactive system at cold temperatures in winter (Köllner 2002, personal communication). The fish immune system is further influenced by sexual development. This factor has an especially strong influence in fish species, which have been shown to down-regulate their immune system before spawning. The manifestation of additional influences on the immune system, e.g. through environmental contamination, can vary considerably on the background of the diverse endogenous and exogenous modulating factors mentioned above. Detailed knowledge about the influence of these factors is therefore necessary to differentiate between various causes, where modifications of the immune system are observed.

Fish immunology is moreover intricate, due to the diversity in species investigated. Fish constitute by far the broadest vertebrate class, occupying considerably diverse environments,

ranging from arctic to tropical fresh and seawaters. Immune functions are only slightly comparable between different fish species. Therefore, results, which can be found in one species, can usually not be transferred to other fish species. Investigations on disease manifestation also vary considerably with the fish species and pathogen or parasite causing the disease.

In general, it can be stated that to date the usefulness of immune parameters for fast screening of environmental pollution remains questionable with the state-of-the-art. Further insights into mechanisms and functioning of the fish immune system are inevitable for the successful use of immune parameters. A better knowledge of mechanisms in immunotoxicology could enable the establishment of clearer cause-effect relationships for environmental pollution. However, even with a better understanding of immune mechanisms, experimental designs to assess effects on the fish immune system will most likely remain work intensive, if achievement of a detailed, valid estimation of the situation is sought. A tiered approach might be appropriate to address immunotoxicological effects in fish. The use of up-to-date methods, like gene array analysis in the field of fish toxicology could thereby enable a first identification of adversely affected reactions at the level of gene expression (Koellner et al., in press). This idea also takes into consideration, that detection of molecules, such as cell surface markers, is often not yet possible in fish, due to a lack of specific antibodies, while gene- and mRNA-sequences of a broad array of molecules have already been elucidated. First indications of effects on certain molecules at the RNA level, could then be followed by corresponding functional assays, provided that appropriate test systems are available.

Immune competence of fish, which constitutes an important factor for the survival of our fish populations, might be affected by the present contamination of our surface waters with a cocktail of low level pollutants. Therefore, investigations on fish immunity should be brought forward, in order to improve methods and applicability of immune parameters in the field of environmental toxicology.

Aquatic pollution: objectives and remediation

In most civilized western countries, direct contamination of water bodies is widely prevented nowadays. Polluted water usually undergoes more or less thorough cleaning treatment, before it is led back into rivers, lakes and oceans. However, a variety of substances, including pharmaceutical residues, ingredients of health care and other household products and industrial chemicals, still reach our surface waters. Main intake route of such pollutants into

our surface waters is through sewage treatment plants (Ternes, 1998; Hirsch et al., 1999; Bennie, 1999; Daughton & Ternes, 1999; Desbrow et al., 1998). Leakages in landfills also play a role. The discovery of a potential of various substances in sewage to disrupt endocrine regulation in aquatic animals has raised concerns about the influence of such contamination on the health and survival of our fish populations and the integrity of whole aquatic ecosystems. Investigations on immunomodulating potential of several pollutants in the aquatic environment now contribute further indications of adverse effects on aquatic organisms.

Several studies have investigated the degradability of substances of concern in sewage and demonstrated a high persistence of a variety of chemicals, including various pharmaceuticals (Kümmerer et al., 1997; Kummerer, 2001; Bennie, 1999). The sewage treatment plants operating to date were designed to remove nutrients and pathogens. These plants do not have the potential to remove the broad array of man-made substances, which can be found in sewage nowadays. Even if removal of the majority of pollutants was possible, extravagant and expensive methods would have to be developed and applied. Thus, at present, the only applicable way to deal with the contamination of our surface waters with the above-mentioned substances might be prevention rather than remediation. Drugs e.g. are often disposed by flushing them down the toilet or throwing them into the garbage (Kuspis & Krenzelok, 1996). This results in additional pollution of surface waters with pharmaceutical residues, on top of excretion after therapeutic use. Discovery of their potential to adversely affect non-target organisms suggests a different handling of left-over pharmaceuticals, in order to avoid contamination of the environment, specifically waters (Daughton, 2003).

Where conflicts of interests exist, like with the use of pesticides or the intake of pharmaceuticals, avoidance might not be practicable. However, possibilities to diminish the use of substances with potential to adversely affect aquatic organisms should be considered. In the case of drug intake, Daughton described several possibilities to optimise the use of pharmaceuticals, which could enable application of lower therapeutic doses (Daughton, 2003). Thereby, environmental protection could moreover be combined with benefits for patients, including more effective therapies and lower expenses on drugs. Chemicals, for which serious effects on the health of our aquatic environments are observed, might be legally banned from use, as has happened with organochlorine pesticides in the 1970s.

The results of this thesis give further evidence of adverse effects of sewage treatment effluent on the endocrine and immune system of fish. Although the causing substances could not be identified within the scope of this work, avoidance of intake of the multitude of man-made

substances into our surface waters is desirable. Careful and thorough monitoring of environmental pollution, including possible effects of water contamination on the immune competence of fish, is inevitable to guarantee the survival and health of aquatic communities.

Summary

A clean and healthy aquatic environment is not only important for humans as a source for drinking water. We also depend on the well-being of aquatic organisms, especially fish, as important food source. In recent years, surface waters throughout the world have been observed to be polluted with a wide variety of man-made substances. At least in civilized western countries, wastewater usually goes through more or less thorough cleaning treatment before it is led back into rivers, lakes and oceans. However, even state-of-the-art sewage treatment plants do not have the potential to remove the broad array of man-made substances, present in sewage. Therefore, intake of sewage treatment plant effluent is nowadays the main source of contamination of our surface waters.

The focus of attention within aquatic toxicology has shifted from acutely toxic contaminations to low-level pollution, causing more subtle adverse effects, like e.g. disruption of endocrine regulation mechanisms. Investigations on endocrine-reproductive alterations in fish, caused by hormonally active contaminants, has recently been complemented by studies on immunotoxicological effects. Negative influences on the fish immune system have been shown for a broad array of compounds. Adverse effects of environmental pollution on immune reactions in fish can lead to a decrease in immune competence and consequently higher susceptibility to diseases, possibly resulting in higher mortality rates among fish populations.

In order to get an insight into possible effects of immunologically active compounds, trout were exposed to two model substances, with known immunosuppressive activity, namely cortisol and rifampicin, over a period of 21 days. For both substances, suppressive effects on immune parameters could be shown. Exposure of juvenile rainbow trout to cortisol via the water, resulted in lower leucocyte counts in peripheral blood and slightly decreased serum lysozyme activity. Water-borne rifampicin was shown to decrease serum lysozyme activity and to slightly reduce leucocrit values in peripheral blood.

As the main subject, the thesis at hand has addressed the influence of sewage treatment effluent on immune functions in rainbow trout. In order to investigate effects of wastewater on the trout immune system, trout were exposed to high concentrations of sewage effluent in an acute situation, as well as chronically to lower, more realistic concentrations. In the acute exposure experiment with juvenile trout, lasting over a period of 4 weeks, high effluent concentrations (30 and 70 %) resulted in decreased peripheral blood leucocyte counts, with lymphocytes being the main white blood cell population affected. Exposure to these high

concentrations also decreased erythrocyte viability, resulting in a considerable high prevalence of degrading erythrocytes in peripheral blood. The other immune parameters, measured in this experiment, including serum lysozyme activity, lymphocyte proliferation, macrophage phagocytosis and oxidative burst, as well as serum antibody levels against the fish pathogen *A. salmonicida*, were not influenced by exposure to effluent. Beside the immune parameters mentioned, liver MFO activity was also assessed and found to be increased after exposure to effluent.

Chronic exposure of mature rainbow trout to 15 % effluent over a period of 32 weeks, resulted in alterations of general physiological and endocrine-reproductive, as well as immunological parameters. Liver and gonad weight were increased in both mature male and female trout, whereby in female fish the higher gonad weight was reflected in a higher fecundity. Exposure to effluent also resulted in higher plasma estradiol level in female and lower plasma 11-ketotestosterone levels in male trout. Moreover, liver MFO activity in male fish was increased after exposure to 15 % effluent, reflecting enhanced detoxification activity. Immune parameters measured in this experiment were serum lysozyme activity, differential blood cell counts, macrophage activity (oxidative burst and phagocytosis), prevalence and distribution of different leucocyte populations in spleen and production of specific antibodies against *A. salmonicida*. Serum antibody level against *A. salmonicida* were lower in mature female trout exposed to effluent compared to control fish, but not in mature male fish. Effluent-exposed immature trout (males and females pooled) and to some extend mature females moreover showed decreased lysozyme activity.

In spite of the possibility to show adverse effects of STP effluent on the immune system of rainbow trout, demonstrated in this thesis, the suitability of immune parameters for monitoring of environmental pollution remains questionable. With the state-of-the-art in fish immunotoxicology, the measurement of a suite of immune parameters leaves many open questions. Alterations in single immune reactions can be used as “first indicators” for adverse effects of environmental contamination. However, an ultimate comprehensive evaluation of immune competence in fish cannot be deduced from the assessment of a few parameters, but is only possible with the demonstration of e.g. lower disease resistance, in the case of a suppressed immune system. A better knowledge of basic fish immunology and mechanisms of immunotoxicity, would enable a better risk assessment for pollution of aquatic environments. As immune competence is an important factor for the health and survival of our fish populations, the field of immunotoxicology should be brought forward, in order to enable a successful inclusion into investigations on environmental toxicology.

Zusammenfassung

Saubere und gesunde Gewässer sind für uns Menschen nicht nur als Trinkwasserquelle wichtig. Aquatische Organismen, insbesondere Fische, stellen für uns auch eine wichtige Nahrungsquelle dar und ihre Gesundheit kann somit auch unsere Gesundheit beeinflussen. In den letzten Jahren rückte die weltweite Verschmutzung von Oberflächengewässern mit einer Vielzahl von anthropogenen Stoffen in den Blickpunkt. Abwässer werden, zumindest in den modernen westlichen Ländern, mehr oder weniger ausgeprägten Reinigungsprozessen unterzogen, bevor sie in Flüsse, Seen oder das Meer eingeleitet werden. Allerdings können sogar die fortschrittlichsten Kläranlagen nicht die Vielzahl der Substanzen anthropogenen Ursprungs entfernen, die heute in Abwässern enthalten sind. Daher stellt der Eintrag von Abwasser über Kläranlagen mittlerweile die Hauptquelle der Gewässerverschmutzung in den westlichen Industrienationen dar.

Das Hauptaugenmerk in der aquatischen Toxikologie hat sich zunehmends von akut-toxischer Gewässerverschmutzung zu Substanzen verlagert, die in den geringen Mengen, in denen sie in den Gewässern auftauchen, subtilere Effekte hervorrufen können. So wurde für verschiedene Substanzen ein Eingriff in hormonelle Regulationsmechanismen gezeigt, der zu einer Beeinflussung der Fortpflanzungsfähigkeit führen kann. Zu den Untersuchungen zur hormonellen Aktivität in verschmutzten Oberflächengewässern kam die Erkenntnis, dass verschiedene Substanzen anthropogenen Ursprungs auch das Immunsystem von aquatischen Organismen beeinträchtigen können. Eine negative Beeinflussung von Immunreaktionen in Fischen kann zu einer erhöhten Anfälligkeit gegenüber Krankheitserregern und somit schließlich zu höheren Sterblichkeitsraten in unseren Fischpopulationen führen.

Um einen Einblick in mögliche Auswirkungen von immunaktiven Substanzen zu bekommen, wurden Forellen zwei Modellsubstanzen mit bekannter immunsuppressiver Aktivität ausgesetzt, nämlich Cortisol und Rifampicin. Für beide Substanzen konnte eine suppressive Wirkung auf einzelne Immunparameter gezeigt werden. Die Exposition juveniler Regenbogenforellen mit Cortisol über einen Zeitraum von 21 Tagen führte zu erniedrigten Leukozyten-Zahlen im peripheren Blut, sowie einer leichten Abnahme der Serum-Lysozym-Aktivität. Exposition mit Rifampicin führte zur Abnahme der Serum-Lysozym-Aktivität, sowie zu leicht erniedrigten Leukokrit-Werten.

Das Hauptaugenmerk der vorliegenden Arbeit lag auf der Untersuchung potentieller immunotoxikologischer Aktivität in kommunalem Kläranlagenabwasser. Regenbogenforellen wurden zum einen über einen relativ kurzen Zeitraum (28 Tage) hohen Abwasserkonzentrationen ausgesetzt (akute Exposition), zum anderen in einem chronischen Expositionsversuch niedrigeren, realitätsnahen Konzentrationen.

Akute Exposition von juvenilen Forellen mit hohen Abwasserkonzentrationen führte zu einer verstärkten Degradation von Erythrozyten, sowie zu einer Abnahme der Leukozyten-Zahlen im peripheren Blut, was vor allem auf ein vermindertes Vorkommen von Lymphozyten zurückzuführen war. Serum-Lysozym-Aktivität, Lymphozyten-Proliferation, Phagozytose-Aktivität und Produktion von reaktiven Sauerstoffspezies in Kopfnieren-Makrophagen, sowie Serum-Antikörper-Level gegen das Fisch-Pathogen *A. salmonicida*, wurden durch die Exposition mit Abwasser nicht beeinflusst. Zusätzlich zu Veränderungen in Immunreaktionen konnte eine erhöhte MFO-Aktivität in Leberproben festgestellt werden.

Chronische Exposition von geschlechtsreifen Regenbogenforellen mit 15 % Abwasser über einen Zeitraum von 32 Wochen führte zu Veränderungen in allgemeinen physiologischen und endokrin-reproduktiven, sowie immunologischen Parametern. Leber- und Gonadengewicht waren sowohl in männlichen, als auch in weiblichen Forellen erhöht, wobei das erhöhte Gonadengewicht in Weibchen auf eine erhöhte Fekundität zurückzuführen war. Weiter konnten erhöhte Plasma-Östradiol-Level in weiblichen, sowie niedrigere 11-Ketotestosteron-Level in männlichen Fischen gezeigt werden. In Leberproben von männlichen Fischen konnte darüber hinaus eine verstärkte MFO-Aktivität nachgewiesen werden, was auf eine Aktivierung von Entgiftungsmechanismen hinweist. Eine Beeinflussung von Immunreaktionen zeigte sich in niedrigeren *A. salmonicida*-spezifischen Antikörper-Level im Serum von weiblichen Forellen, nicht jedoch in männlichen Fischen, einer Abnahme in der Serum-Lysozym-Aktivität in nicht geschlechtreifen Forellen (männlich und weibliche Fische zusammengenommen) und zu einem geringeren Maße in weiblichen Fischen, sowie in Veränderungen in Auftreten und Verteilung verschiedener Leukozyten-Populationen in der Milz.

Wie in der vorliegenden Doktorarbeit gezeigt wurde, können Veränderungen in einzelnen Immunreaktionen als erste Indikatoren für eine Gewässerverschmutzung erfasst werden. Für die Bestimmung der tatsächlichen Immunkompetenz der untersuchten Tiere reicht die Erfassung einzelner Immunparameter allerdings nicht aus. Eine negative Beeinflussung der Immunkompetenz zeigt sich erst, wenn eine geringere Krankheitsresistenz auftritt, die letztendlich zu höheren Sterblichkeitsraten führt, was hinsichtlich von Laboruntersuchungen

für den Einsatz von Belastungsversuchen spricht. Da die Immunkompetenz ein wichtiger Faktor für die Gesundheit und das Überleben unserer Fischpopulationen ist, sollte die Immunotoxikologie eine stärkere Beachtung innerhalb der Umwelttoxikologie finden. Ein besseres Verständnis grundlegender Mechanismen der Immunotoxizität in Fischen wäre dabei von großem Nutzen.

References

Aaltonen, T. M., Jokinen, E. I., Lappivaara, J., Markkula, S. E., Salo, H. M., Leppanen, H. & Lammi, R. 2000a. Effects of primary- and secondary-treated bleached kraft mill effluents on the immune system and physiological parameters of roach. *Aquatic Toxicology*, **51,** 55-67.

Aaltonen, T. M., Jokinen, E. I., Salo, H. M., Markkula, S. E. & Lammi, R. 2000b. Modulation of immune parameters of roach, *Rutilus rutilus*, exposed to untreated ECF and TCF bleached pulp effluents. *Aquatic Toxicology*, **47,** 277-289.

Aaltonen, T. M., Valtonen, E. T. & Jokinen, E. I. 1997. Immunoreactivity of roach, *Rutilus rutilus*, following laboratory exposure to bleached pulp and paper mill effluents. *Ecotoxicology and Environmental Safety*, **38,** 266-71.

Ahmad, I., Fatima, M., Athar, M., Khan, N. Z. & Raisuddin, S. 1998. Responses of circulating fish phagocytes to paper mill effluent exposure. *Bulletin of Environmental Contamination & Toxicology*, **61,** 746-753.

Alexander, J. B. & Ingram, G. A. 1992. Noncellular nonspecific defence mechanisms of fish. *Annual Review of Fish Diseases***,** 249-279.

Allen, Y., Scott, A. P., Matthiessen, P., Haworth, S., Thain, J. E. & Feist, S. 1999. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environmental Toxicology and Chemistry*, **18,** 1791-1800.

Anderson, D. P., Dixon, O. W., Bodammer, J. E. & Lizzio, E. F. 1989. Suppression of antibody-producing cells in rainbow trout spleen sections exposed to copper *in vitro*. *Journal of Aquatic Animal Health*, **1,** 57-61.

Andreozzi, R., Raffaele, M. & Nicklas, P. 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere*, **50,** 1319-30.

Arkoosh, M. R., Clemons, E., Myers, M. & Casillas, E. 1994. Suppression of B-cell mediated immunity in juvenile chinook salmon (*Oncorhynchus tshawytscha*) after exposure to either a polycyclic aromatic hydrocarbon or to polychlorinated biphenyls. *Immunopharmacology and Immunotoxicology*, **16,** 293-314.

Ashfield, L. A., Pottinger, T. G. & Sumpter, J. P. 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environmental Toxicology & Chemistry*, **17,** 679-686.

Balakhnin, I. A. & Dudka, I. A. 1990. The interaction of fish mycopathogens with anti-B lectin of *Salmo gardneri* eggs. *Mikol. Fitopatol.*, **24,** 224-228.

Balakhnin, I. A., Dudka, I. A. & Isaeva, N. M. 1990. Testing of funi on their specific interaction with fish egg lectins. *Mikol. Fitopatol.*, **24,** 416-420.

Barron, M. G., Anderson, M. J., Cacela, D., Lipton, J., Teh, S. J., Hinton, D. E., Zelikoff, J. T., Dikkeboom, A. L., Tillitt, D. E., Holey, M. & Denslow, N. 2000. PCBs, liver lesions, and biomarker responses in adult walleye (*Stizostedium vitreum vitreum*) collected from Green Bay, Wisconsin. *Journal of Great Lakes Research.*, **26,** 250-271.

Beaman, J. R., Finch, R., Gardner, H., Hoffmann, F., Rosencrance, A. & Zelikoff, J. T. 1999. Mammalian immunoassays for predicting the toxicity of malathion in a laboratory fish model. *Journal of Toxicology and Environmental Health*, **56,** 523-42.

Bellahsene, A. & Forsgren, A. 1980. Effect of rifampin on the immune response in mice. *Infection and Immunity*, **27,** 15-20.

Bennett, R. & Wolke, R. E. 1987a. The effect on sublethal endrin exposure on rainbow trout, *Salmo gairdneri* Richardson. I. Evaluation of serum cortisol concentrations and immune responsiveness. *Journal of Fish Biology*, **31,** 378-385.

Bennie, D. T. 1999. Review of the environmental occurance of alkylphenols and alkylphenol ethoxylates. *Water Quality Research Journal of Canada*, **34,** 79-122.

Bersani, C., Bertoletti, R., Colombo, M. L., Merlini, C., Uccelli, M., Fietta, A. & Gialdroni Grassi, G. 1987. *In vitro* and *ex vivo* influence of rifamycins on human phagocytes. *Chemioterapia*, **6,** 420-5.

Betoulle, S., Duchiron, C. & Deschaux, P. 2000. Lindane increases *in vitro* respiratory burst activity and intracellular calcium levels in rainbow trout (*Oncorhynchus mykiss*) head kidney phagocytes. *Aquatic Toxicology*, **48,** 211-221.

Blohm, U., Siegl, E. & Koellner, B. 2003. Rainbow trout (*Oncorhynchus mykiss*) sIgM-leucocytes secrete an interleukin-2 like growth factor after mitogenic stimulation *in vitro*. *Fish & Shellfish Immunology*, **14,** 449-466.

Bly, J. E., Quiniou, S. M. & Clem, L. W. 1997. Environmental effects on fish immune mechanisms. *Developments in Biological Standardization*, **90,** 33-43.

Bols, N. C., Brubacher, J. L., Ganassin, R. C. & Lee, L. E. 2001. Ecotoxicology and innate immunity in fish. *Developmental & Comparative Immunology*, **25,** 853-73.

Bucheli, T. D. & Fent, K. 1995. Induction of cytochrom P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology*, **25,** 201-268.

Bucher, F. & Hofer, R. 1993. The effects of treated domestic sewage on three organs (gills, kidney, liver) of brown trout (*Salmo trutta*). *Water Research*, **27,** 255-261.

Bucke, D., Vethaak, A. D. & Lang, T. 1992. Quantitative assessment of melanomacrophage centres (MMCs) in dab *Limanda limanda* along a pollution transect in the German Bight. *Marine Ecology-Progress Series*, **91,** 193-196.

Burkhardt-Holm, P., Escher, M. & Meier, W. 1997. Wast-water management plant effluents cause cellular alterations in the skin of brown trout. *Journal of Fish Biology*, **50,** 744-758.

Carlson, R. E., Anderson, D. P. & Bodammer, J. E. 1993. *In vivo* cortisol administration suppresses the *in vitro* primary immune response of winter flounder lymphocytes. *Fish & Shellfish Immunology*, **3,** 299-312.

Caruso, D., Schlumberger, O., Dahm, C. & Proteau, J. P. 2002. Plasma lysozyme levels in sheatfish *Silurus glanis* (L.) subjected to stress and experimental infection with *Edwardsiella tarda*. *Aquaculture Research*, **33,** 999-1008.

Choi Sang, H. & Oh, C.-H. 2003. The effect of cortisol on proliferative properties of flounder (*Paralychthys olivaceus*) B lymphocytes. *Korean Journal of Biological Sciences*, **7,** 57-62.

Christensen, F. M. 1998. Pharmaceuticals in the environment - a human risk? *Regulatory Toxicology and Pharmacology*, **28,** 212-21.

Cossarini-Dunier, M., Damael, A. & Siwicki, A. K. 1990. *In vivo* effect of the organophosphorus insecticide trichlorphon on the immune response of carp (*Cyprinus carpio*): effect of contamination on antibody production in relation to residue level in organs. *Ecotoxicology and Environmental Safety*, **19,** 93-98.

Cossarini-Dunier, M., Monod, G., Demael, A. & Lepot, D. 1987. Effect of gamma-hexachlorocyclohexane (lindane) on carp (*Cyprinus carpio*). I. Effect of chronic intoxication on humoral immunity in relation to tissue pollutant levels. *Ecotoxicology and Environmental Safety*, **13,** 339-45.

Cousinou, M., Nilsen, B., Lopez-Barea, J. & Dorado, G. 2000. New methods to use fish cytochrome P4501A to assess marine organic pollutants. *The Science of the Total Environment*, **247,** 213-25.

Crain, D. A., Guillette, L. J., Jr., Rooney, A. A. & Pickford, D. B. 1997. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and

experimentally to environmental contaminants. *Environmental Health Perspectives*, **105,** 528-33.

Daughton, C. G. 2003. Cradle-to-cradle stewardship of drugs for minimizing their environmental disposition while promoting human health. I. Rationale for and avenues toward a green pharmacy. *Environmental Health Perspectives*, **111,** 757-74.

Daughton, C. G. & Ternes, T. A. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental Health Perspectives*, **107,** 907-38.

Davis, K. B., Griffin, B. R. & Gray, W. L. 2003. Effect of dietary cortisol on resistance of channel catfish to infection by *Ichthyopthirius* multifiliis and channel catfish virus disease. *Aquaculture*, **218,** 121-130.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P. & Waldock, M. 1998. Identification of estrogenic chemicals in STW effluent: 1. Chemical fractionation and in vitro biological screening. *Environmental Science & Technology*, **32,** 1549-1558.

Dijkstra, J. M., Koellner, B., Aoyagi, K., Sawamoto, Y., Kuroda, A., Ototake, M., Nakanishi, T. & Fischer, U. 2003. The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules. *Fish & Shellfish Immunology*, **14,** 1-23.

Dunier, M. & Siwicki, A. K. 1993. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. *Fish & Shellfish Immunology*, **3,** 423-438.

Dunier, M. & Siwicki, A. K. 1994. Effects of lindane exposure on rainbow trout (*Oncorhynchus mykiss*) immunity. I. Effect of lindane on antibody-secreting cells (ASC) measured by ELISPOT assay. *Ecotoxicology and Environmental Safety*, **27,** 1-6.

Dunier, M., Siwicki, A. K., Scholtens, J., Dal Molin, S., Vergnet, C. & Studnicka, M. 1994. Effects of lindane exposure on rainbow trout (*Oncorhynchus mykiss*) immunity. III. Effect on nonspecific immunity and B lymphocyte functions. *Ecotoxicology and Environmental Safety*, **27,** 324-34.

Ellis, A. E. 1990. Lysozyme Assays. In: *Techniques in Fish Immunology* (Ed. by Van Muiswinkel, W. B.), pp. 101-103. Fair Haven: SOS Publications.

Ellis, A. E. 2001. Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, **25,** 827-39.

Ellsaesser, C. F. & Clem, L. W. 1987. Cortisol-induced hematologic and immunologic changes in channel catfish (*Ictalurus punctatus*). *Comparative Biochemistry & Physiology A*, **87,** 405-408.

Elsasser, M. S., Roberson, B. S. & Hetrick, F. M. 1986. Effects of metals on the chemiluminescent response of rainbow trout (*Salmo gairdneri*) phagocytes. *Veterinary Immunology & Immunopathology*, **12,** 243-50.

Elskus, A. A., Stegeman, J. J., Susani, L. C., Black, D., Pruell, R. J. & Fluck, S. J. 1989. Polychlorinated biphenyls concentration and cytochrome P-450E expression in winter flounder from contaminated environments. *Marine Environmental Research*, **28,** 25-30.

Espelid, S., Lokken, G. B., Steiro, K. & Bogwald, J. 1996. Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology*, **6,** 95-110.

Evans, D. L., Leary, J. H., 3rd & Jaso-Friedmann, L. 2001. Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. *Developmental & Comparative Immunology*, **25,** 791-805.

Faisal, M., Cooper, E. L., El-Mofty, M. & Sayed, M. A. 1988. Immunosuppression of *Clarias lazera* (Pisces) by a moluscicide. *Developmental & Comparative Immunology*, **12,** 85-97.

Faisal, M. & Huggett, R. J. 1993. Effects of polycyclic aromatic hydrocarbons on the lymphocyte mitogenic responses in spot (*Leiostomus xanthurus*). *Marine Environmental Research*, **35,** 121-124.

Fatima, M., Ahmad, I., Sayeed, I., Athar, M. & Raisuddin, S. 2000. Pollutant-induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. *Aquatic Toxicology*, **49,** 243-250.

Fatima, M., Ahmad, I., Siddiqui, R. & Raisuddin, S. 2001. Paper and pulp mill effluent-induced immunotoxicity in freshwater fish *Channa punctatus* (Bloch). *Archives of Environmental Contamination and Toxicology*, **40,** 271-6.

Fischer, U. & Köllner, B. 1994. Preparation of a B-cell and monocyte free thymocyte suspension. In: *Techniques in Fish Immunology* (Ed. by Stolen, J. S., Fletcher, T. C., Anderson, D. P., Roberson, B. S. & Van Muiswinkel, W. B.), pp. 27-33. Fair Haven, USA: SOS Publications.

Fischer, U., Utke, K., Ototake, M., Dijkstra, J. M. & Kollner, B. 2003. Adaptive cell-mediated cytotoxicity against allogeneic targets by CD8-positive lymphocytes of rainbow trout (*Oncorhynchus mykiss*). *Developmental & Comparative Immunology*, **27,** 323-37.

Fletcher, T. C., White, A. & Baldo, B. A. 1977. C-reactive protein-like precipitin and lysozyme in the lumpsucker *Cyclopterus lumpus* L., during the breeding season. *Comparative Biochemistry and Physiology. B*, **57,** 353-357.

Folmar, L. C., Denslow, N. D., Kroll, K., Orlando, E. F., Enblom, J., Marcino, J., Metcalfe, C. & Guillette, L. J., Jr. 2001. Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. *Archives of Environmental Contamination and Toxicology*, **40,** 392-8.

Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., Enblom, J., Marcino, J. & Guillette, L. J., Jr. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives*, **104,** 1096-101.

Fournier, M., Lacroix, A., Voccia, I. & Brousseau, P. 1998. Phagocytic and metabolic activities of macrophages from mummichog naturally exposed to pulp mill effluents in the Miramichi River. *Ecotoxicology and Environmental Safety*, **40,** 177-83.

Gill, T. S. & Pant, J. C. 1985. Mercury-induced blood anomalies in the freshwater teleost *Barbus conchonius* Ham. *Water, Air, and Soil Pollution*, **24,** 165-171.

Gooch, J. W., Elskus, A. A., Kloepper-Sams, P. J., Hahn, M. E. & Stegeman, J. J. 1989. Effects of ortho- and non-ortho-substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicology and Applied Pharmacology*, **98,** 422-33.

Graham, S. & Secombes, C. J. 1988. The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. *Immunology*, **65,** 293-7.

Graham, S. & Secombes, C. J. 1990a. Cellular requirements for lymphokine secretion by rainbow trout *Salmo gairdneri* leucocytes. *Developmental & Comparative Immunology*, **14,** 59-68.

Graham, S. & Secombes, C. J. 1990b. Do fish lymphocytes secrete interferon gamma? *Journal of Fish Biology*, **36,** 563-573.

Grusby, M. J. & Glimcher, L. H. 1995. Immune responses in MHC class II-deficient mice. *Annual Review of Immunology*, **13,** 417-35.

Guillette, L. J., Jr. & Guillette, E. A. 1996. Environmental contaminants and reproductive abnormalities in wildlife: implications for public health? *Toxicology and Industrial Health*, **12,** 537-50.

Hahn, M., E., Lamb, T. M., Schultz, M. E., Smolowitz, R. M. & Stegemann, J. J. 1993. Cytochrome P450 1A induction and inhibition by 3,3´,4,4´-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquatic Toxicology*, **26,** 185-208.

Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lutzhoft, H. C. & Jorgensen, S. E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment - a review. *Chemosphere*, **36,** 357-93.

Harries, J., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. & Tyler, C. R. 1999. Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environmental Toxicology & Chemistry*, **18,** 932-937.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T. & Zaman, N. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology & Chemistry*, **16,** 534-542.

Hatten, F., Fredriksen, A., Hordvik, I. & Endresen, C. 2001. Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. *Fish & Shellfish Immunology*, **11,** 257-68.

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters*, **131,** 5-17.

Hemming, J. M., Waller, W. T., Chow, M. C., Denslow, N. D. & Venables, B. 2001. Assessment of the estrogenicity and toxicity of a domestic wastewater effluent flowing through a constructed wetland system using biomarkers in male fathead minnows (*Pimephales promelas* Rafinesque, 1820). *Environmental Toxicology & Chemistry*, **20,** 2268-75.

Henschel, K. P., Wenzel, A., Diedrich, M. & Fliedner, A. 1997. Environmental hazard assessment of pharmaceuticals. *Regulatory Toxicology and Pharmacology*, **25,** 220-5.

Hetrick, F. M., Knittel, M. D. & Freyer, J. L. 1979. Increased susceptibility of rainbow trout to infectious hematopoietic necrosis virus after exposure to copper. *Applied Environmental Microbiology*, **37,** 198-201.

Hirsch, R., Ternes, T., Haberer, K. & Kratz, K. L. 1999. Occurrence of antibiotics in the aquatic environment. *The Science of the Total Environment*, **225,** 109-18.

Hitzfeld, B. C., Prietz, A. & Dietrich, D. R. 2000. Gesundheitszustand der Fische im Rheintal, Expositionsversuche 1999: Untersuchungen zum Immunstatus., pp. 87pp: Department Environmental Protection, Canton St. Gallen, Switzerland.

Houghton, G. & Matthews, R. A. 1990. Immunosuppression in juvenile carp, *Cyprinus carpio* L.: the effects of the corticosteriods triamcinolone actonide and hydrocortisone 21-hemisuccinate (cortisol) on acquired immunity and the humoral antibody response to *Ichthyophthirius multifiliis* Fouquet. *Journal of Fish Diseases*, **13,** 269-280.

Hrubec, T. C., Robertson, J. L., Smith, S. A. & Tinker, M. K. 1996. The effect of temperature and water quality on antibody response to *Aeromonas salmonicida* in sunshine bass (*Morone chrysops X Morone saxatilis*). *Veterinary Immunology & Immunopathology*, **50,** 157-166.

Hurvitz, A., Bercovier, H. & Van Rijn, J. 1997. Effect of ammonia on the survival and the immune response of rainbow trout (*Oncorhynchus mykiss*, Walbaum) vaccinated against Streptococcus iniae. *Fish & Shellfish Immunology*, **7,** 45-53.

Hutchinson, T. H. & Manning, M. J. 1996. Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L.) sampled from Lyme Bay, U.K. *Fish & Shellfish Immunology*, **6,** 473-482.

Ibrahim, M. S., Maged, Z. A., Haron, A., Khalil, R. Y. & Attallah, A. M. 1987. Antibiotics and immunity: effects of antibiotics on natural killer, antibody dependent cell-mediated cytotoxicity and antibody production. *Chemioterapia*, **VI,** 426-430.

Iwama, G. & Nakanishi, T. 1996. *The fish immune system. Organisms, pathogen, and environment*. San Diego: Academic Press.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G. C. & Sumpter, J. P. 1998. Widespread sexual disruption in wild fish. *Environmental Science & Technology*, **32,** 2498-2506.

Jobling, S., Reynolds, T., White, R., Parker, M. G. & Sumpter, J. P. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives*, **103,** 582-7.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P. & Sumpter, J. P. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environmental Toxicology & Chemistry*, **15,** 194-202.

Jobling, S. & Sumpter, J. P. 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology*, **27,** 361-372.

Johnson, S. C. & Albright, L. J. 1992. Effects of cortisol implants on the susceptibility and the histopathology of the responses of naive coho salmon *Oncorhynchus kisutch* to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Diseases of Aquatic Organisms*, **14,** 195-205.

Jokinen, E. I., Aaltonen, T. M. & Valtonen, E. T. 1995. Subchronic effects of pulp and paper mill effluents on the immunoglobulin synthesis of roach, *Rutilus rutilus*. *Ecotoxicology and Environmental Safety*, **32,** 219-25.

Jones, D. H., Lewis, D. H., Eurell, T. W. & Cannon, M. S. 1979. Alteration of the immune response of channel catfish (*Ictalurus punctatus*) by polychlorinated biphenyls. Animals as monitors of environmental pollutants. In: *Symposium on Pathobiology of Environmental Pollutants: Animal Models and Wildlife as Monitors*, pp. 385-386.

Jones, O. A., Voulvoulis, N. & Lester, J. N. 2001. Human pharmaceuticals in the aquatic environment a review. *Environmental Technology*, **22,** 1383-94.

Jones, S. R. 2001. The occurrence and mechanisms of innate immunity against parasites in fish. *Developmental & Comparative Immunology*, **25,** 841-52.

Kakuta, I. 1997. Effect of sewage on blood parameters and the resistance against bacterial infection of goldfish, *Carassius auratus*. *Environmental Toxicology & Water Quality*, **12,** 43-51.

Karrow, N. A., Boermans, H. J., Dixon, D. G., Hontella, A., Solomon, K. R., Whyte, J. J. & Bols, N. C. 1999. Characterizing the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*): a microcosm study. *Aquatic Toxicology*, **45,** 223-239.

Karrow, N. A., Bols, N. C., Whyte, J. J., Solomon, K. R., Dixon, D. G. & Boermans, H. J. 2001. Effects of creosote exposure on rainbow trout pronephros phagocyte activity and the percentage of lymphoid B cells. *Journal of Toxicology & Environmental Health*, **63,** 363-81.

Kelly-Reay, K. & Weeks, B. A. 1994. Determination of the macrophage chemiluminescent response in *Fundulus heteroclitus* as a function of pollution stress. *Fish & Shellfish Immunology*, **4,** 95-105.

Koellner, B., Dijkstra, J. M., Fischer, U., Schuetze, H. & Nakanishi, T. 2000. Influence of cultivation temperature and viral infection of RTG-2 cells on the expression of

rainbow trout classical MHC class I molecules. *Developmental & Comparative Immunology*, **24**.

Koellner, B., Wasserrab, B., Fischer, U., Kotterba, G. & van den Heuvel, M. R. in press. How can toxic effects of pollution of the aquatic envrionment on the immuno-competence of fishes be detected? A discussion on the relevance of biomarkers philosophy. In: *Hot spot pollutants: pharmaceuticals in the environment* (Ed. by Dietrich, D. R., Webb, S. F. & Petry, T.).

Köllner, B., Blohm, U., Kotterba, G. & Fischer, U. 2001. A monoclonal antibody recognising a surface marker on rainbow trout (*Oncorhynchus mykiss*) monocytes. *Fish & Shellfish Immunology*, **11,** 127-142.

Köllner, B., Fischer, U., Rombout, J. H. W. M., Taverne, A. & Hansen, J. D. submitted for publication. Indications for the involvement of rainbow trout thrombocytes in immune functions. A study using a panel of monoclonal antibodies and RT-PCR. *Fish & Shellfish Immunology*.

Köllner, B. & Kotterba, G. 2002. Temperature dependent activation of leucocyte populations of rainbow trout, *Oncorhynchus mykiss*, after intraperitoneal immunisation with *Aeromonas salmonicida*. *Fish & Shellfish Immunology*, **12,** 35-48.

Kollner, B., Wasserrab, B., Kotterba, G. & Fischer, U. 2002. Evaluation of immune functions of rainbow trout (*Oncorhynchus mykiss*) - how can environmental influences be detected? *Toxicology Letters*, **131,** 83-95.

Koppang, E. O., Hordvik, I., Bjerkas, I., Torvund, J., Aune, L., Thevarajan, J. & Endresen, C. 2003. Production of rabbit antisera against recombinant MHC class II beta chain and identification of immunoreactive cells in Atlantic salmon (*Salmo salar*). *Fish & Shellfish Immunology*, **14,** 115-132.

Kranz, H. & Gercken, J. 1987. Effects of sublethal concentrations of potassium dichromate on the occurrence of splenic melano-macrophage centres in juvenile plaice, *Pleuronectes platessa,* L. *Journal of Fish Biology*, **31 (Supplement A),** 75-80.

Kummerer, K. 2000. Drugs, diagnostic agents and disinfectants in wastewater and water - a review. *Schriftenreihe des Vereins fur Wasser-, Boden-, und Lufthygiene*, **105,** 59-71.

Kummerer, K. 2001. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere*, **45,** 957-69.

Kummerer, K., Erbe, T., Gartiser, S. & Brinker, L. 1998. AOX-emissions from hospitals into municipal waste water. *Chemosphere*, **36,** 2437-45.

Kümmerer, K., Steger-Hartmann, T. & Meyer, M. 1997. Biodegradability of the anti-tumour agent ifosfamide and its occurence in sewage water. *Ecotoxicology and Environmental Safety*, **31**.

Kuroda, A., Okamoto, N. & Fukuda, H. 2000. Characterization of monoclonal antibodies against antigens shared with neutrophils and macrophages in neutrophils in rainbow trout *Oncorhynchus mykiss*. *Fish Pathology*, **35,** 205-213.

Kuspis, D. A. & Krenzelok, E. P. 1996. What happens to expired medications? A survey of community medication disposal. *Veterinary and Human Toxicology*, **38,** 48-9.

Lappivaara, J. & Oikari, A. 1999. Altered challenge response in whitefish subchronically exposed in areas polluted by bleached kraft mill effluents. *Ecotoxicology & Environmental Safety*, **42,** 212-222.

Larsson, D., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P.-E. & Förlin, L. 1999. Ethinyloestradiol - an undesired fish contraceptive? *Aquatic Toxicology*, **45,** 91-97.

Le Morvan, C., Troutaud, D. & Deschaux, P. 1998. Differential effects of temperature on specific and nonspecific immune defences in fish. *Journal of Experimental Biology*, **201,** 165-8.

Le Morvan-Rocher, C., Troutaud, D. & Deschaux, P. 1995. Effects of temperature on carp leukocyte mitogen-induced proliferation and nonspecific cytotoxic activity. *Developmental & Comparative Immunology*, **19,** 87-95.

Lee, H. B., Weng, J., Peart, T. E. & Maguire, R. J. 1998. Occurrence of alkylphenoxyacetic acids in canadian sewage treatment plant effluent. *Water Quality Research Journal of Canada*, **33,** 19-29.

Loghothetis, P. N. & Austin, B. 1994. Immune response of rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Aeromonas hydrophila*. *Fish & Shellfish Immunology*, **4,** 239-254.

Luebke, R. W., Hodson, P. V., Faisal, M., Ross, P. S., Grasman, K. A. & Zelikoff, J. 1997. Aquatic pollution-induced immunotoxicity in wildlife species. *Fundamental and Applied Toxicology*, **37,** 1-15.

Lunden, T. & Bylund, G. 2002. Effect of sulphadiazine and trimethoprim on the immune response of rainbow trout (*Oncorhynchus mykiss*). *Veterinary Immunology & Immunopathology*, **85,** 99-108.

Lundén, T. & Bylund, G. 2000. The influence of *in vitro* and *in vivo* exposure to antibiotics on mitogen-induced proliferation of lymphoid cells in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, **10,** 395-404.

Mathew, J. A., Guo, Y. X., Goh, K. P., Chan, J., Verburg-van Kemenade, B. M. & Kwang, J. 2002. Characterisation of a monoclonal antibody to carp IL-1beta and the development of a sensitive capture ELISA. *Fish & Shellfish Immunology*, **13,** 85-95.

Matthiessen, P. & Sumpter, J. P. 1998. Effects of estrogenic substances in the aquatic environment. *Fish Ecotoxicology*, **86,** 319-35.

Mayer, K. S., Mayer, F. L. & Witt, A., Jr. 1985. Waste transformer oil and PCB toxicity to rainbow trout. *Transactions of the American Fisheries Society*, **114,** 869-886.

Mazur, C. F. & Iwama, G. K. 1993. Handling and crowding stress reduces number of plaque-forming cells in Atlantic salmon. *Journal of Aquatic Animal Health*, **5,** 98-101.

McMaster, M. E., Munkittrick, K. R. & Van Der Kraak, G. J. 1992. Protocol for measuring circulating levels of gonadal sex steroid in fish. In: *Canadian Technical Report of Fisheries and Aquatic Sciences 1836. Fisheries and Oceans*. Burlington, ON, Canada.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E. & Potter, T. 2000. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese Medaka (*Oryzias latipes*). *Environmental Toxicology & Chemistry*, **20,** 297-308.

Miller, N. W., Bly, J. E., van Ginkel, F., Ellsaesser, C. F. & Clem, L. W. 1987. Phylogeny of lymphocyte heterogeneity: identification and separation of functionally distinct subpopulations of channel catfish lymphocytes with monoclonal antibodies. *Developmental & Comparative Immunology*, **11,** 739-47.

Miller, N. W., Sizemore, R. C. & Clem, L. W. 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* antibody responses of channel catfish leucocytes. *The Journal of Immunology*, **134,** 2884-2888.

Möck, A. & Peters, G. 1990. Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. *Journal of Fish Biology*, **37,** 873-885.

Monosson, E. & Stegeman, J. J. 1991. Cytochrome P450E (P450IA) induction and inhibition in winter flounder by 3 3' 4 4' tetrachlorobiphenyl comparison of response in fish from

Georges Bank and Narragansett Bay Rhode Island USA. *Environmental Toxicology & Chemistry*, **10,** 765-774.

Moyner, K., Roed, K. H., Sevatdal, S. & Heum, M. 1993. Changes in non-specific immune parameters in Atlantic salmon, *Salmo salar* L. induced by *Aeromonas salmonicida* infection. *Fish & Shellfish Immunology*, **3,** 253-265.

Murad, A. & Houston, A. H. 1988. Leukocytes and leucopoietic capcity on goldfish, *Carassius auratus*, exposed to sublethal levels of cadmium. *Aquatic Toxicology*, **13,** 141-154.

Nagae, M., Fuda, H., Ura, K., Kawamura, H., Adachi, S., Hara, A. & Yamauchi, K. 1994. The effect of cortisol administration on blood plasma immunoglobulin M (IgM) concentrations in masu salmon (*Oncorhynchus masou*). *Fish Physiology & Biochemistry*, **13,** 41-48.

Narnaware, Y. K. & Baker, B. I. 1996. Evidence that cortisol may protect against the immediate effects of stress on circulating leukocytes in the trout. *General & Comparative Endocrinology*, **103,** 359-366.

Narnaware, Y. K., Baker, B. I. & Tomlinson, M. G. 1994. The effect of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout *Oncorhynchus mykiss*. *Fish Physiology & Biochemistry*, **13,** 31-40.

Nielsen, H. 1989. Antibiotics and human monocyte function. II. Phagocytosis and oxidative metabolism. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, **97,** 447-51.

Nunomura, W. 1991. C-Reactive protein in eel: purification and agglutinating activity. *Biochimica et Biophysica Acta*, **1076,** 191-196.

O´Neill, J. G. 1981a. The humoral immune response of *Salmo trutta* L. and *Cyprinus carpio* L. exposed to heavy metals. *Journal of Fish Biology*, **19,** 297-306.

O´Neill, J. G. 1981b. Effects of intraperitoneal lead and cadmium on the humoral immune response of *Salmo trutta*. *Bulletin of Environmental Contamination & Toxicology*, **27,** 42-48.

Ottinger, C. A. & Kaattari, S. L. 1998. Sensitivity of rainbow trout leucocytes to aflatoxin B_1. *Fish & Shellfish Immunology*, **8,** 515-530.

Partula, S. 1999. Surface markers of fish T-cells. *Fish & Shellfish Immunology*, **9,** 241-257.

Partula, S., De, G. A., Fellah, J. S. & Charlemagne, J. 1995. Structure and diversity of the T cell antigen receptor beta-chain in a teleost fish. *Journal of Immunology*, **155,** 699-706.

Payne, J. F. & Fancey, L. F. 1989. Effects of polycyclic aromatic hydrocarbons on immune responses in fish: change in melanomacrophage centers in flounder (*Pseudopleuronectes americanus*) exposed to hydrocarbon-contaminated sediments. *Marine Environmental Research*, **28,** 431-435.

Peakall, D. B., Miller, D. S. & Kinter, W. B. 1975. Blood calcium levels and the mechanism of DDE-induced eggshell thinning. *Envrionmental Pollution*, **9,** 289-294.

Peakall, D. B. & Walker, C. H. 1994. The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology*, **3,** 173-179.

Porter, C. M. & Janz, D. M. 2003. Treated municipal sewage discharge affects multiple levels of biological organization in fish. *Ecotoxicology and Environmental Safety*, **54,** 199-206.

Press, C. M. 1998. Immunology of fishes. In: *Handbook of vertebrate immunology* (Ed. by Pastoret, P. P., Griebel, P., Bazin, H. & Govaerts, A.), pp. 3-62: Academic Press.

Price, M. A., Jurd, R. D. & Mason, C. F. 1997. A field investigation into the effect of sewage effluent and general water quality on selected immunological indicators in carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology*, **7,** 193-207.

Prietz, A., Fleischhauer, V., Hitzfeld, B. & Dietrich, D. R. 2000. Effects of stream water on immune parameters of brown trout (*Salmo trutta f. fario*). In: *3rd SETAC (Society of Environmental Toxicology and Chemistry) World Conference, Mai 2000, Poster Presentation*.

Reynaud, S., Duchiron, C. & Deschaux, P. 2003. 3-methylcholanthrene inhibits lymphocyte proliferation and increases intracellular calcium levels in common carp (*Cyprinus carpio* L). *Aquatic Toxicology*, **63,** 319-31.

Rice, C. D., Banes, M. M. & Ardelt, T. C. 1995. Immunotoxicity in channel catfish, *Ictalurus punctatus*, following acute exposure to tributyltin. *Archives of Environmental Contamination and Toxicology*, **28,** 464-70.

Rice, C. D., Kergosien, D. H. & Adams, S. M. 1996. Innate immune function as a bioindicator of pollution stress in fish. *Ecotoxicology and Environmental Safety*, **33,** 186-92.

Rice, C. D. & Schlenk, D. 1995. Immune function and cytochrome P4501A activity after acute exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in channel catfish. *Journal of Aquatic Animal Health*, **7,** 195-204.

Rice, C. D. & Weeks, B. A. 1990. The influence of *in vivo* exposure to tributyltin on reactive oxygen formation in oyster toadfish macrophages. *Archives of Environmental Contamination and Toxicology*, **19,** 854-857.

Robinson, C. D., Brown, E., Craft, J. A., Davies, I. M., Moffat, C. F., Pirie, D., Robertson, F., Stagg, R. M. & Struthers, S. 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus minutus*, Pallas). *Aquatic Toxicology*, **62,** 119-34.

Robohm, R. A. 1986. Paradoxical effects of cadmium exposure on antibacterial antibody responses in two fish species: inhibition in cunners (*Tautogolabrus adspersus*) and enhancement in striped bass (*Morone saxatilis*). *Veterinary Immunology & Immunopathology*, **12,** 251-62.

Rodsaether, M. C., Olafsen, J., Raa, J., Myhre, K. & Steen, J. B. 1977. Copper as an initiating factor in vibriosis (*Vibrio anguillarum*) in eel (*Anguilla anguilla*). *Journal of Fish Biology*, **10,** 17-21.

Rohn, W. M., Lee, Y. J. & Benveniste, E. N. 1996. Regulation of class II MHC expression. *Critical Reviews in Immunology*, **16,** 311-30.

Ruane, N. M., Nolan, D. T., Rotllant, J., Tort, L., Balm, P. H. M. & Bonga, S. E. W. 1999. Modulation of the response of rainbow trout (*Oncorhynchus mykiss* Walbaum) to confinement, by an ectoparasitic (*Argulus foliaceus* L.) infestation and cortisol feeding. *Fish Physiology & Biochemistry*, **20,** 43-51.

Sacher, F., Lange, F. T., Brauch, H. J. & Blankenhorn, I. 2001. Pharmaceuticals in groundwaters Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *Journal of Chromatography A*, **938,** 199-210.

Sahoo, P. K. & Mukherjee, S. C. 2002. Influence of high dietary alpha-tocopherol intakes on specific immune response, nonspecific resistance factors and disease resistance of healthy and aflatoxin B1-induced immunocompromised Indian major carp, *Labeo rohita* (Hamilton). *Aquaculture Nutrition*, **8,** 159-167.

Scapigliati, G., Romano, N. & Abelli, L. 1999. Monoclonal antibodies in fish immunology: identification, ontogeny and activity of T- and B-lymphocytes. *Aquaculture*, **172,** 3-28.

Schmidt, H., Bernet, D., Wahli, T., Meier, W. & Burkhardt-Holm, P. 1999. Active biomonitoring with brown trout and rainbow trout in diluted sewage plant effluents. *Journal of Fish Biology*, **54,** 585-596.

Schuwerack, P. M., Lewis, J. W., Hoole, D. & Morley, N. J. 2001. Ammonia-induced cellular and immunological changes in juvenile *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis*. *Parasitology*, **122,** 339-45.

Secombes, C., Zou, J., Daniels, G., Cunningham, C., Koussounadis, A. & Kemp, G. 1998. Rainbow trout cytokine and cytokine receptor genes. *Immunological Reviews*, **166,** 333-40.

Secombes, C. J. 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: *Techniques in Fish Immunology* (Ed. by Van Muiswinkel, W. B.), pp. 101-103. Fair Haven: SOS Publications.

Secombes, C. J., Bird, S., Cunningham, C. & Zou, J. 1999. Interleukin-1 in fish. *Fish & Shellfish Immunology*, **9,** 335-343.

Secombes, C. J., Fletcher, T. C., O'Flynn, J. A., Costello, M. J., Stagg, R. & Houlihan, D. F. 1991. Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. *Comparative Biochemistry and Physiology. C: Comparative Pharmacology and Toxicology*, **100,** 133-6.

Secombes, C. J., Fletcher, T. C., White, A., Costello, M. J., Stagg, R. & Houlihan, D. F. 1992. Effects of sewage sludge on immune responses in the dab, *Limanda limanda* (L.). *Aquatic Toxicology*, **23,** 217-229.

Secombes, C. J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K. J., Cunningham, C. & Zou, J. 2001. Cytokines and innate immunity of fish. *Developmental & Comparative Immunology*, **25,** 713-23.

Secombes, C. J., White, A., Fletcher, T. C., Stagg, R. & Houlihan, D. F. 1995. Immune parameters in plaice, *Pleuronectes platessa*, L. along a sewage sludge gradient in the Firth of Clyde, Scotland. *Ecotoxicology*, **4,** 329-340.

Serrou, B. C., Solassol, L., Karcenty, H., Joyeux, X. & Pujol, H. 1972. Action immunodepressive de la rifampicin. *La Nouvelle Presse Medicale*, **1,** 1295.

Sharma, R. P. & Zeeman, M. G. 1980. Immunologic alterations by environmental chemicals: relevance of studying mechanisms versus effects. *Journal of Immunopharmacology*, **2,** 285-307.

Sizemore, R. C., Miller, N. W., Cuchens, M. A., Lobb, C. J. & Clem, L. W. 1984. Phylogeny of lymphocyte heterogeneity: the cellular requirement for *in vitro* mitogenic responses of channel catfish leukocytes. *The Journal of Immunology*, **133,** 2920-2924.

Slater, C. & Schreck, C. B. 1993. Testosterone alters the immune response of chinook salmon (*Oncorhynchus tshawytscha). General & Comparative Endocrinology*, **89,** 291-298.

Slater, C. H., Fitzpatrick, M. S. & Schreck, C. B. 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. *General & Comparative Endocrinology*, **100,** 218-25.

Smith, D. A., Schurig, G. G., Smith, S. A. & Holladay, S. D. 1999. Inhibited cytotoxic leukocyte activity in tilapia (*Oreochromis niloticus*) following exposure to immunotoxic chemicals. *International Journal of Toxicology*, **18,** 167-172.

Sole, M., Barcelo, D. & Porte, C. 2002. Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in carp, *Cyprinus carpio*, in relation to sewage treatment plants. *Aquatic Toxicology*, **60,** 233-48.

Spies, R. B. & Rice, D. W. 1988. Effects of organic contaminants on reproduction of the starry flounder, *Platichthys stellatus*, in San Francisco Bay. II. Reproductive success of fish captured in San Francisco Bay and spawned in the laboratory. *Marine Biology*, **98,** 191-200.

Stegeman, J. J. & Hahn, M. E. 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: *Aquatic Toxicology: Molecular, Biochemical*

and Cellular Perspectives (Ed. by Ostrander, G. K. & Malins, D.), pp. 87. Boca Raton, FL: Lewis Publishers.

Steger-Hartmann, T., Kummerer, K. & Hartmann, A. 1997. Biological degradation of cyclophosphamide and its occurrence in sewage water. *Ecotoxicology and Environmental Safety*, **36,** 174-9.

Steger-Hartmann, T., Lange, R. & Schweinfurth, H. 1999. Environmental risk assessment for the widely used iodinated X-ray contrast agent iopromide (Ultravist). *Ecotoxicology and Environmental Safety*, **42,** 274-81.

Stumpf, M., Ternes, T. A., Wilken, R. D., Rodrigues, S. V. & Baumann, W. 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. *The Science of the Total Environment*, **225,** 135-41.

Tahir, A., Fletcher, T. C., Houlihan, D. F. & Secombes, C. J. 1993. Effect of short-term exposure to oil-contaminated sediments on the immune response of dab, *Limanda limanda* (L.). *Aquatic Toxicology*, **27,** 71-82.

Ternes, T. A. 1998. Occurence of drugs in German sewage treatment plants and rivers. *Water Research*, **32,** 3245-3260.

Thuvander, A. 1989. Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. *Journal of Fish Biology*, **35,** 521-529.

Thuvander, A., Fossum, C. & Lorenzen, N. 1990. Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays. *Developmental & Comparative Immunology*, **14,** 415-23.

van den Heuvel, M. R. & Ellis, R. J. 2002. Timing of exposure to a pulp and paper effluent influences the manifestation of reproductive effects in rainbow trout. *Environmental Toxicology & Chemistry*, **21,** 2338-2347.

van den Heuvel, M. R., Power, M., MacKinnon, M. D. & Dixon, D. G. 1999. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. chemical and biochemical indicators of exposure to oil sands related waters. *Canadian Journal of Fisheries and Aquatic Sciences*, **56,** 1226-1233.

Van der Auwera, P. & Husson, M. 1989. Influence of rifampicin and ansamycin on motility and adherence of human neutrophils studied *in vitro*. *Journal of Antimicrobial Chemotherapy*, **24,** 347-353.

Van Muiswinkel, W. B. 1992. Fish immunology and fish health. *Netherlands Journal of Zoology*, **42,** 494-499.

Verburg-van Kemenade, B. M., Weyts, F. A., Debets, R. & Flik, G. 1995. Carp macrophages and neutrophilic granulocytes secrete an interleukin-1- like factor. *Developmental & Comparative Immunology*, **19,** 59-70.

Verburg-van Kemenade, B. M. L., Nowak, B., Engelsma, M. Y. & Weyts, F. A. A. 1999. Differential effects of cortisol on apoptosis and proliferation of carp B-lymphocytes from head kidney, spleen and blood. *Fish & Shellfish Immunology*, **9,** 405-415.

Vethaak, A. D., Bucke, D., Lang, T., Wester, P. W., Jol, J. & Carr, M. 1992. Fish disease monitoring along a pollution transect: A case study using dab *Limanda limanda* in the German Bight. *Marine Ecology-Progress Series*, **91,** 173-192.

Vial, T., Nicolas, B. & Descotes, J. 1996. Clinical immunotoxicity of pesticides. *Journal of Toxicology & Environmental Health*, **48,** 215-29.

Vladimirov, V. L. 1968. Immunity in fish. *Bulletin de l´Office International des Epizooties*, **69,** 1365-72.

Wahli, T., Knuesel, R., Bernet, D., Segner, H., Pugovkin, D., Burkhardt-Holm, P., Escher, M. & Schmidt-Posthaus, H. 2002. Proliferative kidney disease in Switzerland: Current state of knowledge. *Journal of Fish Diseases*, **25,** 491-500.

Wang, R. & Belosevic, M. 1995. The *in vitro* effects of estradiol and cortisol on the function of a long-term goldfish macrophage cell line. *Developmental & Comparative Immunology*, **19,** 327-336.

Wechsler, S. J., McAllister, P. E., Hetrick, F. M. & Anderson, D. P. 1986. Effect of exogenous corticosteroids on circulating virus and neuralising antibodies in striped bass (*Morone saxatilis*) infected with infectious pancreatic necrosis virus. *Veterinary Immunology & Immunopathology*, **12,** 305-311.

Weeks, B. A. & Warinner, J. E. 1986. Functional evaluation of macrophages in fish from a polluted estuary. *Veterinary Immunology & Immunopathology*, **12,** 313-20.

Wester, P. W., Vethaak, A. D. & van Muiswinkel, W. B. 1994. Fish as biomarkers in immunotoxicology. *Toxicology*, **86,** 213-32.

Weyts, F. A. A., Verburg-van Kemenade, B. M. L., Flik, G., Lambert, J. G. D. & Wendelaar Bonga, S. E. 1997. Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. *Brain, Behaviour, and Immunity*, **11,** 95-105.

Wilkinson, L. 1990. SYSTAT: The System for Statistics. Evanston, IL, USA: SYSTAT Inc.

Wilson, M. R., Zhou, H., Bengten, E., Clem, L. W., Stuge, T. B., Warr, G. W. & Miller, N. W. 1998. T-cell receptors in channel catfish: structure and expression of TCR alpha and beta genes. *Molecular Immunology*, **35,** 545-57.

Wishkovsky, A., Mathews, E. S. & Weeks, B. A. 1989. Effect of tributyltin on the chemiluminescent response of phagocytes from three species of estuarine fish. *Archives of Environmental Contamination and Toxicology*, **18,** 826-831.

Wojtaszek, J., Dziewulska-Szwajkowska, D., Lozinska-Gabska, M., Adamowicz, A. & Dzugaj, A. 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): cortisol effect on the carp blood. *General and Comparative Endocrinology*, **125,** 176-83.

Wolke, R. E. 1992. Piscine macrophage aggregates: A review. *Annual Review of Fish Diseases*, **2,** 91-108.

Wolke, R. E., Murchelano, R. A., Dickstein, C. D. & George, C. J. 1985. Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. *Bulletin of Environmental Contamination and Toxicology*, **35,** 222-7.

Yamaguchi, T., Watanuki, H. & Sakai, M. 2001. Effects of estradiol, progesterone and testosterone on the function of carp, *Cyprinus carpio*, phagocytes *in vitro*. *Comparative Biochemistry & Physiology C Toxicology and Pharmacology*, **129,** 49-55.

Yin, Z., Lam, T. J. & Sin, Y. M. 1995. The effects of crowding stress on the non-specific immune response in fancy carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology*, **5,** 519-529.

Yousif, A. N., Albright, L. J. & Evelyn, T. P. T. 1991. Occurrence of lysozyme in the eggs of coho salmon *Oncorhynchus kisutch*. *Diseases of Aquatic Organisms*, **10,** 45-49.

Yousif, A. N., Albright, L. J. & Evelyn, T. P. T. 1994a. *In vitro* evidence for the antibacterial role of lysozyme in salmonid eggs. *Diseases of Aquatic Organisms*, **19,** 15-19.

Yousif, A. N., Albright, L. J. & Evelyn, T. P. T. 1994b. Purification and characterization of a galactose-specific lectin from the eggs of coho salmon *Oncorhynchus kisutch* and its interaction with bacterial fish pathogens. *Diseases of Aquatic Organisms*, **20,** 127-136.

Zelikoff, J. T. 1993. Metal pollution-induced immunomodulation in fish. *Annual Review of Fish Diseases***,** 305-325.

Zelikoff, J. T. 1998. Biomarkers of immunotoxicity in fish and other non-mammalian sentinel species: Predictive value for mammals? *Toxicology*, **129,** 63-71.

Zelikoff, J. T., Bowser, D., Squibb, K. S. & Frenkel, K. 1995. Immunotoxicity of low level cadmium exposure in fish: An alternative animal model for immunotoxicological studies. *Journal of Toxicology & Environmental Health*, **45,** 235-248.

Zelikoff, J. T., Raymond, A., Carlson, E., Li, Y., Beaman, J. R. & Anderson, M. 2000. Biomarkers of immunotoxicity in fish: From the lab to the ocean. *Toxicology Letters***,** 325-331.

Zou, J., Cunningham, C. & Secombes, C. J. 1999a. The rainbow trout *Oncorhynchus mykiss* interleukin-1 beta gene has a different organization to mammals and undergoes incomplete splicing. *European Journal of Biochemistry*, **259,** 901-8.

Zou, J., Grabowski, P. S., Cunningham, C. & Secombes, C. J. 1999b. Molecular cloning of interleukin 1beta from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ice cut site. *Cytokine*, **11,** 552-560.

Zou, J., Holland, J., Pleguezuelos, O., Cunningham, C. & Secombes, C. J. 2000a. Factors influencing the expression of interleukin-1 beta in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Developmental & Comparative Immunology*, **24,** 575-82.

Zou, J., Wang, T., Hong, S., Cunningham, C. & Ch.J., S. 2000b. Role of interleukin-1ß in immune responses of rainbow trout. *Fish & Shellfish Immunology*, **10,** 289.

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