JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE

84

Tuula Aaltonen

Effects of Pulp and Paper Mill Effluents on Fish Immune Defence

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2000

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 84

Tuula Aaltonen

Effects of Pulp and Paper Mill Effluents on Fish Immune Defence

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Villa Ranan Blomstedt-salissa
toukokuun 26. päivänä 2000 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Natural Sciences of the University of Jyväskylä,
in the Building Villa Rana, Blomstedt Hall, on May 26, 2000 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2000

Effects of Pulp and Paper Mill Effluents on Fish Immune Defence

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 84

Tuula Aaltonen

Effects of Pulp and Paper Mill Effluents on Fish Immune Defence

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2000

Editors
Jukka Särkkä
Department of Biological and Environmental Science, University of Jyväskylä
Kaarina Nieminen
Publishing Unit, University Library of Jyväskylä

URN:ISBN:978-951-39-8333-8
ISBN 978-951-39-8333-8 (PDF)
ISSN 1456-9701

ISBN 951-39-0724-4
ISSN 1456-9701

Jyväskylä University Printing House,
Jyväskylä and ER-Paino, Lievestuore 2000

ABSTRACT

Aaltonen, Tuula
Effects of pulp and paper mill effluents on fish immune defence
Jyväskylä: University of Jyväskylä, 2000, 62 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 84)
ISBN 951-39-0724-4
Yhteenveto: Metsäteollisuuden jätevesien aiheuttamat immunologiset muutokset kaloissa
Diss.

Discharges from pulp and paper mills cause a marked contamination in recipient waters. A variety of biological effects in fish have been associated with exposures to bleached kraft mill effluents (BKMEs).

Lately emphasis has been placed on the development of tests in which fish serve as sentinel animals or biomarkers of immunotoxicity in contaminated waters. In order to study the effects of BKMEs on fish immune defence, a panel of assays was developed for roach (*Rutilus rutilus*). The fish were challenged by immunising and the numbers of antigen-specific antibody-secreting cells (ASC) in lymphoid organs and blood were enumerated, and the levels of the antigen-specific antibodies in the circulation were determinated. In addition, immunoglobulin-secreting cells (ISC), the concentration of immunoglobulin and mitogen driven proliferation of lymphocytes *in vitro* were assayed. Furthermore, phagocytosis and migration of leukocytes were studied.

The antibody-mediated immunity of roach was suppressed after exposure, both on-site in a contaminated lake and in an aquarium, to BKME from a mill with elemental chlorine/chlorine dioxide (C/D) bleaching. Exposure to diluted untreated bleach liquor from C/D process suppressed immunoreactivity more than liquors from elemental chlorine-free (ECF) or totally chlorine-free (TCF) processes. Primary-treated and also secondary-treated BKME from a mill using ECF/TCF bleaching affected immune parameters of fish. Sex-related differences were evident in certain immune functions.

Response to immunisation proved to be a sensitive indicator of effects of BKMEs on fish immune system. The results indicate that BKMEs are potentially immunotoxic in fish. Recent changes in pulping and bleaching processes have resulted in less immunomodulatory effects suggesting lower toxicity.

Key words: Aquatic pollution; biomarkers for immunotoxicology; BKME; ECF; fish immune system; immunotoxicity; TCF.

T. Aaltonen University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FIN-40351 Jyväskylä, Finland

Author´s address

Tuula Aaltonen
Department of Biological and Environmental Science
University of Jyväskylä
P.O. Box 35, FIN-40351
Jyväskylä, Finland
E-mail tumiaa@jyu.fi

Supervisor

Docent Ilmari Jokinen
Department of Biological and Environmental Science
University of Jyväskylä
P.O. Box 35, FIN-40351
Jyväskylä, Finland
E-mail eijokine@jyu.fi

Reviewers

Professor Mikko Nikinmaa
Department of Biology
FIN-20014 University of Turku, Finland
E-mail mikko.nikinmaa@utu.fi

Dr Anthony Ellis
FRS, Marine Laboratory, P.O. Box 101
Victoria Road, Aberdeen AB11 9DB, UK
E-mail t.ellis@marlab.ac.uk

Opponent

Docent Harry Björklund
Department of Biology,
FIN-20014 University of Turku, Finland
E-mail harry.bjorklund@utu.fi

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Aaltonen T.M., Jokinen E.I. & Valtonen E.T., 1994: Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. Fish Shellfish Immunol. 4: 129-140.

II 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*. Ecotoxicol. Environ. Safety 32: 219-225

III 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. Ecotoxicol. Environ. Safety 38: 266-271

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

V Aaltonen, T.M., Jokinen, E.I., Lappivaara, J., Markkula, S.E., Salo, H.M. , Leppänen, H., & Lammi R., 2000: Effects of primary- and secondary-treated bleached kraft mill effluents on the immune system and physiological parameters of roach. Aquat. Toxicol. 50(4) (in press)

ABBREVIATIONS

AOX	absorbable organic halogen
ASC	antibody-secreting cells
BGG	bovine γ-globulin
BKME	bleached kraft mill effluent
BOD	biological oxygen demand
BSA	bovine serum albumin
COD	chemical oxygen demand
ConA	concanavalin A
C/D	chlorine/chlorine dioxide
DTPA	diethylenetriaminepentaacetic acid
ECF	elemental chlorine-free
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immuno spot
EROD	7-ethoxyresorufin O-deethylase
FCA	Freund´s complete adjuvant
Ig	immunoglobulin
ISC	immunoglobulin-secreting cells
LPS	lipopolysaccharide
NBT	nitro blue tetrazolium
NCC	natural cytotoxic cell
NK cell	natural killer cell
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PFC	plaque forming cell
PHA	phytohemagglutinin
p.i.	post immunisation
PMA	phorbol 12-myristate 13-acetate
SI	stimulation index
TCF	totally chlorine-free

1 INTRODUCTION

The effluents from pulp and paper mills are one of the major contaminants of lakes in countries with a wood-processing industry. Bleached kraft mill effluent (BKME) is a complex mixture of soluble chemicals and insoluble particles and fibres (Sjöström & Westermark 1999). The characteristics of each effluent are dependent on wood furnish and technology as well as final effluent treatment. The main effluent streams from the kraft pulp process are filtrates from bleaching, waste waters from debarking, a part of the condensates from cooking and evaporation, black liquor residues and spills from different process departments (LaFleur 1996; Anonymous 1997).

During the period 1945-1980 the pulp mill industry caused substantial waste water discharges into receiving waters. The effects observed were sometimes of a dramatic character with oxygen depletion and fish kills. Therefore more attention was paid to removing high discharges of oxygen-consuming suspended and dissolved solids, and mills started to treat their waste waters before discharging effluent. However, a variety of biological effects, including structural, physiological and biochemical responses in fish, were still associated with exposures to discharges from a mill using chlorine/chlorine dioxide (C/D) bleaching (Andersson et al. 1988; Södergren 1989; Lindström-Seppä & Oikari 1989b; Hodson et al. 1992). From the end of the 1970s until recently, the main emphasis in studies of pulp mill effects was put on the role of chlorinated substances formed in the bleaching (Turoski 1998). During the last decade, rapid development in bleaching processes has occurred and most mills have substituted chlorine dioxide for chlorine. The main types of bleaching practised today in the Nordic countries are the elemental chlorine-free (ECF) bleaching and the totally chlorine-free (TCF) bleaching. The toxicity of effluents from mills with activated sludge treatment has drastically reduced. However, alterations has still been observed in biochemical and reproductive status of fish exposed to effluents from mills using ECF/TCF bleaching (Sandström 1995; Larsson et al. 1997; Karels 2000; Soimasuo et al. 2000).

Knowledge about the effects of pulp mill effluents on the immune defence of fish has been very limited. However, the increased prevalence of parasites on roach in waters polluted by BKME (Valtonen & Koskivaara 1989; Valtonen & Koskivaara 1994) as well as an altered white blood cell picture of exposed perch

(Andersson et al. 1988) has suggested impaired immunity in fish. Recently, a reduced level of plasma IgM (Soimasuo et al., 1995a; b) and disturbed macrophage activity (Fournier et al. 1998) in fish exposed to effluents from a pulp mill using C/D bleaching has been reported.

In the present study, the effects of treated and untreated BKMEs on the immune defence of fish were investigated. The effects of secondary-treated BKME were studied on-site in a lake and the results verified under laboratory conditions. The role of different bleaching processes as well as the influence of waste water treatment concerning the immunotoxicity of fish were also evaluated.

2 REVIEW OF LITERATURE

2.1 Pulp and paper mill effluents

The dominant pulp producers are the industrialised countries of the northern hemisphere, i.e. the USA, Canada, Sweden, Japan and Finland. In mechanical pulping the structure of wood is broken up by applying a mechanical force. In chemical pulping a mixture of wood chips and aqueous solution of chemicals is heated and the lignin is dissolved. Chemical pulping is conducted by two processes, the sulphite process and the kraft (sulphate) process. The kraft process has become the main process both for unbleached and bleached grades because it more easily allows the recovery of valuable chemicals and, as a result, minimises discharges into the environment (Kleppe 1970; Solomon et al. 1993).

In kraft pulping, the cooking liquor is separated from the fibres after cooking. This so-called weak black liquor holds the cooking chemicals and approximately 50% of the original wood (Anonymous 1997). The lignin cannot be completely removed in cooking without damaging the fibre. In a modern kraft pulp mill wood fibre is further delignified prior to final bleaching. In order to recover additional recoverable dissolved organic solids and used chemicals, the pulp is washed using counter current flow and the liquid is combined with the weak black liquor, evaporated and burnt in a recovery boiler. Extended delignification is performed by applying oxygen delignification or, preferably, a combination of extended cooking and oxygen delignification. The use of efficient washing equipment is an essential part of the process (Anonymous 1997; McDonough 1998). The residual lignin affects the whiteness of the pulp and further processing, called bleaching, removes the dark colour. In bleaching today, elemental chlorine has been replaced by chlorine dioxide (ECF bleaching) or by totally chlorine-free (TCF bleaching) bleaching agents such as oxygen, ozone and hydrogen peroxide (Anonymous 1997).

During the period 1945-1979, the pulp and paper industry caused substantial waste water discharges into the receiving waters. During that time these discharges had dramatic effects such as oxygen depletion and fish kills.

The reason for such effects was the high amount of oxygen-consuming suspended and dissolved solids (Tana & Lehtinen 1996). More attention was paid to removing these substances and mills started treating their waste waters before discharge. In twelve years (1980-1992) the suspended solids and oxygen-demanding loads (BOD) from the forestry industry fell by 65% and 80%, respectively. Yet, at the same time the output of paper and boards rose by 50% and that of pulp by 20% (Karessuo 1992). From the end of the 1970s until recently, the main emphasis was put on the role of chlorinated substances formed during the bleaching (Turoski 1998). All chlorinated organic substances were regarded as man-made, non-degradable, bioaccumulative and hazardous to biological system. However, it has been shown that a significant generation of halogenated -mainly chlorinated and brominated- compounds occurs naturally in both terrestrial and marine environments as a result of activity by several organisms like bacteria, fungi, algae, lichen and higher plants (see Axegård et al., 1993).

The environmental control authorities in many countries, including Finland, have set restrictions on the discharges of chlorinated organics measured as Absorbable Organic Halogen (AOX) into the aquatic environment. The official limit in 1995 in Finland was 1.4 kg AOX/t pulp, although the mean discharge of AOX that year was in fact much lower (0.34 kg/t pulp) (Karessuo 1996). The decrease in AOX has been achieved by a combination of several means. First of all elementary chlorine has been replaced by chlorine dioxide in the bleaching process, and other oxygen-containing chemicals such as molecular oxygen, peroxide and ozone have also been introduced. Elemental chlorine has not been used as a bleaching agent since 1993 in Finland (Anonymous 1997) and most of the pulp in western Europe is also produced without elementary chlorine (Axegård et al. 1993). Another factor contributing to the decreased emissions of AOX into receiving waters is the installation of external treatment plants. Finally, most mills have decreased their waste water emissions by increased closure of processes, improved spill control, sewer design, handling of condensates and improved process control (Figure 1) (Tana & Lehtinen 1996). Current trends within the pulp and paper industry, at least in Scandinavia and Canada, is towards the increased closure of bleach plants by using either ECF (elemental chlorine-free) or TCF (totally chlorine-free) bleaching (Ericson et al. 1998).

2.1.1 Treatment of BKME

The effluent treatment implemented in the pulp and paper industry consists of primary (mechanical) treatment, and secondary (biological) treatment. The first step in the effluent treatment sequence, in which suspended solids (fibres, bark particles and inorganic materials such as fillers) are removed, known as primary treatment. The separation of suspended solids is generally carried out mechanically by sedimentation, flotation and filtration. The efficiency of clarification is improved by the addition of coagulants and flocculants (Anonymous 1997).

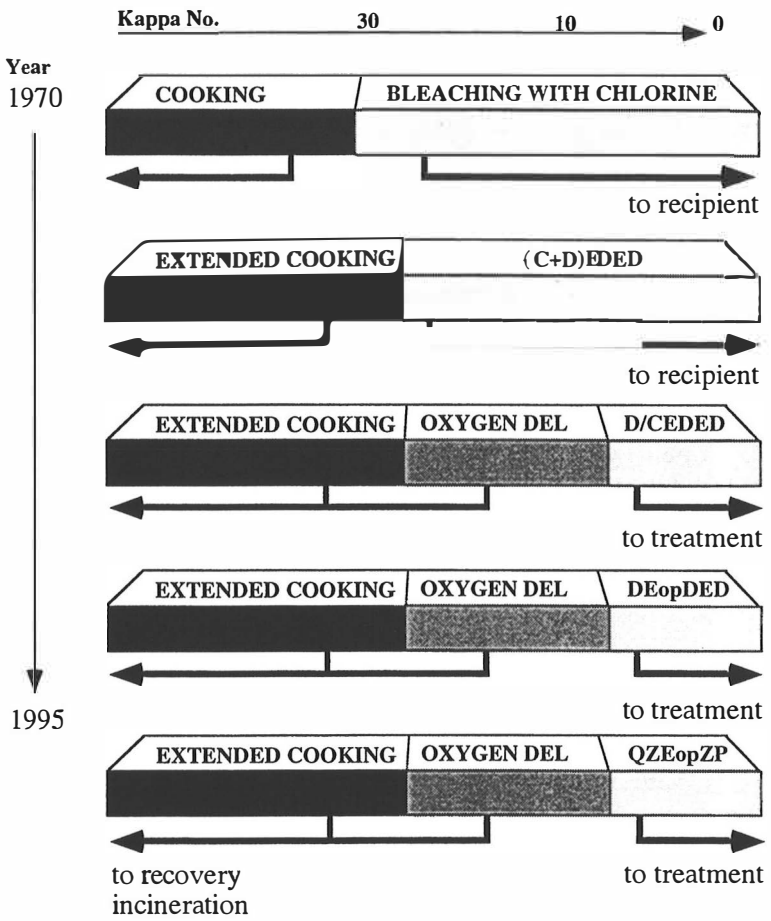


FIGURE 1 Development in pulping , bleaching and discharges (modified from Annergren, 1993).

The secondary treatment of waste waters utilises natural biological process, where micro-organisms utilise dissolved organic material in the water as a source of energy. Two types of methods are used: aerobic and anaerobic treatment. Although anaerobic treatment has advantages such as lower demands on electrical power, a lower nutrient demand and lower sludge production, it is suitable only for effluents with a relatively high concentration of easy degradable organic substances and thus its utilisation is very limited. The aerobic treatment methods used in the pulp and paper industry are activated sludge and aerated lagoon-type installations. In 1984 the first activated sludge plants in the Finnish pulp and paper mill industry came into

operation, and today almost all Finnish kraft mills are equipped with this efficient effluent treatment system (Anonymous 1997; Ruonala & Lammi 1997).

The biological treatment of effluents has found to be efficient in reducing acute toxicity and BOD; more than 95% of the BOD in the water phase can be eliminated by activated sludge. COD and AOX are reduced by up to about 60% (Georgov et al. 1988). The reduction in AOX is higher for effluents from ECF-bleaching than for effluents from chlorine bleaching (Dahlman et al. 1993). This is probably due to a higher percentage of low molecular weight AOX in the effluent when the pulp is bleached with chlorine dioxide instead of elemental chlorine (O´Connor et al. 1994). TCF processes lead to the discharge of metal chelating agents which are susceptible to biodegradation under aerobic conditions, although the rate of biodegradation is too slow to ensure efficient removal during the biological treatment of sewage. Secondary treatment is also efficient in removing wood extractives (LaFleur et al. 1998). Sterol removal was found to range from 13 to 95% (Cook et al. 1997; LaFleur et al. 1998). Concentrations of unchlorinated resin acids usually decreased by 90% or more following treatment, whereas chlorinated resin acids were more resistant to biological biodegradation (McLeay 1987).

2.1.2 Characterisation of BKME

Pulp and paper mill effluents are very complex mixtures of organic and inorganic compounds varying in chemical structure and molecular weight. The characteristics of each effluent are dependent on numerous factors, including wood furnish and process technology (washing, condensates, cooking, bleaching etc.) as well as final effluent treatment (Axegård et al. 1993). Organic substances originate mainly from the natural chemical constituents of wood: polysaccharides, lignin and extractives. Resin acids, fatty acids and phytosterols are the most common wood extractives found and are shared by many pulping processes (Sjöström & Alén 1999).

The compounds identified can be separated into three main classes according to their chemical properties: acids, phenolic compounds and neutral compounds. The organic material can also separated according to molecular weight. Hundreds of low molecular weight organic compounds have been identified in effluents from production of bleached kraft pulp (Mikkelson & Paasivirta 1997; Dahlman et al. 1999). The high molecular weight material carries a large part of the COD and AOX found in kraft bleach plant effluents. (Dahlman et al. 1999). The molecular weight distribution will vary depending on the wood species and bleaching processes used. The effluents from the production of bleached hardwood kraft pulp contain organic material of lower average molecular weight than the corresponding softwood effluents (Mörck et al. 1991). Effluent from modern bleaching with oxygen delignification also exhibits lower molecular weight (Yin et al. 1989; Yin et al. 1990; O´Connor et al. 1994). Biological treatment shifts the molecular weight distribution of the organic material towards higher molecular weight because the bacteria preferably remove organic material of low molecular weight (Yin et al. 1989; Yin et al. 1990; Dahlman et al. 1993).

The release of chlorinated compounds has decreased dramatically following ClO_2 removal. Effluents from bleaching kraft pulp by ECF bleaching contains the same compounds (resin acid, fatty acid, chlorinated acetic acid, chlorinated and nonchlorinated phenolic compounds, chloroform, terpenes, terpenoids and sterols) but considerably lower quantities of chlorinated compounds and with a lower degree of chlorination than the effluent from earlier kraft mills with chlorine-based bleaching processes(Bright et al. 1997; LaFleur & Barton 1997). Mikkelson & Paasivirta (1997) have identified and quantified the relative abundance of more than 300 compounds in effluents from pine pulp process with ECF or TCF bleaching.

Other chemical constituents common to many pulping processes include process additives (LaFleur & Barton 1997). Alkylphenol polyethoxylates are constituents in the surfactants and detergents used by the industry. The most abundantly used is nonylphenol ethoxylate. The original surfactant can break down into several metabolites that are more biologically active. Other process additives that are receiving greater attention due to the increased use of ozone and peroxide bleaching are the chelants ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). The increased use of EDTA/DTPA, although not considered toxic, may result in the increased discharge of some metals.

2.1.3 Effects of BKME on fish

Conventional pulp and paper mill effluents exhibited toxicity that were acutely lethal to fish, but when diluted in recipient waters toxicity decreased below lethal levels (Miettinen et al. 1982; Oikari et al. 1984; McLeay 1987). Fish kills associated with pulp and paper mill discharges have been seldom observed and no fish kills have been reported in any waterbodies receiving discharges of biotreated mill effluent (McLeay 1987). The toxicity of effluents from modern mills using ECF bleaching has been drastically reduced and acute lethal effects, even with untreated effluent, are no longer observed (Priha 1996; Verta et al. 1996).

In the 1980s increased attention began to be paid to the sublethal effects of BKME. The effects of BKME using chlorine-based bleaching have been associated with habitat alterations and subsequent effects on fish population and community levels, e.g. lowered fish biomass and the differences in the species composition of the fish community close to the discharge outlet (Adams et al. 1992; Hakkari 1992; Södergren 1993). There are, however, other studies which have found BKME to have only minor effects at the population and community levels (Hodson et al. 1992; Swanson et al. 1994). Reproductive effects such as decreased steroid hormone levels, delayed maturity, reduced gonad size and fecundity have been reported in several fish species exposed to BKME (Lindström-Seppä & Oikari 1989a; Munkittrick et al. 1991; Södergren 1993; Gagnon et al. 1994; Munkittrick et al. 1994).

Various physiological and biochemical disturbances have been noted in fish after exposure to BKME. Typical symptoms have been altered liver function, such as liver enlargement, reduced liver RNA and protein concentration, hepatocellular changes, and strong induction of hepatic MFO

activity (Lehtinen & Oikari 1980; Oikari & Niittylä 1985; Hodson et al. 1992). The induction of liver MFO activity measured as EROD activity probably is the most prominent response to pulp and paper mill effluent and has been well revealed e.g. in studies in Southern Lake Saimaa (Lindström-Seppä & Oikari 1989b; Soimasuo et al. 1995b; Oikari & Holmbom 1996). Exposure to conventional BKME has also led to altered carbohydrate metabolism and osmo/ionoregulation in fish (Andersson et al. 1988; Lindström-Seppä & Oikari 1990). Changes in blood cellular parameters and alterations in blood hemoglobin and plasma protein concentrations occurred in fish under the influence of BKME (Oikari et al. 1985; Andersson et al. 1988; Larsson et al. 1988; Sandström & Thoresen 1988; Lindström-Seppä & Oikari 1989b; Lindström-Seppä & Oikari 1990; Sandström et al. 1992). Fish also suffered from a higher frequency of vertebral deformities and fin erosion (Andersson et al. 1987; Härdig et al. 1988; Lindesjöö & Thulin 1990; Lindesjöö & Thulin 1992; Lindesjöö & Thulin 1994). Furthermore, the white blood cell pattern suggested a suppressed immune defence (Andersson et al. 1988). Studies in Finland, in Lake Saimaa, showed decreased IgM concentrations in fish caged near a pulp and paper mill using chlorine-based bleaching (Soimasuo et al. 1995b).

The results after exposure to single defined chlorinated chemical, such as tetra-chloro-1,2-bensoquinone (Bengtsson et al. 1988b), were similar to those observed in fish exposed to bleached kraft mill effluents in the laboratory or in fish living in the receiving waters from a bleach pulp mill. This suggests that chlorinated compounds in the bleached kraft mill effluents contributed to the symptoms in the exposed fish. However, changes in fish performance could also be associated with a variety of other factors in effluents. In fact, some changes were evident at sites not receiving bleaching effluents (Munkittrick et al. 1994) and, for example, exposure to unbleached kraft mill effluents in a long-term laboratory experiment with fourhorn sculpins (*Myoxocephalus quoadricornus*) resulted in increased frequency of vertebral deformities, although the responses were considerably lower compared to those following exposures to bleached effluents (Bengtsson et al. 1988a). Thus, there is growing awareness that some of the biological effects observed in pulp mill effluents may be attributable to natural wood extractives. Extractives acutely toxic to fish include juvabione (Leach et al. 1975), resin and fatty acids (Leach & Thakore 1977), and diterpene alcohols (Leach & Thakore 1977). A mixture of resin and fatty acids has also been found to cause sublethal effects in fish, such as liver dysfunction, increased plasma bilirubin and major effects on respiration and the energy metabolism (Oikari & Nakari 1982). As early as in the middle of the 1970s, a Canadian study showed that sterols were also acutely toxic to fish (Leach et al. 1975). More recently it has been found that wood-derived sterols interfere with the hormonal status of fish (MacLatchy & Van Der Kraak 1994; Tremblay & Van Der Kraak 1998).

The rapid development and introduction of new techniques for bleaching of kraft pulp in Scandinavia has resulted in a substantial reduction in chlorinated organic materials discharged into the receiving waters (Södergren 1993; Oikari & Holmbom 1996). Recovery studies were performed both in Finland and in Sweden after alterations to the pulping and bleaching and waste water treatment system. Recovery studies in Finland showed that EROD

induction was only 4-20% of that measured before the alterations and that blood IgM levels and hematocrit had returned to normal (Soimasuo et al. 2000). Despite the decreased exposure of fish to BKME, reproductive status, measured by serum steroid hormone levels, gonad size and fecundity, was affected in perch, and to a lesser extent in roach (Karels 2000). Recovery studies in Sweden demonstrated a pronounced reduction or elimination of the previously observed effects in the receiving waters (Sandström 1995; Larsson et al. 1997). Fish biomass and the species composition of the fish community had successfully returned to normal. The fin erosions and other histopathological changes in perch as well as many biochemical responses had disappeared. Signs of liver enlargement were not detected after 1990. Induced hepatic EROD activity, slightly elevated hematocrit and reduced gonad size continued to be observed in 1993 in fish near a pulp mill. In 1995, gonad size and red blood cell picture were normal, while delay in sexual maturity and slight EROD induction indicated incomplete recovery (Sandström 1995; Larsson et al. 1997).

Recently, the differences in toxicity between untreated and secondary treated effluents from TCF and ECF processes have been studied. In the study by Priha (1996) the total effluents from all 15 Finnish pulp mills evaluated by laboratory short-term tests. In general, the biologically treated bleached kraft mill effluents showed little or no toxicity regardless of the type of bleaching process used. The mechanically treated effluents were toxic whereas none of the secondary treated effluents at a 100% concentration affected the survival of zebra fish embryos and larvae. Many studies have found some degree of toxicity in both ECF and TCF effluents, and thus it cannot be concluded that one modern bleaching technique is superior to another in terms of its environmental effects (Kovacs et al. 1994; Verta et al. 1996).

2.2 Immunotoxicity in fish

Immunotoxicology in fish has received increasing attention in recent years. There are several reasons for developing this field: many fish diseases are related to environmental quality, various environmental pollutants have immunotoxic potential and many fish diseases have an immunological component (Wester et al. 1994). This development has been aided by progress in related scientific fields, such as fish immunology and rodent immunotoxicology.

2.2.1 The fish immune system

The immune defence mechanisms of fish have not been studied as extensively as those of mammals, but obvious progress has taken place in fish immunology in the last two decades (reviewed by several authors e.g. Anderson and Zeeman, 1995; Iwama and Nakanishi, 1996; Bernstein et al., 1998; Nakanishi et al., 1999). Fish are the oldest and most diverse of the vertebrate groups; their immune systems are quite varied and appear to be associated with fish phylogeny. In general, the immune system of teleost, the most studied fish,

appears to be fairly well developed and in various aspects is comparable to that of the mammalian immune system. Although the teleosts do not have bone marrow or lymph nodes, these fish posses functionally equivalent hematopoietic tissues primarily in the areas of the spleen, head kidney and thymus. The immune system in fish is a highly evolved system that functions to provide the organism with the ability to resist infectious agents, destroy neoplastic cells, and reject nonself components. The immune responses in fish also involve both humoral and cellular components.

The system is divided into two functional entities; nonspecific resistance and specific acquired resistance. Nonspecific defence mechanisms (reviewed by Secombes, 1996) are important in fish, because fish have fewer specific immune capabilities than higher vertebrates. These nonspecific defences have to respond quickly, even at low temperatures. Indeed, fish place a greater emphasis on physiological availability or energy spent on nonspecific defence mechanisms than mammals. Nonspecific responses are mediated by mononuclear phagocytes (macrophages and monocytes) and granulocytes. Phagocytosis and inflammation are two nonspecific responses that appear to be universal in fish. Another important group involved in immune surveillance are nonspecific cytotoxic cells (NCC) resembling mammalian natural killer (NK) cells (Evans & Jaso-Friedmann 1992). A number of nonspecific humoral factors and body secretions are thought to contribute to the natural resistance of fish (reviewed by Yano, 1996). These include lectins, lytic enzymes, transferrin, enzyme inhibitors, interferons and C-reactive protein. The complement system is an important part of the organism's defence against micro-organisms and in the teleost it is essentially similar to that of the higher vertebrates.

Specific immune responses are directed against a specific agent to which the organism has previously been sensitised. The specific immune mechanisms of fish, resembling those of mammals, include cell- and humoral-mediated responses. Cell-mediated immunity (reviewed by Manning and Nakanishi, 1996; Nakanishi et al., 1999) involves e.g. the generation of cytotoxic T-lymphocytes against intracellular viruses and the up and down regulation of immune responses. Fish produce a number of cytokine-like soluble products that act to orchestrate the events in an immune response. Most of these have been identified in biological assays on the basis of their functional similarity to mammalian cytokine activities. Recently interleukin-1' has been cloned in several teleost species (Secombes et al. 1999). Antibody molecules (immunoglobulins) produced by plasma cells are involved in humoral-mediated immunity (Kaattari & Pagancli 1996). Thc rolc of immunoglobulins is to protect the host from infectious diseases involving virus neutralisation, opsonization and complement-mediated lysis. The major immunoglobulin class produced in fish is IgM.

2.2.2 Survey of immunotoxicology

Immunotoxicology originated in the early 1970s when immunologists and toxicologists began investigating the immunotoxic potential of prominent environmental chemicals. The World Health Organisation (WHO) defines "immunotoxicology" as concerned with the adverse effects resulting from the interaction of the immune system with xenobiotics, and includes the

consequences of an action (i.e., either suppression or enhancement) by a substance (or its metabolite) on the immune system, as well as the immunological response to such a substance (Karras & Holsapple 1995). Unlike most target organs that are confined anatomically within a host, the immune system is dispersed throughout the body and can be exposed to xenobiotics at many locations and during various metabolic states. This factor in itself acknowledges the immune system as a highly sensitive organ for detecting toxicosis (Koller 1996). In addition to direct effects, xenobiotics may have indirect effects on immune functions (Figure 2). The immune system is regulated by a number of other organ systems such as various nervous and endocrine systems, as well as the activity of the liver. Input from these organs can constitute indirect pathways for xenobiotics perturbation of immune function. These interactions can result in no change in immune function, suppression of immunity, or immune enhancement (Karras & Holsapple 1995).

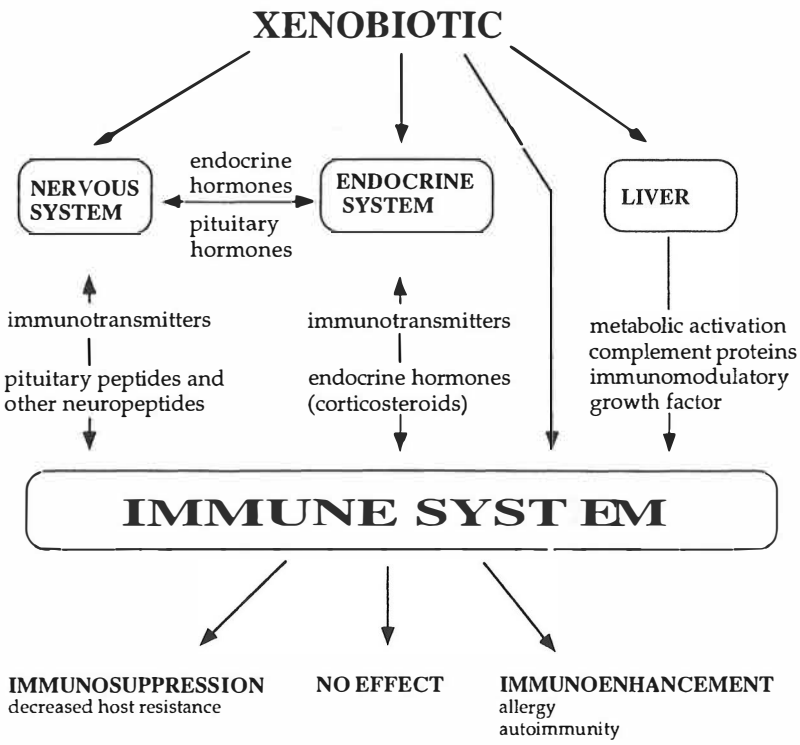


FIGURE 2 Scheme showing direct or indirect effects of xenobiotics on immunity and potential outcomes (Source: Karras and Holsapple, 1995).

The mechanisms of immunity are extremely intricate and there are a number of potential sites where immunotoxicants could lead to failure of immune functions. Defective phagocytosis could result in impaired antigen recognition and processing with failure of antigen presentation to the receptors on lymphocytes. Another potential site of immunosuppression, in addition to this so called afferent limb, would be at the level of lymphocyte antigen recognition and cell activation. This could occur due to impaired expression of antigen receptor and/or failure of biochemical mechanisms that would cause failure of activation and subsequent failure of proliferation or differentiation of the lymphocytes. This would result in immunosuppression because of failure to form a specific antibody or specific effector cells (Tucker 1994). On the other hand, immunotoxicants may also affect non-specific defence mechanisms which also provide resistance to infections and tumours. These include non-specific cells such as macrophages, natural killer (NK) cells, granulocytes, mast cells and probably even endothelial cells. Macrophages, neutrophils and natural killer cells are particularly important "front-line" effector cells in non-specific defence mechanisms which are independent of specific immune responses. Research in the field of immunotoxicology will therefore also involve the study of these types of cells (Vos & van Loveren 1996).

Due to the complexity of the immune system, many assays have been developed to examine either the integrated functioning of the entire system or its various component parts. Several authors have organised these tests into a tiered system for the most effective approach to assessing the effect of chemicals on the immune system (Vos 1980; Luster et al. 1988; Luster et al. 1992). Tier I provides a general screening of immune functions including e.g. leukocyte analysis, the histopathological examination of major lymphoid organs, the measurement of serum immunoglobulin and detecting the organ weights. These assays are usually easy and inexpensive and require only little laboratory equipment. If the screening study has provided data that suggest immunotoxicity, function studies, Tier II, are performed to confirm and further identify the nature of the immunotoxic effect. Tier II comprises a comprehensive testing of all components of the immune response, which can usually be carried out only in the laboratory. Tests include e.g. cell surface markers, immunoglobulin quantitation, lymphocyte blastogenesis and chemotaxis. The most relevant end point for immunological dysfunction requires testing the host resistance (challenge to bacteria, virus, parasites or tumours) occasionally called Tier III.

2.2.3 Effects of xenobiotics on the immune system of fish

In mammalian immunotoxicology a variety of effects are considered, not only immunosuppression, but also immunostimulation, allergy, and autoimmunity (Descotes 1999). The major interest of immunotoxicology in rodents lies in the evaluation of the results of toxicity testing for the purpose of risk assessment in humans. In fish, the suppression of the immune system seems mainly to be the target of the study (reviewed by Dunier and Siwicki, 1993; Zelikoff, 1994; Anderson and Zeeman, 1995). The effects of xenobiotics can be investigated on different biological levels by different immunological and disease-resistance

assays (Figure 3). In the field it is possible to study fish either by catching them in the wild or doing monitored cage tests. Both wild and cultured fish have been used in *in vivo* and *in vitro* laboratory exposures. Several species, although mainly teleost, have been used in field and laboratory studies, which makes research in this domain rather diffuse and extended. However, there are some preferred species such as trout, salmon and carp, and moreover, homozygous inbred strains of these fish are available (Komen et al. 1991).

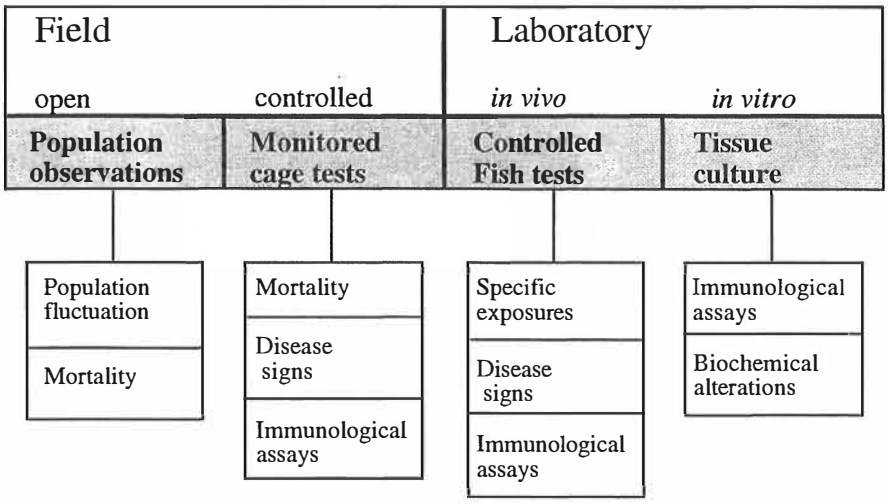


FIGURE 3 Biological levels for the investigation of the effects of pollutants on fish by immunological and disease-resistance assays (Source: Anderson, 1990)

Many immunotoxicological studies in fish have been performed under field conditions (Faisal et al. 1991; Pulsford et al. 1995; Rice et al. 1996; Fournier et al. 1998). Exposure in the wild is mainly chronic and fish may also be exposed via contaminated food, sediment and particles which are not present in aquaria. Although exposures in natural waters give the most relevant results, there are also many disadvantages in field studies. They often lack the specificity and sensitivity to allow the drawing of conclusion as to immunosuppressive effects, while stress related to handling and temperature changes may cause major fluctuations. Moreover, a deficient case history and lack of knowledge of the migratory patterns of feral fish hamper the interpretation of results (Vethaak et al. 1992). The disadvantages of field study can be minimised by doing on-site types of exposure e.g. keeping fish in cages in a lake. However, even on-site exposure resembling natural exposure suffers from inadequately controlled conditions. The effects observed in field studies are generally related to non-specific pollution (e.g. harbour sediment, sewage sludge, pulp mill effluents) whereas in laboratory it is also possible to study one xenobiotic at a time. Function tests under controlled conditions yield the most

reliable and sensitive methods of assessing immunological stress. However, risk assessment at the ecosystem level is difficult because laboratory findings do not necessarily apply in the field.

Because of the complexity of the immune system, no single immune function assay can provide an adequate evaluation of the adverse effects of xenobiotics on the immune system. Many different parameters have been used for studying immunotoxicology in fish e.g. structural parameters, functional response of the immune system and susceptibility to disease.

Parameters of nonspecific defence

Blood monitoring is one of the most popular approaches in assessing immunotoxicity. Blood cell counts and differential cells count have been widely used because these are easy to perform. White blood cells play a major role in the immune response. However, many other environmental factors not related to defence may modify the status of white blood cells, and therefore its function as an indication is limited (Anderson 1990). The white blood cell picture has been affected e.g. after exposure to industrial waste waters (Pulsford et al. 1995), pulp mill effluent (Andersson et al. 1988) organophosphate insecticides (Areechon & Plumb 1990) and tributyltin (Rice et al. 1995). A dose-dependent decrease in red and white blood cell counts were observed after exposure to copper (Khangarot & Tripathi 1991). A reduction in total leukocyte counts together with a decrease in lymphocyte and thrombocyte numbers was seen after exposure to cadmium (Murad & Houston 1988). Exposure to oil-contaminated sediments increase lymphocyte numbers at low diesel oil doses, whereas high doses decreased these values (Tahir et al. 1993).

Other nonspecific measures used in immunotoxicological studies for fish are organ weight and morphology. Spleen weight has been used as a biomarker because the spleen is easy to excise. It is, however, not very sensitive due the fact that a major and variable portion of the spleen consists of storage blood or erythropoietic tissue (Fänge & Nilsson 1985). Experimental immunotoxicology in mammals has demonstrated the weight of the thymus as a very sensitive parameter in the case of thymus effects. However, this parameter is not commonly used in fish because the thymus in many fish species has a complex localisation, which makes clean dissection almost impossible. Splenic somatic index has been demonstrated to decrease in fish living in polluted lakes e.g. as a result of industrial and urban development (Pulsford et al. 1995).

Phagocytes constitute an important cell population for both specific and non-specific defence. These parameters are widely used in measuring the immunotoxicity of xenobiotics; a possible reason for this is that these tests are quite easy to perform and do not require species-specific reagents. At present assays for measuring phagocytes are one of the most widely used tests in fish immunotoxicology. Various methods are used to measure the uptake of particular or soluble materials by phagocytic cells in investigating the health of fish. Direct observation by microscopy to determinate phagocytosis is used when counting engulfed bacteria. Phagocytosis of granulocytes or macrophages can also be assessed by measuring the production of oxygen radicals with chemiluminescence assay or nitro blue tetrazolium (NBT) reaction. Migration and chemotaxis of phagocytes can be followed e.g. by migration under agarose

assay or in a Boyden chamber. The suppression of macrophage function by environmental contaminants has been well documented (Rice & Weeks 1989; Weeks et al. 1990; Rice et al. 1996). Phagocyte oxidative burst activity is sensitive in several fish species to a variety of contaminants including tributyltin (Rice et al. 1995), metals (Zelikoff 1993), planar PCBs (Rice & Schlenk 1995), polycyclic aromatic hydrocarbon (PAH) (Kelly-Reay & Weeks-Perkins 1994) and pentachlorophenol (Anderson 1994).

Specific acquired resistance

Functional tests are widely used parameters in mammalian immunotoxicology in which lymphocytes are stimulated *in vitro* by exposure to mitogenic agents. Proliferation is monitored by measuring the incorporation of radioactive thymidine in DNA. This test yields information about immunotoxic mechanisms but the usefulness of these *in vitro* -tests to predict the immunomodulating effects of xenobiotics in fish is difficult. Suppression of the response to the T cell mitogens (Concanavalin A, ConA or phytohemagglutinin, PHA) and a sharp augmentation of the response to the B cell mitogens (lipopolysaccharide, LPS or pokeweed mitogen, PWM) in spot (*Leiostomus xanthurus*) has been demonstrated under field conditions (Faisal et al. 1991). *In vitro* effects of metal ions (Ghanmi et al. 1989) and organotin (O'Halloran et al. 1998) on the mitogenic response of fish lymphocytes have also been found. High concentrations of organotin inhibited both ConA- and LPS-stimulated mitogenesis while manganese inhibited only T lymphocyte mitogenesis, B lymphocyte mitogenesis remaining unaffected.

Determination of circulating immunoglobulin levels in serum is a useful test of the net result of the functioning of an immunoglobulin pathway *in vivo*. It can be measured in "naive" animals (total Ig) or after immunisation with an antigen. Nowadays the most commonly used method of measuring immunoglobulins is the enzyme-linked immunosorbent assay (ELISA). A related test, enzyme-linked immunospot assay (ELISPOT), which identifies the total number of immunoglobulin secreting cells (ISC) or specific antibody secreting cells (ASC) after immunisation, has recently also become more common in fish immunology and has replaced the hemolytic plaque-forming (PFC) assay (Secombes et al., 1991b; Waterstrat et al., 1991; Davidson et al., 1993; Siwicki et al., 1994). Studies with rodents have shown antibody production (PFC assay) to have the highest association with immunotoxicity (host resistance-test) (Luster et al. 1992). Studies in fish concerning the influence of pollutants on antibody formation have produced contradictory results. Anderson (Anderson et al. 1990) found reduced numbers of antibody-producing cells in phenol-pretreated rainbow trout immunised by bacterin antigen bath. On the other hand, the PFC response was not significantly altered in trout receiving halogenated aromatic hydrocarbons or PCB (Cleland et al. 1988a), and an increase in the number of PFC has been reported after exposure to dioxin (Spitsbergen et al. 1986) and after exposure to oil-contaminated sediments (Tahir et al. 1993).

3 AIMS OF THE STUDY

The interaction of environmental contaminants with the components of the immune defence may alter the function and balance of the immune system and result in undesirable effects such as immunosuppression, leading to the increasing prevalence of neoplasia or altered host defence against pathogens.

The present study assessed immunomodulatory and immunotoxic effects in fish after exposure to pulp and paper mill effluents.

The specific aims were:

1) to develop methods, for non-specific and specific immune parameters, suitable for studying immunotoxicity in the fish model (I, II, IV).

2) to determine the effects of exposure to BKME on piscine immune functions (II, III).

3) to investigate the significance of type of bleaching process on immunotoxicity (III, IV).

4) to study the influence of the secondary treatment of BKME on immunotoxicity in fish (V).

4 SUMMARY OF MATERIALS AND METHODS

Materials and methods are described detail in the original articles (I-V).

4.1 Fish and exposure to effluents

Roach used in studies (I-V) were caught from Lake Peurunka, an uncontaminated oligotrophic lake in Central Finland. Studies (I, III-V) were performed under controlled aquarium circumstances, except for study (II) where fish were exposed to bleached kraft mill effluent (BKME) on-site in Lake Vatia downstream from Mill A and control fish were caged simultaneously in uncontaminated Lake Peurunka. In all the laboratory experiments fish were exposed to effluents diluted with tap water or, in the case of the controls, fish were kept in aerated tap water. Oxygen concentration, temperature, illumination regime and feeding were maintained the same in control and exposure groups.

In the on-site study in a lake (II) and in the first of the laboratory studies (III) fish were exposed to BKME from mill A. At the time of these studies (1990-91) elemental chlorine and chlorine dioxide were used in the bleaching of softwood pulp. Exposures to untreated bleach liquors were performed using the effluents from the elemental chlorine /chlorine dioxide (C/D) bleaching (III), elemental chlorine-free (ECF) bleaching (IV) or the totally chlorine-free (TCF) bleaching (IV) from Mill B situated on the western coast of Finland. Primary and secondary treated BKME (V) came from Mill B.

Effluents and exposures of fish are described in detail in Table 1.

4.2 Immunisation of fish

In order to study the capability of roach to respond to foreign antigens the fish were immunised with a single intraperitoneal injection of bovine γ-globulin (BGG) emulsified in Freund's complete adjuvant (FCA). Each fish received 200 µl of antigen solution containing 500 µg BGG.

TABLE 1 Summary of effluents and exposures of fish in original publications II-V.

	II	IIIa	IIIb	IV	V
Year	1990	1991	1993	1995	1998
Exposure	On-site in lake	Aquarium	Aquarium	Aquarium	Aquarium
Mill	A	A	B	B	B
Bleaching	C/D	C/D	C/D	ECF softwood or	ECF and TCF
Raw material	Softwood and hardwood	Softwood	Softwood	TCF hardwood	Softwood and hardwood
Effluent	Secondary treated BKME	Secondary treated BKME	Untreated bleach liquor	Untreated bleach liquor	Primary treated or secondary treated BKME
Exposure concentration %	1*	3.5	25	0.6, 2, 6, 20	20
Exposure time (weeks)					
before immunization	5	3		2	
after immunization	0, 2, 3, 4	0, 2, 3, 4	0, 1, 2, 3, 4, 5	3	3
total	5, 7, 8, 9	3, 5, 6, 7	0, 1, 2, 3, 4, 5	5	3

* Calculated from mean discharge of Lake Vatia and waste water volume from mill A in June 1990 (Priha & Paavilainen 1991).

The kinetics of immune responses were followed for 28 (II-IIIa) or for 35 days (IIIb). In studies IV and V fish were sampled on day 21 post immunisation (p.i.) at the time of peak response against the antigen.

4.3 Sampling

A blood sample was drawn from the caudal vein of each fish either without heparin (I-III) or with heparin (IV-V). Serum or plasma was stored frozen until determination of immunoglobulin (IgM) concentration. The gills and liver tissue were excised immediately, frozen in liquid nitrogen and stored at -70°.

Cells were isolated from the spleen, blood or the head kidney with Percoll density gradients after disrupting the spleen or the head kidney against a nylon net. The cell culture medium used in all the experiments were modified for roach. The viability and the numbers of the isolated cells were counted by trypan blue exclusion in a haemocytometer (viability >95%).

4.4 Immune function assays

4.4.1 Antibody against roach IgM (I-II)

Immunoglobulin was isolated from roach serum with ammonium sulphate precipitation and anion exchange chromatography. Antibodies against purified roach IgM were raised in rabbits (I). The specificity of antibodies was tested by immunoelectrophoresis of roach serum (II) and only a single precipitation arch appeared when reacted with rabbit anti-roach IgM antibody.

4.4.2 Enumeration of secreting lymphocytes (I-V)

The enzyme-linked immunospot (ELISPOT) assay was used in the enumeration of ISC or antigen-specific ASC. The ELISPOT assay for roach (I) was based on the methods developed for human lymphocytes (Czerkinsky et al. 1983; Sedgwik & Holt 1983). Flat-bottomed 96-well microtiter plates were coated either with BGG, for the determination of specific ASC, or with rabbit anti-roach IgM antibody for ISC. Cells were dispensed into bovine serum albumin (BSA) -saturated wells and allowed to secrete antibodies for 3 hours. Trapped antibodies were detected with biotin-conjugated anti-roach IgM antibody followed by alkaline phosphatase-conjugated avidin. The substrate, bromo-chloro-indolyl phosphate, was mixed with warm agarose and added to the wells. Finally, blue spots were counted using a stereo microscope.

4.4.3 Quantification of immunoglobulin in blood (I-V)

The levels of total IgM and anti-BGG specific antibodies in roach serum (I-III) or plasma samples (IV-V) were determined by enzyme-linked immunosorbent assay (ELISA). The assay of serum immunoglobulin was standardised with known concentrations of purified roach IgM, and in the case of the specific antibody, a calibration curve was constructed using a pool of high titer plasmas obtained from fish immunised with several injections of BGG. The concentrations of anti-BGG specific antibodies in the samples were then expressed as artificial units/ml (U/ml).

4.4.4 Proliferation assay (IV)

A method for assaying of proliferative responses of lymphocytes after mitogen activation was developed for roach (IV). Lymphocytes from the blood or spleen were added in triplicate to 96-well plates, 4×10^5 cells/well, and activated with Concanavalin A (ConA). After 5 days incubation, ^{3}H-thymidine was added and the cultures were incubated for 18 hours and harvested by water lysis and adherence to glass fiber filters. The results were expressed as radioactivity counts per second (cpm) and as a stimulation index (SI) calculated according to the formula: SI = Mean cpm of stimulated cultures / Mean cpm of nonstimulated cultures.

4.4.5 Respiratory burst (IV)

Head kidney phagocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) and the respiratory burst activity was determined by the luminol-enhanced chemiluminescense method (Salo et al. 1998). A model 1250 LKB-Wallac luminometer with temperature control (IV) or a microplate luminometer (Victor^2, 1420 Multilabel counter, Wallac, Finland) (V) was used to monitor the chemiluminescense at 25°C. The peak value in millivolts (IV) or counts per minutes (V) was determined from each reaction.

4.4.6 Migration (IV)

The capability of head kidney granulocytes to move was assayed by a migration-under-agarose technique (Salo et al. 1998) modified from the method of Nelson et al. (1975). The middle wells, punched in an agar layer cast on microscope slides, were filled with casein solution and the outer wells received granulocytes. Cells were allowed to migrate under the agarose in a humidified environment at 25°C for 3 hr and then fixed overnight with methanol. The agarose was removed and the slides with migrated cells were stained. The distance the cells had migrated from the margin of the well towards the well containing casein (directed migration) and in the opposite direction (random migration) were measured under the microscope.

4.5 Other physiological methods (V)

4.5.1 Cortisol

Plasma cortisol was measured with a commercial kit Gamma Coat [^{125}I] Cortisol (INCSTAR Co., Stillwater, Minnesota, U.S.A).

4.5.2 Carbohydrate metabolism and osmoregulation

Plasma were mixed with 0.6 M $HClO_4$ before measuring the concentration of ions, glucose and lactate. Glucose was measured with the GOD-Period method (Boehringer Mannheim, Germany, Cat. no. 124036) and lactate with a modified method of Noll (1974) (Boehringer Mannheim, Germany. Cat. no. 256773) using a spectrophotometer iEMS platereader (Labsystems, Finland). Plasma sodium and potassium concentrations were measured with a Corning 435 -flame photometer using lithium as an internal standard.

Glycogen was extracted and purified by a method modified from Lim & Ip (1989). The amount of glucose released from the original glycogen was measured using the GOD-Period method.

Total and Na^+-K^+-ATPase analyses were carried out with the method described by Schrock et al., (1994). Inorganic phosphate liberated in the reaction was measured in a malachite green solution using spectrophotometer. The concentration of total proteins in the gill homogenates were measured by a Bio-Rad DC Protein Assay Kit.

4.5.3 The activity of EROD

The preparation of hepatic microsomes is described in detail in paper V. Microsomal fractions were stored in liquid nitrogen until assayed. The activity of 7-ethoxyresorufin O-deethylase (EROD) was measured fluorometrically from the microsomal fractions of liver using resorufin as the internal standard according to the method of Burke & Mayer (1985), adapted for the microplate method by Soimasuo et al. (1998).

4.6 Statistics

The data were analysed for statistically significant differences by the Mann Whitney U-test, student´s t-test or two-way ANOVA. A statistically significant difference from controls is expressed as $^*P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$. The relationships between variables were assessed by Pearson correlation with Bonferroni adjusted probability.

5 REVIEW OF THE RESULTS

5.1 Immunoglobulin synthesis in roach (I-V)

The concentration of immunoglobulin M (IgM), analysed with ELISA, in the serum of wild roach caught in the middle of May from Lake Peurunka was 15.5 ± 6.6 mg/ml (II, mean ± SD, n= 22). Mean plasma IgM concentrations in immunised control fish kept in aquaria ranged from 12 to 20 mg/ml (IV, V).

A large proportion of cells isolated with Percoll gradient from the spleen, the kidney or the blood produced immunoglobulins. The proportion of immunoglobulin-secreting cells (ISC) among lymphocytes isolated from the spleen was rather constant 20-25% (I, III-V). The number of ISC in the kidney was higher than in the spleen but no significant differences in ISC between these organs were detected (I, III). The ISC count of cells isolated from the blood was significantly higher than in those from the spleen (IV, V).

The kinetics of the appearance of anti-BGG specific antibodies in the serum were studied on days 0, 14, 21, 28 and 35 post immunisation (p.i.) with BGG (I-III). Antibody concentration increased after day 14 and reached its plateau value on day 28 p.i.. The kinetics of the formation of antibody-secreting cells (ASC) in the spleen or in the kidney following immunisation with BGG was determined by ELISPOT (I-III). Anti-BGG-specific antibody-secreting cells were not detected earlier than 14 days p.i., and the peak ASC response was noted on day 21 p.i.. The kinetics of the ASC responses in the spleen and in the kidney were identical (I, III). The number of ASC in the cell fraction isolated from the kidney was greater than the number of ASC in the cells from the spleen but did not reach statistical significance at any time of sampling. A highly significant correlation was found between the responses in the spleen and the kidney. Furthermore, correlation between cellular responses and serum antibody levels after immunisation were also detected (I). The number of lymphocytes secreting anti-BGG-specific antibodies in the blood varied widely (10-fold difference) between experiments (IV, V).

5.2 Effects of pulp mill effluents on immune functions

5.2.1 Immunoreactivity (II-V)

Changes in the number of ASC in roach exposed to effluents from different pulping and bleaching processes indicate that BKMEs negatively affect the immune functions of fish (Fig. 4). Exposure of roach to secondary-treated BKME from a mill with C/D bleaching significantly impaired the immunoreactivity of the fish as evidenced by the low number of splenic ASC in the fish kept in cages on-site in a lake receiving BKME (II) or exposed to BKME in an aquarium (III). A decreased number of ASC in the spleen and blood was also noted in fish exposed under laboratory circumstances to secondary- or primary-treated BKME from mill with ECF/TCF bleaching (V).

The effects of untreated bleach liquors from the different methods of kraft pulp bleaching (III, IV) on the number of ASC in the spleen and in the blood are summarised in Fig. 5. Exposure to the bleach liquors from C/D bleaching suppressed the number of splenic ASC (III). Exposure to ECF bleach liquors had contradictory effects: exposure to 2% liquor increased and to 20% liquor decreased the ASC response in the spleen (IV). However, the number of ASC in the blood decreased regardless of the concentration of ECF bleach liquor used in exposures (2, 6 and 20%). Exposure to TCF bleach liquors had no significant effects on splenic ASC. The number of blood ASC decreased after exposure to 2 and 20% TCF bleach liquors but exposure to 6% liquor resulted in an increased ASC count.

The plasma concentration of anti-BGG antibody on day 21 p.i. of fish caged in a lake receiving BKME (II) was lower than that of the control fish kept in clean lake, but on day 28 p.i. the concentration was higher than that of controls. Thus, exposure on-site to BKME from mill with C/D bleaching appeared to slow down the production of serum antibody but not prevent it entirely. Exposure to primary or secondary-treated effluent from mill with ECF/TCF bleaching rather increased than decreased the anti-BGG antibodies (V). Bleach liquors from ECF bleaching, particularly in high concentration (20%), decreased the anti-BGG antibody level on day 21 p.i., whereas liquors from TCF bleaching had no impairing effect (IV).

5.2.2 Immunoglobulin M (IgM) production (II-V)

A significant decrease in serum IgM concentration was noted in the non-immunised fish exposed in cages for 5 weeks in a lake receiving BKME when compared with fish kept in captivity in an unpolluted reference lake (II). The serum IgM levels of the exposed fish also remained low later on week 8 . Laboratory exposure to 20% secondary-treated BKME from a mill using ECF/TCF bleaching slightly increased the concentration of IgM (V) but 20% primary-treated BKME from the same mill had no effect. Exposures to ECF and TCF bleach liquors had no consistent effects on the plasma IgM concentration of

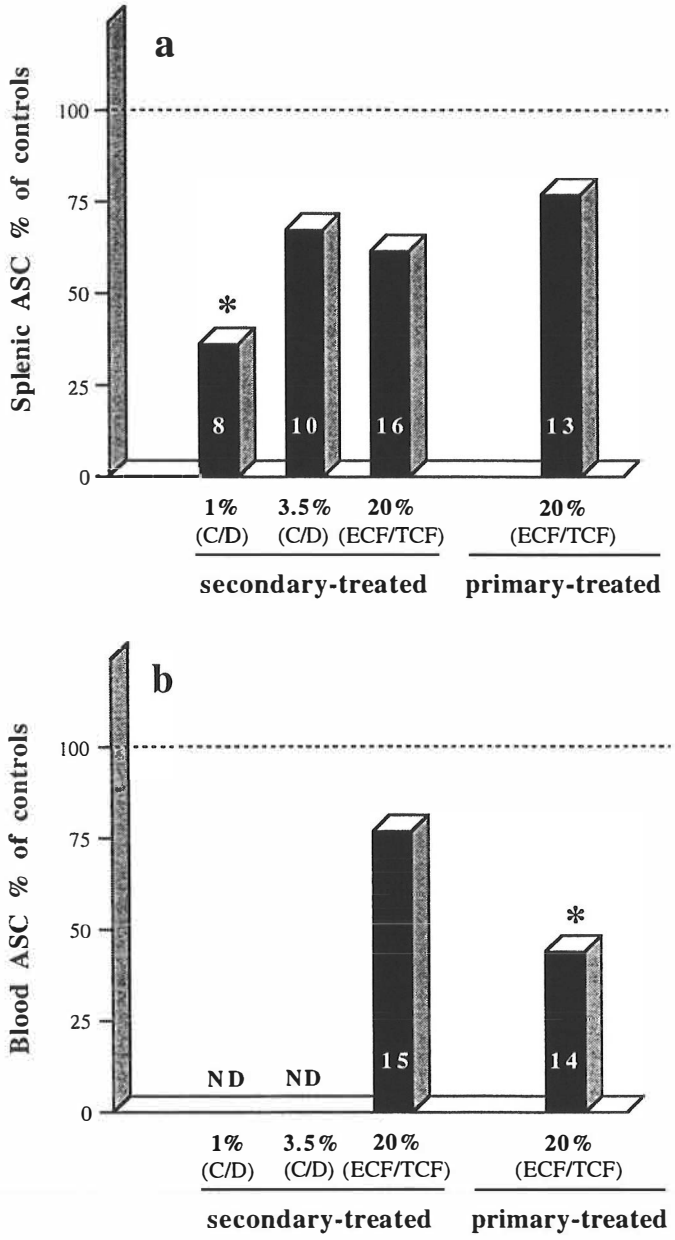


FIGURE 4 Anti-BGG antibody-secreting cell (ASC) response a) in the spleen and b) in the blood after exposure to BKME. BKME in the lake study (II) and in the laboratory exposure to 3.5% effluent (III) were from Mill A. Primary and secondary-treated BKMEs from mill with ECF/TCF bleaching came from Mill B (V). Bars represent the mean of exposed fish expressed as % of unexposed controls. Significant difference when compared to controls of the same study (for SE see figures in articles II, III and V), *$P \leq 0.05$. The number of exposed fish are marked in the columns. ND = not done.

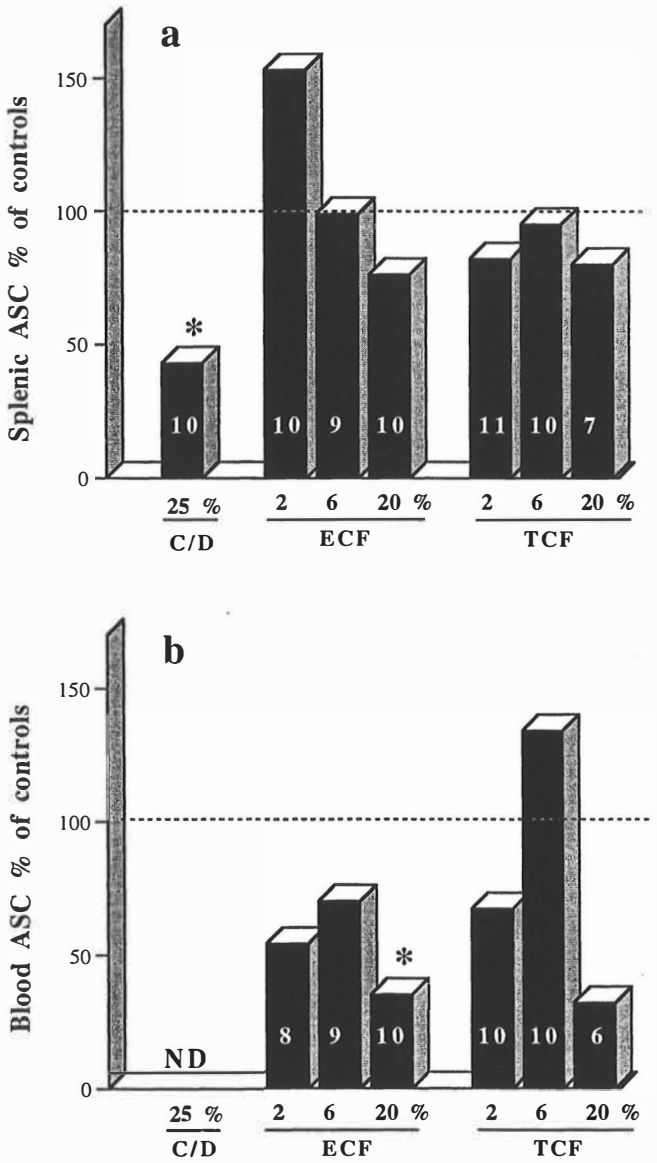


FIGURE 5 ASC response a) in the spleen and b) in the blood after exposure to bleach liquors from C/D (III), ECF (IV) or TCF (IV) bleaching. Bars represent the mean of exposed fish expressed as % of unexposed controls in each study. Significant difference when compared to controls (for SE see figures in articles III and IV), $*P \leq 0.05$. The number of fish are marked in the columns. ND = not done.

immunised fish (IV). Exposure to low concentration (0.6%) of both ECF and TCF bleach liquors increased IgM levels rather than suppressed them.

The number of splenic ISC in non-immunised fish (III) as well as the splenic and blood ISC counts in immunised fish were determined (III-V). The 3-week exposure to 3.5% BKME from a mill with C/D bleaching diminished the number of ISC by 53% compared to the unexposed controls (III). However, after immunisation the number of ISC in the spleen of exposed fish increased gradually and reached the level of controls on day 21 p.i. (III). The number of splenic ISC on day 21 p.i. of fish exposed to BKME from a mill with ECF/TCF bleaching was not affected (V). The results did not differ between exposures to primary- or secondary-treated BKME.

After exposure to 20% ECF bleach liquor the number of ISC decreased in the spleen and in the blood (IV). No statistically significant changes after exposure to TCF bleach liquor in the splenic ISC count were noted (IV) and the number of ISC in the blood indicated inconsistent results: exposure to 6% TCF bleach liquor decreased the number of circulatory ISC, but when exposed to 2% and 20% TCF bleach liquors the number of ISC increased but did not reach statistical significance when compared to controls.

5.2.3 Proliferation of lymphocytes *in vitro* (IV)

Low concentrations (2 and 6%) of ECF or TCF bleach liquors decreased ConA-driven proliferative responses of cultured lymphocytes by 30- 44% (IV). By contrast, when exposed to a high concentration (20%) of ECF bleach liquor, the ConA-stimulated proliferation was increased by 48%. Similar results were obtained in nonstimulated cultures as well. A downward trend in the stimulation index (SI) occurred after exposure to ECF bleach liquor (IV), reaching statistical significance in fish exposed to 20% liquor.

5.2.4 Granulocyte functions (IV, V)

Bleach liquors (0.6, 2, 6, 20%) from ECF bleaching (IV) and primary-treated BKME (20%) from a mill using ECF/TCF bleaching (V) reduced the respiratory burst but no statistically significant decreases were observed. Secondary-treated BKME from a mill using ECF/TCF bleaching or TCF bleach liquor had no effect on the respiratory burst (IV, V).

Exposure of fish to primary- or secondary-treated BKME from a mill using ECF/TCF bleaching had no effect on the migration of the granulocytes (V). Exposure of fish to low concentrations (2%) of ECF and TCF bleach liquors inhibited migration, whereas high concentrations (20%) of bleaching liquors had no effect (IV).

5.2.5 Depuration (III)

Fish exposed in an aquarium for 3 weeks to 3.5% BKME from a mill with C/D bleaching (III) were transferred to clean water at the time of immunisation and the recovery of antibody-mediated immunity was studied. A marked increase in the number of splenic ISC compared to the controls and to the fish exposed to BKME also after immunisation (III) was noted. The number of splenic anti-

BGG-specific ASC in the preexposed fish were higher than in fish exposed to BKME also after immunisation, or in unexposed control fish (III).

5.3 Effects on other physiological and biochemical parameters (IV, V)

Physiological and biochemical parameters known to be affected by BKME were studied after exposures to primary- and secondary treated BKME from a mill with ECF/TCF bleaching (V). Only slightly increased EROD activities were found in roach exposed to BKME. Exposure to primary-and secondary-treated BKME increased cortical levels. The level of gill total ATPase activity decreased after exposure to the secondary- or primary-treated BKME (V). The plasma glucose, lactate, potassium and sodium balance remained unchanged after exposures. Hematocrit values were remained unchanged after exposure to either primary- or secondary-treated BKME (V). In contrast, a significant decrease in hematocrit was seen in fish exposed to 2, 6, and 20% ECF bleach liquors (IV).

5.4 Sex-related differences (V)

The roach in studies I-IV were mainly female (~85%). Gender differences were not studied in I-IV due to the insufficient number of males in the population. The results reported in these studies can be considered to represent female data. For example, if males are removed in study IV the results remain unchanged. In contrast to studies I-IV, in study V more than 70% of the roach were male.

In the immunised control roach, sex-differences were seen in the number of blood ISC and plasma cortisol levels (V). In males, the blood ISC counts were 2-fold and cortisol value 2.6-fold compared to female roach. Sex-related differences were also noted after exposure to BKME from mill with ECF/TCF bleaching (Fig. 6). Exposure to primary-treated BKME impaired the splenic ASC counts in male roach only, but exposure to secondary-treated BKME decreased ASC counts in both sexes. A clear sex-related difference was obvious in the number of blood ISC: exposure to both effluents decreased the blood ISC count of male fish but increased female values. Differences were also noted in cortisol levels between the sexes. A drastic increase was noted in female roach after exposure to secondary-treated BKME but in males cortisol increased only slightly. Further, a difference in hepatic EROD activity was noted between the sexes: exposure to the primary-treated effluent resulted in a 2-fold increase in EROD activity in males, whereas in females an increase was noted only after exposure to the secondary-treated BKME.

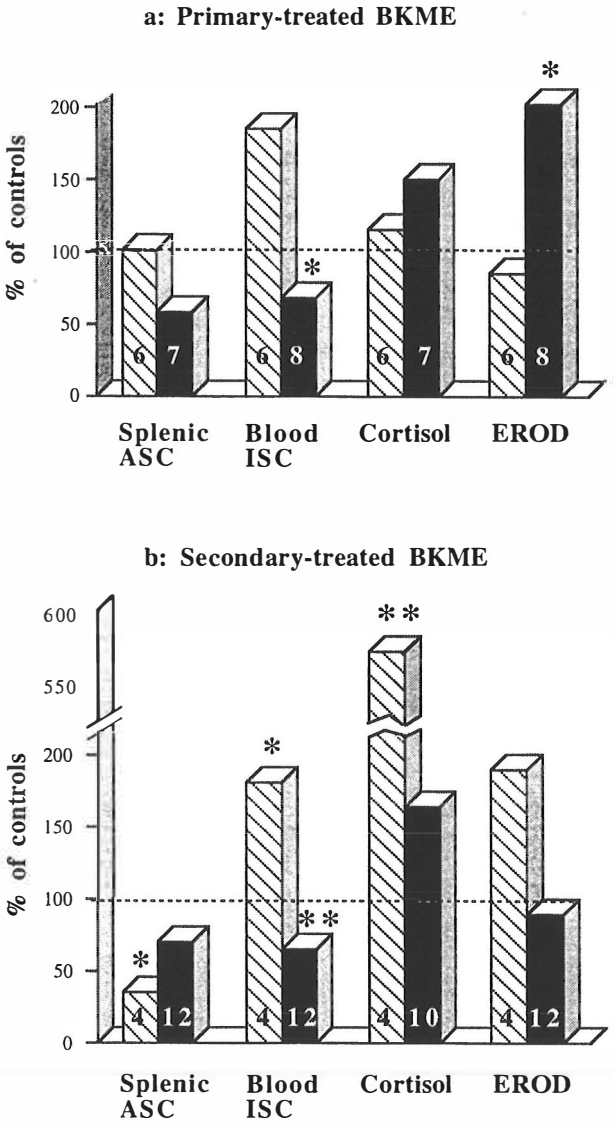


FIGURE 6 Sex-related differences in roach exposed to a) primary-treated or b) secondary-treated BKME from mills with ECF/TCF bleaching (V). Bars represent the mean of exposed fish (females: striped bars; males: black bars) expressed as % of controls of the same sex. Significant difference when compared to controls (for SE see figure in article V), $*P \leq 0.05$, $**P \leq 0.01$. The number of exposed fish are marked in the columns.

6 DISCUSSION

6.1 Roach as a model for immunotoxicological studies

The development and application of biomarkers for immunotoxicology in fish have became of interest in the last decade and at the same time knowledge on the immune system of fish and techniques in the field of fish immunology have developed significantly (Stolen et al. 1990; Stolen et al. 1992; Stolen et al. 1994; Stolen et al. 1995; Iwama & Nakanishi 1996). Only few out of more than 20,000 known fish species have been examined for their immune structure and/or functions. In immunological studies trout, salmon and carp have been used as model fish (Wester et al. 1994) but some information is also available about the immune system of roach. Zapata (1981a;b; 1982) has studied the structure of roach lymphoid organs. Antibodies (Williams & Hoole 1992; Aaltonen et al. 1997) and cellular responses (Hoole and Arme, 1983a; b; Taylor and Hoole, 1989) against parasites have also been studied. Taylor and Hoole have investigated leukocyte chemoattraction (1993) and chemiluminescence activity (1995) and, in addition, they have reported on mitogen-induced lymphocytes proliferation in roach (1994).

The increased prevalence on roach parasites in polluted waters, also examined in the present study (II), was interpreted as being due to the impaired immunity of fish (Valtonen & Koskivaara 1989; Valtonen & Koskivaara 1994). In addition, to studying the immune system of roach for this reason, roach offer a number of advantages in immunotoxicological studies. The roach is a common wildlife species in European waters and is easy to catch. Although the roach is a wild fish, it is easily adapted to laboratory conditions and can thus be exposed to toxicants in well-defined laboratory conditions in addition to open field studies and controlled on-site caging. Predicting toxicological hazards in a feral population has more relevance when studies are carried out with wild fish than with cultured fish. Roach offer also the opportunity to study gender-differences as roach of adequate size for experiments are sexually mature. Roach proved to be a good model for immunotoxicological studies also due to its immunological properties. Roach demonstrate a good immune response to protein antigens; 3-8 ‰ of cells isolated from lymphoid organs or blood with density gradients

secreted specific antibodies on the day of peak response (I-V). The proportion of ISC in isolated cells was 20-40%, i.e. 10 times higher than e.g. in dab (1.5%) (Secombes et al., 1991b) .

6.2 Differences in effluents used in exposures (II-V)

BKME contains hundreds of substances derived from raw materials or pulping and bleaching processes (LaFleur 1996; Sjöström & Alén 1999). The complexity of the effluents makes studies of BKMEs difficult. Indeed, very few toxicant-specific events in BKME exposed fish have been described. The toxicological effects of some of the major single components of BKME, e.g. resin acids (Nikinmaa & Oikari 1982) and chlorinated compounds such as tetra-chloro-1,2-bensoquinone (Bengtsson et al. 1988b) , have been studied. The compounds responsible for immunomodulation were not determined in the present study, but raw materials, bleaching processes and the treatment of waste waters are relevant to immunotoxicity. During the last decade, the pulp and paper industry has made a number of changes to manufacturing processes and waste water treatment all over the world (Axegård et al. 1993). The primary emphasis has been placed on the effects of chlorinated organic compounds formed during bleaching. Today, discharges of chlorinated organic compounds in effluents, e.g. the amount of absorbed organic chlorine (AOX), have decreased markedly due to use of less or terminated use of elemental chlorine in bleaching. During the period in which the present studies were carried out the use of elemental chlorine was replaced by chlorine dioxide and the introduction of oxygen-containing chemicals (Turoski 1998). At the time of the lake study in 1990 (II) as well as at the time of the first laboratory experiment in 1991 (IIIa) Mill A was using elemental chlorine in the first stage of bleaching. In 1993 Mill A replaced elemental chlorine with chlorine dioxide. The bleaching effluent used in experiment (IIIb) in 1993 from Mill B were from a process similar to that earlier used in mill A (IIIa). The use of elemental chorine in Finland was stopped in 1993 and, consequently, effluents from elemental chlorine bleaching were no longer available for studies.

The effluents used in the present studies differ in their composition in many ways. First of all, the concentration of chlorinated compounds and degree of chlorination varies between effluents. The differences in the concentration of AOX in exposures between diluted bleach liquors are obvious. The AOX concentration was 13 mg/ml between exposure to C/D bleach liquor (IIIb) and 0.15-5 mg/ml in exposure to ECF liquor (IV) but only 0.002-0.08 mg/ml in exposure to TCF liquor (IV). In contrast, no such differences were noted between the BKMEs. In the lake study (II) and in the exposure to secondary-treated BKME from a mill with ECF/TCF bleaching (V) the AOX values were 0.4 and 0.6 mg/ml , respectively. The AOX concentration were similar but the compounds were definitely of a lower degree of chlorination in BKME from ECF/TCF bleaching (V) than from chlorine-based bleaching (II) (Bright et al. 1997). The degree of chlorination has a clear influence on the toxicity of compounds (Voss et al. 1980; Lancaster et al. 1999).

Secondly, differences in the composition of effluents are caused also by the raw material used in pulping. Effluents contain organic substances originating from wood: polysaccharides, lignin and extractives e.g. resin acids, fatty acids and sterols (LaFleur 1996; Strömberg et al. 1996; Sjöström & Alén 1999) which are known to be harmful to fish (Leach et al. 1975; Leach & Thakore 1977; Nikinmaa & Oikari 1982; Oikari & Nakari 1982). It is well documented that softwood and hardwood differ in composition. For example, effluents from the production of bleached hardwood kraft pulp contain organic material of lower average molecular weight than corresponding softwood effluents (Dahlman et al. 1999). In contrast, softwood contains more resin acid than hardwood (Strömberg et al. 1996). Softwood was the raw material used in the present studies with one exception: in TCF bleach liquor (IV) only hardwood was used. In studies II and V, the BKMEs were combined effluents from both softwood and hardwood pulping. Differences in the composition of the effluents can also be clearly seen in the present study (IV) where the concentration of resin acids in the ECF bleach liquor from softwood process was thirty times higher than that in the TCF bleach liquors from hardwood pulping. Steroids and fatty acids occur in both softwood and hardwood (Dahlman et al. 1999) as was also noted in the present study. Consequently, the difference in their effects between TCF and ECF bleach liquors may be due to both raw material and bleaching sequence.

Thirdly, the effluents were either untreated bleach liquors or treated BKMEs. Predicting the effects of exposure to untreated bleach liquors can not be directly generalised to the hazards of exposures to secondary-treated effluents as the treatment effectively reduces the load of organic material in pulp mill effluents (Strömberg et al. 1996; LaFleur et al. 1998). However, studies conducted with untreated bleach liquors enable the comparison of immunotoxicity between different bleaching processes.

6.3 Effects of pulp and paper mill effluents

A variety of biological effects, including structural, physiological and biochemical responses in fish, have been associated with subchronic exposures to discharges from the pulp and paper industry (Andersson et al. 1988; Södergren 1989; Lindström-Seppä & Oikari 1989b; Hodson et al. 1992; Södergren 1993). The knowledge of the effects of pulp mill effluents on fish immunological parameters, however, has been very scarce. An altered white blood cell picture in perch (*Perca fluviatilis)* due to exposure to pulp mill effluent has been found (Andersson et al. 1988). A reduced level of plasma IgM has been reported in the whitefish (*Coregonus lavaretus* L.) exposed to effluent from a mill using C/D bleaching (Soimasuo et al., 1995a; b). Macrophage activity in the mummichog (*Fundulus heteroclitus*) from waters contaminated by BKME was affected (Fournier et al. 1998). However, the effects of exposure to BKMEs on immunoreactivity have not been studied earlier.

6.3.1 Effects on immunoreactivity

Subchronic exposure of fish in a lake receiving treated effluents from a bleached kraft mill affected the antibody-mediated immunity, resulting in weak responsiveness against antigen (II). Effects after exposures were more prominent on the cellular level (ASC counts) than in the concentrations of the anti-BGG antibody in the serum. The antibodies against BGG in the serum increased more slowly in the BKME-exposed fish compared to unexposed fish in the reference lake, but the concentrations finally reached the same level (II). In field studies effects are often modified or confounded by numerous factors despite the fact that, the fish were kept in cages in two lakes situated quite near each other, the fish were fed daily and water temperature was monitored regularly and the position of the cages was carefully selected in order to keep the fish in both lakes at the same temperature. The field study was confirmed under controlled laboratory conditions (III) and a decrease in splenic ASC was verified after exposure to BKME from the mill studied in the on-site study (II). Exposure to BKME from the mill with ECF/TCF bleaching (V) also decreased the ASC counts in the spleen and in the blood. An equal decrease in the splenic ASC count was noted after exposures either to BKME from a mill with C/D bleaching (IIIa) or BKME from a mill with ECF/TCF bleaching (V), although the concentration of effluents was very different (3.5% vs. 20%).

Impaired immunoreactivity was also confirmed by a depuration experiment (III). The reactivity against immunogen in fish exposed to BKME was recovered after the fish were transferred into clean water, proving that the decrease in the splenic ASC count was due to BKME. The depuration experiment also indicates that the suppression of antibody-mediated immunity after subchronic exposure to BKME was reversible. Alterations of certain physiological parameters after exposure to BKME have been found to reverse (Södergren 1993). Also during depuration after exposure to aromatic hydrocarbons, the recovery of the lymphocyte response to Con A and LPS (Faisal et al. 1991) and the recovery of macrophage functions (Weeks & Warinner 1984) have been reported; however, there is no previous data concerning the recovery of immunological parameters after exposure to BKME.

Exposure to untreated liquors from C/D bleaching (IIIb) alone suppressed antibody synthesis, suggesting that compounds derived from the bleaching stage were, at least partly, involved in immunotoxic effects. Liquors from the C/D process exerted more prominent effect on the immunoreactivity than liquors from the ECF or the TCF bleaching processes. The suppression was not noted in the number of splenic ASC (Fig. 5a) but blood ASC count were clearly decreased suggesting, however, some immunotoxicity in ECF and TCF bleach liquors.

Quantitating the antibody response following immunisation is regarded as a good indicator of immunotoxicity as it provides information on multiple components (i.e. antigen-presenting cells, helper T lymphocytes and B lymphocytes) of the immune system (Wester et al. 1994). Indeed, one of the highest association with immunotoxicity in rodents has shown to be the splenic antibody plaque forming cell (PFC) response (Luster et al. 1992). Studies concerning the influence of pollutants on antibody formation in fish have, however, yielded contradictory results. Anderson et al. (1990) found reduced

numbers of antibody-producing cells in immunised phenol-pretreated rainbow trout. Also Rice et al (1995) noted that number of ASC was a sensitive parameter and was affected after exposure to tributyltin. On the other hand, the PFC response was not significantly different in trout receiving halogenated aromatic hydrocarbons or PCB (Cleland et al. 1988b), and an increase in the number of PFC in rainbow trout was reported after exposure to dioxin (Spitsbergen et al. 1986). In the present study the ASC count proved to be a suitable parameter to examine the immunotoxicity of pulp mill effluents. Blood ASC count (IV, V) was even more sensitive to pollutants than the number of ASC in the spleen.

6.3.2 Effects on other immunoparameters

The contaminants in the lake receiving effluent from the pulp and paper mill with C/D bleaching decreased the level of serum immunoglobulin in roach (II). Reduced plasma IgM have also been reported in whitefish (*Coregonus lavaretus* L.) exposed on-site in lake or in an aquarium to BKME from a mill with C/D bleaching (Soimasuo et al., 1995a; b). Changes in the bleaching process together with the installation of the secondary-treatment system abolished the decrease in IgM in whitefish (Soimasuo et al. 2000). Changes in IgM concentration were not noted either in field studies with roach from an area contamined by BKME from mills using ECF bleaching compared to roach in reference sites (Karels et al. 1998). In the present study, consistent with aforementioned studies, no changes in IgM level were noted after laboratory exposure to BKME from a mill with ECF/TCF bleaching (IV,V).

Further evidence on the immunotoxic effect of BKME on antibody-mediated immunity was obtained as a lowered number of ISC in the spleen (III-V) and in the blood (IV,V). ISC enumeration is rarely used in immunotoxicology, probably because species-specific antibodies against immunoglobulin are required. However, the number of ISC proved to be sensitive to the influence of BKMEs (III-V). The 3-week exposure to BKME diminished the number of ISC by 53% in exposed fish compared to controls (III). Afterwards, post immunisation, the number of ISC increased to the level of that of the control fish, but no such increase was seen in unexposed fish. The increase in secreting lymphocytes in immunised fish is probably due to the adjuvant used in the immunisation. General stimulation of the immune system by mycobacteria in Freund's complete adjuvant can be reflected as an increased ISC count in fish with suppressed immunity (Anderson 1992).

Lymphoid cell-related functions were also investigated by studying ConA-stimulated proliferative responses. Plant-derived lectins induce activation and maturation of lymphocytes *in vitro* and serve as indicators of lymphocyte functioning (Maluish & Strong 1986). Controversial results on ConA-stimulated proliferative responses, as well as on non-stimulated proliferation, were obtained (IV): decreased responses in the fish exposed to low effluent concentrations but increased responses in the fish exposed to high concentrations of ECF/TCF bleach liquor. Several pollutants, e.g. dioxin and PAH, impair mitogen-stimulated proliferation (Spitsbergen et al. 1986; Faisal et al. 1991). On the other hand, stimulation of proliferative responses has been

described, e.g. after exposure to polychlorinated biphenyls (Thuvander et al. 1993).

Nonspecific defence mechanisms are extremely important in fish, because fish place a greater emphasis on energy spent on nonspecific mechanisms than the more elaborate specific defence response used by mammals (Anderson & Zeeman 1995). The successful killing of pathogenic organisms demands a good level of mobility by phagocytes in order to trap the pathogen, and the production of toxic substances is necessary to destroy the invader. In cyprinid fish the head kidney is the major phagocytic organ (Lamers & Parmentier 1985). Although no statistically significant reduction in the production of reactive oxygen species by head kidney granulocytes was observed, exposure of fish to any concentration of ECF liquors (IV) as well as to the primary-treated BKME (V) suppressed respiratory burst activity. Interestingly, the migration of granulocytes was inhibited in fish exposed to low concentrations (2%), but normal migration was noted after exposure to a high (20%) concentration of ECF and TCF effluents. An explanation for this controversial observation is difficult to find, but non-linear responses of immunological parameters to toxic agents are not uncommon (Koller 1996). Tahir et al. (1993), for example, found low migratory indices in fish exposed to small concentrations of oil drilling mud, but the indices were equal to the control values when the fish were exposed to higher concentrations.

6.4 Mechanisms of immunomodulation

The immunological effects of exposure to complex mixtures of chemicals are difficult to interpret. In the presence of several potential immunomodulators in combination, some substances can have agonist and some antagonistic characteristics. Thus, different dosages may lead to opposite effects. Only a few immunological studies have been carried out on fish exposed to complex mixtures of chemicals such as polluted sediments or sludges (Secombes et al., 1991a; Tahir et al., 1993; Secombes et al., 1995; Tahir and Secombes, 1995). The immunological effects of single defined chemicals, such as heavy metals and pesticides, have been studied more widely (Spitsbergen et al. 1986; Bennett & Wolke 1987; Zelikoff 1993; Voccia et al. 1994). In addition, a great variety of testing methods may lead to a situation in which a substance is a suppressor according to one method, and a stimulator according to another, and not immunotoxic at all according to a third. Linear dose-response are not always found in toxicological studies even with a single chemical exposure e.g. U-shaped dose-response curves (hormesis) may occur. In fact, the number of ASC and the concentration of IgM in the present study (IV) represent the U-shaped dose-response curve. Other atypical dose-responses were noted in the proliferation of lymphocytes (IV), where low concentrations of bleach liquors decreased and high concentrations increased the spontaneous proliferation of lymphocytes as also ConA-stimulated proliferation. Further, an atypical dose-response was found in the motility of granulocytes: only low concentrations of bleach liquors impaired the migration.

In addition to components of effluents affecting the fish differently, xenobiotics may have effects on different target organs. First, xenobiotics may directly affect different parts of the immune system. Immunocompetent cells require continued proliferation and differentiation for self-renewal and, therefore, these cells are sensitive to xenobiotics. Chemicals can compromise one or several reactive sites within the immune network. A complete immunological paradigm would include assessment of several compartments of the immune system which are inclusive of humoral and cell-mediated immunity, macrophage activity, NK (NCC) cell cytotoxicity and cytokine production/activity. Because optimum performance of the immune system is dependent upon a cascade of immune events, disruption of any one of the components of this circuit can alter the immune competence. The present study proves that BKME can impair the immunoreactivity of roach. Immune responsiveness provides information on multiple components in the immune system i.e. antigen-presenting cells, helper T lymphocytes and B lymphocytes, working in concert. Defects in any of the cellular pathways contributing to this response may lead to measurable decreases in antibody production. Modulation in the proliferation of lymphocytes and suppression in the stimulation indices of Concanavalin A-stimulated proliferation responses *in vitro* (IV) suggest that, at least partly, impaired immune responsiveness may be due to altered T cell-like functions. On the other hand, compromised macrophage function may also affect immunoreactivity, as exposure for 7 days to BKME from a mill with C/D bleaching resulted in the reduced production of reactive oxygen species of macrophages (our unpublished results). In addition to suppressed immunoreactiviness, effluents from ECF process have some, although slight, effects on nonspecific immune defence. We found a reduction, although not statistically significant, in the production of reactive oxygen species by head kidney granulocytes of fish after exposure to ECF bleach liquors (IV) or primary-treated BKME from a mill with ECF/TCF bleaching (V).

Besides direct effects, xenobiotics can have indirect effects through other organ systems such as the nervous and endocrine systems as well as through the activity of the liver (Karras & Holsapple 1995; Sanders 1995). In addition to metabolic activation of the liver, the complement proteins, immunomodulatory and growth factors produced in liver may also be affected. There is growing evidence of intensive interactions between the neuroendocrine system and the immune system also in fish (Verburg-van Kemenade et al. 1999). Endocrine-immune interaction was also clearly noted in the present study (V). Sex-related differences in immune responses were evident in many immune parameters. The small number of females makes the comparison of the results between the sexes difficult but it is well documented in mammals that females and males have their own characteristic immunological features (Ansar Ahmed & Talal 1990; Talal 1992; Deschaux & Khan 1995). Knowledge about sex-related differences in basic immunological parameters in fishes is scarce and even less is known about the effects of xenobiotics on female and male immune functions. Fournier et al (1998) reported a stronger suppression of phagocytic function of macrophages in female mumminchogs *(Fundulus heteroclitus)* than in males after exposure to BKME. The mechanisms of the sex hormone regulation of the immune system are poorly known. However, receptors for estrogens and androgens on lymphocytes and macrophages (Bhalla 1989;

Savino & Dardenne 1995; Slater et al. 1995) offer a possible route for immunomodulation. One probable reason for hormonal influence may be wood-derived sterols. BKMEs, indeed, have been reported to interfere with hormonal status in fish and, for example, disturbances in reproduction have been noted (McMaster et al. 1991; Munkittrick et al. 1991; Munkittrick et al. 1994).

The results (V) also suggest a connection between cortisol and immune functions. Receptors for corticosteroids have been detected in the leukocytes of coho salmon and carp (Maule & Schreck 1990; Weyts et al. 1998a). Suppressed immunity of fish with increased plasma cortisol is well demonstrated, and high cortisol level is widely used as an indicator of stress (reviewed by Wendelaar Bonga, 1997). Moderately increased plasma cortisol levels of male roach were noted after exposure to both effluents but in females cortisol increased only after exposure to secondary-treated effluents (V). In the case of a chronic stressor, cortisol levels may remain elevated, although well below acute peak values. Such cortisol responses have been reported after prolonged exposures to a variety of pollutants (reviewed by Donaldson, 1981; Barton and Iwama, 1991; Brown, 1993), and in salmonids even a slight chronic elevation in cortisol levels have been implicated in depressed immune functions (Maule & Schreck 1987; Pickering & Pottering 1989; Maule et al. 1993). Indeed, in the present study (V) high cortisol levels appeared to be associated with a low number of splenic ASC (Pearson r= -0.295, P=0.047). The results after exposure to the primary-treated effluent in female roach are difficult to interpret, and the lack of cortisol response in female roach does not necessarily indicate the absence of stressors (Wendelaar Bonga 1997). The impaired cortisol response may be related to enhanced cortisol clearance or down-regulation of the interrenal response to ACTH in chronically stressed fish. For example, Hontela et al (1992; 1997) concluded that exposure to BKMEs may lead to exhaustion of the pituitary-interrenal axis as a result of prolonged hyperactivity of the system.

6.5 The usefulness of immune parameters in predicting the effects of BKME on fish disease resistance and in assessing toxicological hazards in feral fish populations

The present studies (II-V) indicate that certain immune functions are altered after exposure to BKME. A question remains: what is the importance of the suppression of immune functions when considering the resistance of fish to pathogens. In mice a good correlation exists between changes observed in immune tests and altered host resistance: there were no instances when host resistance was altered without affects in the immune parameters, but, in contrast, immune changes were observed in the absence of detectable changes in host resistance (Luster et al. 1993) . This can be interpreted to reflect that immune tests are, in general, more sensitive than host resistance assays. Thomas & Sherwood (1996) estimate that suppression of certain immune parameters by 40-50% or more is required before enhanced susceptibility to infection in laboratory mammals is seen. However, apart from the fact that the

structure of the immune system in teleost fish is similar to that in mammals, there is no evidence that this applies as such to fish. The significance of the results presented in current studies on resistance to pathogens needs to be verified with host resistance studies.

The effects observed in field studies are generally related to unspecified pollution and the effects are often modified or confined by numerous factors, in particular for feral fish with a deficient case history, migratory patterns and limited knowledge of biology (Vethaak et al. 1992). In the present study (II) a controlled on-site type of exposure was chosen because of its close resemblance to the exposure of natural populations present in polluted waters. Thus, the impairment in immunoreactiviness (II) can be supposed to predict the immunomodulating effects of BKME in feral fish. However, it is essential that the results are confirmed by experiments in more controlled conditions in aquaria. The results from laboratory study (III) with BKME from a mill using C/D bleaching produced similar suppression of immunity as that in the on-site study. In these studies the exposures can be compared to each other as the concentrations (3.5%) in laboratory conditions and on-site in the lake (1%) were very similar. The studies with bleach liquors were done in order to compare different bleaching processes in respect of immunotoxicity. The bleaching effluents from pulp and paper mills are almost never released without secondary treatment and thus, there is no reason for extrapolating these results directly to the situation in recipient waters. The decrease in the ASC response after exposure to ECF or TCF bleach liquors was less prominent than after exposure to C/D bleach liquors (Fig. 5), suggesting lower immunotoxic potential in ECF and TCF processes compared to earlier processes with elemental chlorine.

Much emphasis has recently been placed on the development of biological markers that can predict exposure to, and the effects of, environmental pollutants. As monitors for exposure, biomarkers have the advantage of quantifying only biologically-available pollutants. As measures of effects, biomarkers can integrate the effects of multiple stressors and can assist in elucidating particular mechanisms associated with those effects (Weeks et al. 1992). Subtle changes in specific components of the immune system often occur before severe immune suppression, and frequently at chemical doses much lower than those that cause acute toxicity (Luster et al. 1988; Weeks et al. 1992). Consequently, it is typical of immunological biomarkers that they are sensitive and useful, but are often non-specific. Immunological biomarkers are important in monitoring the health of animals and as an early warning about the potential harm of environmental chemicals. The present study offers the applicability of the fish immune response, especially the blood ASC count, as a model for predicting the immunotoxicity of aquatic contaminants like BKME.

7 CONCLUSIONS

The focus of the present study was on the effects of effluents, treated and untreated, from pulp and paper mills on the immune functions of fish. The main conclusions are the following:

1. The antibody-mediated immunity of roach was suppressed after exposure to BKME both on-site in a contaminated lake and under controlled laboratory conditions.

2. Exposure to untreated bleach liquor alone affected immunoreactivity. Liquors from the C/D process exerted more prominent effect on the immunoreactivity than liquors from the ECF or the TCF bleaching processes.

3. Differences between male and female fish were observed, especially in the number of splenic ASC and blood ISC, indicating that sex-related factors are involved in immunomodulation after exposure to BKME.

4. In the present studies immunoreactivity proved to be a sensitive indicator of toxic insult, the most sensitive parameter being the number of blood ASC.

Acknowledgements

This study was carried out at the Department of Biological and Environmental Science, University of Jyväskylä. I am indebted to the Department for providing excellent working facilities. The study was supported by Research Council for Environmental and Natural Resources of the Academy of Finland, the Graduate School on Fish Biology and Fisheries in Finland, and grants from the Emil Aaltonen Foundation and Jenny and Antti Wihuri Foundation.

First and foremost, I want to thank my supervisor, Docent Ilmari Jokinen. He first introduced me to the fascinating field of immunology, and throughout this study has given freely of his time and expertise. During these years his encouragement, support and friendship have become very precious to me.

I would also like to express my deep gratitude to Professor Tellervo Valtonen without whom these studies would never have been started. Her optimism, enthusiasm and efficiency have been essential and have made my life much more exciting, particularly during congresses.

I am indebted to the official referees of the thesis, Dr Anthony Ellis and Professor Mikko Nikinmaa, for their valuable criticism and comments. I am also grateful to Reino Lammi, Harri Leppänen and Jarmo Lappivaara for their co-operation and to the fishermen who supplied the fish, in particular to Olavi Järvinen. I also want to thank Michael Freeman for revising the language of the thesis.

I owe a great deal to the members of our research group, with whom it has been easy to ventilate various problems relating to research and to life in general. I especially want to express my warmest thanks to my closest colleagues and friends, Eveliina Markkula and Harri Salo. My special thanks also go to our graduate students as well as to Arja Mansikkaviita, Laura Pitkänen and Elina Virtanen and all the other individuals who have helped us during these years.

I gratefully acknowledged all the personnel of the Department of Biological and Environmental Science for creating a friendly and convivial atmosphere in which to work. In particular, I have enjoyed the fruitful discussions we have had in the coffee room. My special thanks are due to Marjatta Suhonen, Maija-Leena Kiiveri and Anna-Liisa Kotiranta for help in many ways.

I want to thank my family and friends, especially both grandmothers, Tyyne and Paula, who have always been ready to lend a hand. Last, but not least, I want to thank my husband, Kari, and our children, Riina and Ossi, for their patience and love.

YHTEENVETO (Résumé in Finnish)

Metsäteollisuuden jätevesien aiheuttamat immunologiset muutokset kaloissa

Sellu- ja paperiteollisuus on yksi Suomen suurimmista vesiympäristön kuormittajista. Erityisesti selluloosan valkaisussa syntyviä yhdisteitä on pidetty ympäristön kannalta haitallisina. Vaikutuksia ympäristöön on pyritty vähentämään mm. prosessitekniikan muutoksilla. Esimerkiksi alkuainekloorin käytöstä on luovuttu ja se on korvattu klooridioksidilla (ECF, alkuaineklooriton valkaisu) tai muilla happea sisältävillä kemikaaleilla kuten peroksidi ja otsoni (TCF, klooriton valkaisu). Toinen merkittävä tekijä päästöjen vähentämisessä on ollut tehtaan ulkoisten aktiivilietelaitosten käyttöönotto.

Tutkittaessa selluloosa- ja paperitehtaiden jätevesien vaikutusta kaloihin on todettu, että subletaalit pitoisuudet vaikuttavat kalojen fysiologiaan esim. verenkuvaan, plasman hiilihydraatti- ja ionitasapainoon, maksan toimintaan sekä kalojen kasvuun ja lisääntymiseen. Metsäteollisuuden jätevesien vaikutusta kalojen immunologisiin toimintoihin ei tähän saakka juurikaan ole tutkittu. Tiedetään, että teollisuuden likaamilla vesialueilla kaloissa esiintyy lisääntyneessä määrin kasvaimia ja muita sairauksia. Kemiallisen puunjalostuksen alapuolisilla vesialueilla on todettu myös muutoksia kalojen loisten määrissä ja lajistossa, mikä on tulkittu osoitukseksi jäteveden vaikutuksista kalojen vastustuskykyyn.

Tutkimuksemme osoittavat, että metsäteollisuuden jätevesillä on haitallisia vaikutuksia kalojen immunologiseen puolustukseen. Särjellä subkrooninen altistus kaasuklooria valkaisussa käyttävän sellutehtaan alapuolisessa vesistössä aiheutti veren immunoglobuliinipitoisuuden alenemisen lisäksi huonontuneen vasteen immunisoinnille. Vasta-ainesynteesin heikkeneminen todistettiin myös allasolosuhteissa, jolloin kalat altistettiin tehtaan puhdistetulle jätevedelle. Jätevesien aiheuttama immunovasteiden heikkeneminen oli palautuva: kaloilla, jotka siirrettiin jätevesialtistuksesta puhtaaseen veteen, immunisointivasteet nousivat kontrollien tasolle ja joillakin immuunipuolustuksen osa-alueilla havaittiin jopa stimulaatiota. Samanlainen immunoreaktiivisuuden heikkeneminen kuin altistettaessa koko tehtaan jätevesille todettiin myös pelkästään valkaisujätevesille altistetuissa kaloissa.

Kloorittomien ja klooridioksidia käyttävien valkaisuprosessien jätevesien vaikutuksia kalojen immuunipuolustukseen tutkittiin allasoloissa. Altistaminen laimennetuille käsittelemättömille valkaisujätevesille antoi viitteitä siitä, että ECF- ja TCF-valkaisun jätevedet eivät aiheuta yhtä voimakasta immunisoituvuuden heikkenemistä kuin alkuainekloria käyttävän valkaisun jätevedet. Tutkittaessa ECF/TCF-valkaisua käyttävän tehtaan jätevesien vaikutuksia allasoloissa havaittiin, että biologinen jäteveden puhdistus pääasiassa vähensi, mutta ei kuitenkaan täysin poistanut, jäteveden immunotoksisia vaikutuksia. Lisäksi, jätevesien immunotoksisten vaikutusten havaittiin riippuvan sukupuolesta.

Kalojen immunologiset toiminnat, ja erityisesti veren spesifistä vasta-ainetta erittävien solujen määrä, osoittautuivat herkiksi mittareiksi tutkittaessa metsäteollisuuden jätevesien toksisuutta. Immunologisella tutkimustiedolla on suuri merkitys arvioitaessa vesistöjä rasittavien päästöjen haittoja kalojen hyvinvointiin ja terveyteen. Edelleen, koska immunologiset muuttujat reagoivat herkästi ympäristöön, ne toimivat osoittimena ja varoittimena vaaratekijöistä.

REFERENCES

Aaltonen, T. M., Valtonen, E. T. & Jokinen, E. I. 1997. Humoral response of roach (Rutilus rutilus) to digenean Rhipidocotyle fennica infection. Parasitology 114, 285-291.

Adams, S. M., Crumby, W. D., Greeley, M. S., Shugart, L. R. & Saylor, C. F. 1992. Responses of fish populations and communities to pulp mill effluents: a holistic assessment. Ecotoxicol. Environ. Saf. 24, 347-360.

Anderson, D. P. 1990. Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. Am. Fish. Soc. Symp. 8, 38-50.

Anderson, D. P. 1992. Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. Annu. Rev. Fish Dis. 2, 281-307.

Anderson, D. P. & Zeeman, M. G. 1995. Immunotoxicology in fish. In: Rand, D. P. (ed.), Fundamentals of Aquatic Toxicology. Effects, environmental fate and risk assessment: 371-404. Taylor & Francis, Florida.

Anderson, D. P., Dixon, O. W. & van Muiswinkel, W. B. 1990. Reduction in the numbers of antibody-producing cells in rainbow trout, Oncorhynchus mykiss, exposed to sublethal doses of phenol before bath immunization. In: Landis, W. G. & van der Schalie, W. H. (eds), Aquatic Toxicology and Risk Assessment: 331-337. American Society for Testing and Materials, Philadelphia.

Anderson, R. S. 1994. Modulation of blood cell-mediated oxyradical production in aquatic species: Implications and applications. In: Malins, D. C.& Ostrader, G. K. (eds), Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives: 241-265. Lewis, Boca Raton, FL.

Andersson, T., Bengtsson, B.-E., Förlin, L., Härdig, J. & Larsson, Å. 1987. Long-term effects of bleached kraft mill effluents on carbohydrate metabolism and hepatic xenobiotic biotransformation enzymes in fish. Ecotoxicol. Environ. Saf. 13, 53-60.

Andersson, T., Förling, L., Härdig, J. & Larsson, Å. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. Can. J. Fish Aquat. Sci. 45, 1525-1536.

Annergren, G. 1993. Trends in the development of future bleaching processes. In: Södergren, A. (ed.), Bleached pulp mill effluents. Composition, Fate and Effects in the Baltic Sea. Final Report from the Environment/ Cellulose Project II: 127-133. Swedish National Environmental Protection Board, Solna.

Anonymous 1997. The Finnish background report for the EC documentation of best available techniques for pulp and paper industry. Ministry of the Environment Report, pp. 138. Helsinki.

Ansar Ahmed, S. &Talal, N. 1990. Sex hormones and the immune system-part 2. Animal data. Baillieres Clinical Rheumatology 4, 13-31.

Areechon, N. & Plumb, J. A. 1990. Sublethal effects of malathion on chanel catfish, Ictalurus punctatus. Bull. Environ. Contam. Toxicol. 44, 435-442.

Axegård, P., Dahlman, O., Haglind, I., Jacobsen, B., Mörck, R. & Strömberg, L. 1993. Pulp bleaching and the environment - the situation 1993. Nord. Pulp Pap. Res. J. 4, 365-378.

Barton, B. A. & Iwama, G. K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the responses and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3-26.

Bengtsson, B.-E., Bengtsson, A. & Tjärnlund, U. 1988a. Effects of pulp mill effluents on vertebrate of fourhorn sculpin, Myoxocephalus quadricorns, bleak, Alburnus alburnus, and pearch, Perca fluviatilis. Arch. Environm. Contam. Toxicol. 17, 789-797.

Bengtsson, B.-E., Larsson, Å., Bengtsson, A. & Renberg, L. 1988b. Sublethal effects of tetrachlo-1,2-benzoquinone - a component in bleachery effluents from pulp mill. Ecotoxicol. Environ. Saf. 15, 62-71.

Bennett, R. O. & Wolke, R. E. 1987. The effect of sublethal endrin exposure on rainbow trout, Salmo gairdneri Richardson. I. Evaluation of serum cortisol concentrations and immune responsiveness. J. Fish. Biol. 31, 375-385.

Bernstein, R. M., Schluter, S. F. & Marchalonis, J. J. 1998. Immunity. In: Evans, D. H. (ed.), The physiology of fishes: 217-242. CRC Press LLC.

Bhalla, A. K. 1989. Hormones and the immune response. Ann. Rheum. Dis. 48, 1-6.

Bright, D., Hodson, P. V., Lehtinen, K.-J., McKague, B., Rodgers, J. & Solomon, K. 1997. Evaluation of ecological risks associated with the use of chlorine dioxide for the bleaching of pulp. Scientific progress since 1993. p. 11-20. In: Proceedings of the 3rd International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand.

Brown, J. A. 1993. Endocrine responses to environmental pollutants. In: Rankin, J. C.& Jensen, F. B. (eds), Fish Ecophysiology: 276-296. Chapman and Hall, London.

Burke, M. D., Thompson, S., Elcombe, C. R., Halpert, J., Haaparanta, T. & Mayer, T. T. 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. Biochemical Pharmacology 34, 3337 - 3345.

Cleland, G. B., McElroy, P. J. & Sonstegard, R. A. 1988a. The effect of dietary exposure to Aroclor 1254 and / or mirex in humoral immune expression of rainbow trout (Salmo gairdneri). Aquat. Toxicol. 2, 141-146.

Cleland, G. B., Oliver, B. G. & Sonstegard, R. A. 1988b. Dietary exposure of rainbow trout (Salmo gairdneri) to Great Lakes coho salmon (Oncorhynchus kisuch Wallbaum). Bioaccumulation of halogenated aromatic hydrocarbons and host resistance studies. Aquat. Toxicol. 13, 281-290.

Cook, D. L., LaFleur, L. E., Parrish, A., Jones, J. & Hoy, D. 1997. Characterization of plant sterols from 22 US pulp and paper mills. Water Sci. Technol. 2-3, 297-303.

Czerkinsky, C. C., Nilsson, L.-Å., Nygren, H., Ouchterlony, Ö. & Tarkowsky, A. 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65, 109-121.

Dahlman, O., Haglind, I., Mörck, R., Johansson, L., Sousa de, F. & Strömberg, L. 1993. Chemical composition of effluents from chlorine dioxide bleaching of kraft pulp before and after secondary treatment. p. 193-215. In: Proceedings of the International Environmental Symposium, EUCEPA. Paris, France.

Dahlman, O., Mörck, R. & Knuutinen, J. 1999. Analysis of bleached liquors. In: Sjöström, E. & Alén, R. (eds), Analytical methods in wood chemistry, pulping, and papermaking: 233-268. Springer-Verlag, Heidelberg.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. 1993. Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout (Oncorhynchus mykiss). Dev. Comp. Immunol. 17, 373-376.

Deschaux, P. & Khan, N. A. 1995. Immunophysiology: the immune system as a multifunctional physiological unit. Cell. Mol. Biol. 41, 1-10.

Descotes, J. (ed.) 1999. An introduction to immunotoxicology. 183 p. Taylor & Francis Ltd, London.

Donaldson, E. M. 1981. The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering, A. D. (ed.), Stress in Fish: 11-47. Academic, London.

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 Immunol. 3, 423-438.

Ericson, R. L., Thut, R. N. & Persinger, W. H. 1998. Closing up the bleach plant: Striving for a minimum-impact mill. In: Turoski, V. (ed.), Chlorine and chlorine compounds in the paper industry: 25-38. Ann Arbor Press, Mihigan.

Evans, D. L. & Jaso-Friedmann, L. 1992. Nonspecific cytotoxic cells as effectors of immunity in fish. Annu. Rev. Fish Dis. 2, 109-121.

Faisal, M., Marzouk, M. S. M., Smith, C. L. & Huggett, R. J. 1991. Mitogen induced proliferative responses of lymphocytes from spot (Leiostomus xanthurus) exposed to polycyclic aromatic hydrocarbon contaminated environments. Immunopharmacol. Immunotoxicol. 13, 311-327.

Fänge, R. & Nilsson, S. 1985. The fish spleen: structure and function. Experientia 41, 152-158.

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. Ecotoxicol. Environ. Saf. 40, 177-83.

Gagnon, M. M., Dodson, J. J., Hodson, P. V., Van Der Kraak, G. & Carey, J. H. 1994. Seasonal effects of bleached kraft mill effluent on reproductive parameters of white sucker (Catostomus commersoni) populations of the ST.Maurice River, Quobec, Canada. Can. J. Fish Aquat. Sci. 51, 337-347.

Georgov, M., Priha, M. H., Talka, E., Valttila, O., Kangas, A. & Kukkonen, J. 1988. Chlorinated organic compounds in effluent treatment at kraft mills. Tappi J 71, 175-184.

Ghanmi, Z., Rouabhia, M., Othmane, O. & Deschaux, P. A. 1989. Effects of metal ions cyprinid fish immune response: In Vitro effects of Zn2+ and Mn2+ on the mitogenic response of carp pronephros lymphocytes. Ecotoxicol. Environ. Saf. 17, 183-189.

Hakkari, L. 1992. Effects of pulp and paper mill effluents on fish populations on Finland. Finnish Fisheries Research 13, 93-106.

Härdig, J., Andersson, T., Bengtsson, B.-E., Förlin, L. & Larsson, Å. 1988. Long-term effects of bleached kraft mill effluents on red and white cell status, ion balance, and vertebral structure in fish. Ecotoxicol. Environ. Saf. 15, 96-106.

Hodson, P. V., McWhirther, M., Ralph, K., Gray, B., Thivierge, D., Carey, J. H., Van Der Kraak, G. J., Whittle, D. M. & Levesque, M.-C. 1992. Effects of bleached kraft mill effluent on fish in the St.Maurice river, Quebec. Environm. Toxicol. Chem. 11, 1635-1651.

Hontela, A., Rasmussen, J. B., Audet, C. & Chevalier, G. 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch. Environm. Contam. Toxicol. 22, 278-283.

Hontela, A., Daniel, C. & Rasmussen, J. B. 1997. Structural and functional impairment of the hypothalamo-pituitary-interrenal axis in fish exposed to bleached kraft mill effluent in the St. Maurice River, Quebec. Ecotoxicology 6, 1-12.

Hoole, D. & Arme, C. 1983a. Ligula intestinalis (Cestoda: Pseudophyllidea): an ultrastructural study on the cellular response of roach fry, Rutilus rutilus. Int. J. Parasitol. 13, 359-363.

Hoole, D. & Arme, C. 1983b. Ultrastructural studies on the cellular response of fish hosts following experimental infection with the pleurocercoid of Ligula intestinalis (Cestoda: Pseudophyllidea). Parasitology 87, 139-149.

Iwama, G. & Nakanishi, T. (ed.) 1996. The fish immune system. Organism, pathogen, and environment. 380 p. Academic Press, San Diego.

Kaattari, S. L. & Paganelli, J. D. 1996. The specific immune system: humoral defence. In: Iwama, G.& Nakanishi, T. (eds), The fish immune system. Organism, pathogen, and environment: 207-254. Academic Press, San Diego.

Karels, A. 2000. Ecotoxicity of pulp and paper mill effluents in fish. Responses at biochemical, individual, population and community levels. Jyväskylä Studies in Biological and Environmental Science 83:1-68.

Karels, A., Soimasuo, R., Lappivaara, H., Leppänen, H., Aaltonen, T., Mellanen, P. & Oikari, A. 1998. Effects of ECF-bleached kraft mill effluent on reproductive steroids and liver MFO activity in populations of perch and roach. Ecotoxicology 7, 123-132.

Karessuo, A. (ed.) 1992. Environmental report 1992. 27 p. Finnish Forest Industries Federation, Espoo, Finland.

Karessuo, A. (ed.) 1996. Ympäristönsuojelun vuosikirja 1996. 139 p. Metsäteollisuus ry, Espoo.

Karras, J. G. & Holsapple, M. P. 1995. Structure and function of the immune system. In: Smialowicz, R. J.& Holsapple, M. P. (eds), Experimental immunology: 3-12. CRC Press, Boca Raton.

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

Khangarot, B. S. & Tripathi, D. M. 1991. Changes in humoral and cell-mediated immune response and in skin and respitory surfaces of catfish, Saccoobranchus fossilis, following copper exposure. Ecotoxicol. Environ. Saf. 22, 291-308.

Kleppe, P. J. 1970. Kraft pulping. TAPPI J. 53, 1-35.

Koller, L. D. 1996. Profiling Immunotoxicology: Past, Present, and Future. In: Stolen, J. S., Fletcher, T. C., Bayne, C. J., Secombes, C. J., Zelikoff, J. T., Twerdok, L. E. & Anderson, D. P. (eds), Modulators of Immune Responses: The evolutionary trail: 301-310. SOS Publications, Fair Haven, NJ, USA.

Komen, J., Bongers, A. B. J., Richter, C. J. J., van Muiswinkel, W. B. & Huisman, E. A. 1991. Gynogenesis in common carp (Cyprinus carpio) II. The production of homozygous gynogenetic clones and F1 hybrids. Aquaculture 92, 127-142.

Kovacs, T. G., Tana, J., Lehtinen, K. J. & Sangfårs, O. 1994. A comparison of the environmental quality of elemental chlorine-free (ECF) and totally chlorine-free (TCF) hardwood bleach plant effluents. In: Proceedings of International Non-Bleaching Conference. Miller Freeman Inc. Distribution Center, Gilroy, CA,USA. Amelia islands, FL,USA.

LaFleur, L. E. 1996. Sources of pulping and bleaching derived chemicals in effluents. In: Servos, M. R., Munkittrick, Carey, J. H.& Van Der Kraak, G. J. (eds), Environmental fate and effects of pulp and paper mill effluents: 21-31. St. Lucie Press, Delray Beach, Florida.

LaFleur, L. E. & Barton, D. A. 1997. Pulp mill effluent characterization and treatment: An update. p. 1-10. In: Proceedings of the 3rd International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand.

LaFleur, L. E., Bousquet, T. & Cook, D. 1998. Effect of biological treatment on pulp milleffluent chemical characteristics. In: Turoski, V. (eds), Chlorine and chlorine compounds in the paper industry: 115-136. Ann Arbor Press, Inc., Chelsea, MI, USA.

Lamers, C. H. J. & Parmentier, H. K. 1985. The fate of intraperitoneally injected carbon particles in cyprinid fish. Cell Tissue Res. 242, 499-503.

Lancaster, L. M., Renard, J. J. & Phillips, R. B. 1999. Assuring the environmental safety of kraft pulp bleaching: A cost-benefit analysis. In: Sjöström, E.& Alén, R. (eds), Analytical methods in wood chemistry, pulping, and papermaking: 237-246. Springer-Verlag, Heidelberg.

Larsson, Å., Andersson, T., Förlin, L. & Härdig, J. 1988. Physiological distuebances in fish exposed to bleached kraft mill effluents. Water Sci. Technol. 20, 67-76.

Larsson, Å., Förlin, L., Lindesjöö, E. & Sandström, O. 1997. Monitoring of individual organism responses in fish populations exposed to pulp mill effluents. p. 265-276. In: Proceedings of the 3rd International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand.

Leach, M. J. & T hakorc, Λ. N. 1977. Compounds to toxic to fish in pulp mill waste streams. Prog. Wat. Tech. 9, 787-798.

Leach, M. J., Thakore, A. N. & Manville, J. F. 1975. Acute toxicity to juvenile rainbow trout (Salmo gairdneri) of naturally occuring insect juvenile hormone analogoes. J. Fish. Res. Board Can. 32, 2556-2559.

Lehtinen, K.-J. & Oikari, A. 1980. Sublethal effects of kraft pulp mill waste waters on the perch, Perca fluviatilis, studied by rotary-flow and histological techniques. Ann. Zool. Fennici 17, 255-259.

Lim, A. L. & Ip, Y. K. 1989. Effect of fasting on glycogen metabolism and activities of glycolytic and gluconeogenic enzymes in the mudskipper Boleophthalamus boddaerti. J. Fish. Biol. 36, 349-367.

Lindesjöö, E. & Thulin, J. 1990. Fin erosion of pearch Perca fluviatilis and ruffe Gymnocephalus cernua in a pulp mill effluent area. Dis. Aquat. Org. 8, 119-126.

Lindesjöö, E. & Thulin, J. 1992. A skeletal deformity of nothern pike (Esox lucius) related to pulp mill effluents. Can. J. Fish Aquat. Sci. 49, 166-172.

Lindesjöö, E. & Thulin, J. 1994. Histopathology of skin and gills of wild fish in pulp mill effluents. Dis. Aquat. Org. 18, 81-93.

Lindström-Seppä, P. & Oikari, A. 1989a. Bleached kraft pulp mill effluents and TCDD: Effects of biotransformation and testosterone status of mature male rainbow trout (Salmo gairdneri). Mar. Environm. Res. 28, 99-103.

Lindström-Seppä, P. & Oikari, A. 1989b. Biotransformation and other physiological responses in whitefish caged in a lake receiving pulp and paper mill effluents. Ecotoxicol. Environ. Saf. 18, 191-203.

Lindström-Seppä, P. & Oikari, A. 1990. Biotransformation and other toxicological and physiological responses in rainbow trout (Salmo gairdneri Richardson) caged in a lake receiving efflunts of pulp and paper industry. Aquat. Toxicol. 16, 187-204.

Luster, M. I., Munson, A. E., Thomas, P. T., Holsapple, M. P., Fenters, J. D., White, K. L., Lauer, L. D., Germolec, D. R., Rosenthal, G. J. & Dean, J. H. 1988. Methods evaluation. Development of testing battery to assess chemical-induced immunotoxicity: National toxicology program´s guidelines for immunotoxicity evaluation of mice. Fund. Appl. Toxicol. 10, 2-19.

Luster, M. I., Portier, C., Pait, D. G., White, K. L., Gennings, C., Munson, A. E. & Rosenthal, G. J. 1992. Risk assesment in immunotoxicology 1. Sensitivity and predictability of immune tests. Fund. Appl. Toxicol. 18, 200-210.

Luster, M. I., Portier, C., Pait, D. G., Rosenthal, G. J., Germolec, D. R., Corsini, E., Blaylock, B. L., Pollock, P., Kouchi, Y., Craig, W., White, K. L., Munson, A. E. & Comment, C. E. 1993. Risk assesment in immunotoxicology 2. Relationships between immune and host resistance tests. Fund. Appl. Toxicol. 21, 71-82.

MacLatchy, D. I. & Van Der Kraak, G. J. 1994. The plant sterol ß-sitosterol decreases reproductive fitness in goldfish. In: Proceedings of The 2nd International Conference on Environmental Fate and Effects on Bleached Pulp Mill Effluents. Vancouver, B.C., Canada.

Maluish, A. E. & Strong, D. M. 1986. Lymphocyte proliferation. In: Rose, N. R., Friedman, H.& Fahey, J. L. (eds), Manual of clinical laboratory immunology: 274-281. American Society for Microbiology, Washington, DC.

Manning, M. J. & Nakanishi, T. 1996. The specific immune system: cellular defence. In: Iwama, G.& Nakanishi, T. (eds), The fish immune system. Organism, pathogen, and environment: 159-205. Academic Press, San Diego.

Maule, A. G. & Schreck, C. B. 1987. Changes in the immune system of Coho salmon (Oncorhyncus kisutch) during the parr-to-smolt transformation and after implantation of cortisol. Can. J. Fish Aquat. Sci. 44, 161-166.

Maule, A. G. & Schreck, C. B. 1990. Glucocorticoid receptors in leucocytes and gill of juvenile coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. 77, 448-455.

Maule, A. G., Schreck, C. B. & Sharpe, C. 1993. Seasonal changes in cortisol sensitivity and glucocorticoid receptor affinity and number in leucocytes of coho salmon. Fish Physiol. Biochem 10, 497-506.

McDonough, T. J. 1998. The revolution in bleached chemical pulp manufacturing technology. In: Turoski, V. (eds), Chlorine and chlorine compounds in the paper industry: 25-38. Ann Arbor Press, Mihigan.

McLeay, D. J. 1987. Aquatic toxicity of pulp and paper mill effluents: A rewiev. Environment Canada, Canada Report, pp. 191

McMaster, M. E., Van Der Kraak, G. J., Portt, C. B., Munkittrick, K. R., Sibley, P. K., Smith, I. R. & Dixon, D. G. 1991. Changes in hepatic mixed-function oxygenase (MFO) activity, plasma steroid levels and age at maturity of a white sucker (Catostomus commersoni) population exposed to bleached kraft pulp mill effluents. Aquat. Toxicol. 21, 199-218.

Miettinen, V., Lönn, B.-E. & Oikari, A. 1982. Effects of biological treatment on the toxicity for fish of combined deparking and kraft pulp bleaching effluent. Pap. Puu 4, 251-254.

Mikkelson, P. & Paasivirta, J. 1997. Chemical characterication of total wastewaters from pine pulp mill processwith TCF or ECF bleaching. p. 105-113. In: Proceedings of the 3rd International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand.

Mörck, R., Reimann, A. & Dahlman, O. 1991. Characterization of high molecular weight organic materials in modern softwood and hardwood bleached kraft mill effluents. In: Södergren, A. (eds), Environmental fate and effects of bleached pulp mill effluents: 155-163. Swedish environmental protection agency, Stockholm, Sweden.

Munkittrick, K. R., Portt, C. B., Van Der Kraak, G. J., Smith, I. R. & Rokosh, D. A. 1991. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker (Catostomus commersoni) population. Can. J. Fish. Aquat. Sci. 48, 1371-1380.

Munkittrick, K. R., Van Der Kraak, G. J., McMaster, M. E., Portt, C. B., van der Heuvel, M. R. & Servos, M. R. 1994. Survey of receiving water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity and plasma sex sterols levels in white sucker. Environm. Toxicol. Chem. 13, 1089-1101.

Murad, A. & Houston, A. H. 1988. Leucocytes and leucopoietic capacity in goldfish, Carassius auratus , exposed to sublethal levels of cadmium. Aquat. Toxicol. 13, 141-154.

Nakanishi, T., Aoyagi, K., Xia, C., Dijkstra, J. M. & Ototake, M. 1999. Specific cell-mediated immunity in fish. Vet. Immunol. Immunopathol. 72, 101-109.

Nelson, R. D., Quie, P. G. & Simmons, R. L. 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115, 1650-1656.

Nikinmaa, M. & Oikari, A. 1982. Physiological changes in trout (Salmo gairdneri) during a short-term exposure to resin acids and during recovery. Toxicol. lett. 14, 103-110.

Noll, F. 1974. L-(+)-lactate:Determination with LDH, GPT and NAD. In: Bergmeyer, H. U. (ed.), Methods of Enzymatic Analysis: 1475. Verlag Chemie, Weinheim/ Academic press, London.

O'Halloran, K., Ahokas, J. T. & Wright, P. F. A. 1998. Response of fish immune cells to in vitro organotin exposures. Aquat. Toxicol. 40, 141-156.

Oikari, A. & Nakari, T. 1982. Kraft pulp mill effluents cause liver dysfunction in trout. Bull. Environ. Contam. Toxicol. 28, 266-270.

Oikari, A. & Niittylä, J. 1985. Subacute physiological effects of bleached kraft mill effluent (BKME) on the liver of trout, Salmo gaidneri. Ecotoxicol. Environ. Saf. 10, 159-172.

Oikari, A. & Holmbom, B. 1996. Ecotoxicological effects of process changes implemented in a pulp and paper mill:A Nordic case study. In: Servos, M. R., Munkittrick, Carey, J. H.& Van Der Kraak, G. J. (eds), Environmental fate and effects of pulp and paper mill effluents: 613-625. St. Lucie Press, Delray Beach, Florida.

Oikari, A., Nakari, T. & Holmbom, B. 1984. Sublethal actions of simulated kraft mill effluent (KME) in Salmo gairdneri Richardson, in fresh water. Water Res. 17, 81-89.

Oikari, A., Holmbolm, B., Ånas, E., Miilunpalo, M., Kruzynski, G. & Castrén, M. 1985. Ecotoxicological aspects of pulp and paper mill effluents discharged to an inland water system: distribution in water, and toxicant residues and physiological effects in caged fish (Salmo gairdneri). Aquat. Toxicol. 6, 219-239.

O´Connor, B. I., Kovacs, T. G., Voss, R. H., Martel, P. H. & van Lierop, B. 1994. A laboratory assesment of the environmental quality of alternative pulp bleaching effluents. Pulp Pap. Can. 95, 47-56.

Pickering, A. D. & Pottering, T. G. 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem 7, 253-258.

Priha, M. & Paavilainen, K. 1991. Äänekoski-Vaajakoski -vesireitin velvoite-tarkkailu vuonna 1990. Oy Keskuslaboratorio Report, pp. 46., Espoo, Finland.

Priha, M. H. 1996. Ecotoxicological impacts of pulp mill effluents in Finland. In: Servos, M. R., Munkittrick, Carey, J. H.& Van Der Kraak, G. J. (eds), Environmental fate and effects of pulp and paper mill effluents: 637-650. St. Lucie Press, Delray Beach, Florida.

Pulsford, A. L., Thomas, M. E., Lemaire-Gony, S., Coles, J., Fossato, V. U. & Pipe, R. K. 1995. Studies on the immune system of the Goby, Zosterisessor ophiocephalus, from the Venice Lagoon. Mar. Pollut. Bull. 30, 586-591.

Rice, C. D. & Weeks, B. A. 1989. Influence of tributyltin on in vitro activation of oyster toadfish macrophages. J. Aquat. Animal Health 1, 62-78.

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. J. Aquat. Animal Health 7, 195-204.

Rice, C. D., Banes, M. M. & Ardelt, T. C. 1995. Immunotoxicity in channel catfish, Ictalurus punctatus, following acute exposure to tributyltin. Arch. Environm. Contam. Toxicol. 28, 464-70.

Rice, C. D., Kergosien, D. H. & Adams, S. M. 1996. Innate immune function as a bioindicator of pollution stress in fish. Ecotoxicol. Environ. Saf 33, 186-192.

Ruonala, S. & Lammi, R. 1997. Environmental impacts of pulp bleaching based on oxygen chemicals. p. 79-86. In: Proceedings of the 3rd International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand.

Salo, H. M., Aaltonen, T. M., Markkula, S. E. & Jokinen, E. I. 1998. Ultraviolet B irradiation modulates the immune system of fish (Rutilus rutilus, Cyprinidae). I. Phagocytes. Photochemistry and Photobiology 67, 433-437.

Sanders, V. M. 1995. Neurotransmitters, neuropeptides, and immune function: Implications for immunotoxicology. In: Smialowicz, R. J.& Holsapple, M. P. (eds), Experimental immunology: 169-185. CRC Press, Boca Raton.

Sandström, O. 1995. Incomplete recovery in a costal fish community exposed to effluents from a modernized Swedish bleached kraft mill. Can. J. Fish Aquat. Sci. 51, 2195-2202.

Sandström, O., Neuman, E. & Karas, P. 1992. Bleached pulp mill effects on Baltic costal fish populations. In: Södergren, A. (ed.), Environmental Fate and Effects of Bleached Pulp Mill Effluents: 357-370. Swedish Environmental Protection Agency,

Sandström, O. & Thoresen, G. 1988. Mortality in perch populations in a Baltic pulp mill effluent area. Mar. Pollut. Bull. 19, 564-567.

Savino, W. & Dardenne, M. 1995. Immune-neuroendocrine interactions. Immunol. Today 16, 318-322.

Schrock, R. M., Beeman, J. W., Rondolf, D. W. & Haner, P. V. 1994. A microassay for gill sodium, potassium-activated ATPase in juvenile Pacific salmonids. Trans. Am. Fish. Soc. 123, 223-229.

Secombes, C. J. 1996. The nonspecific immune system: cellular defence. In: Iwama, G.& Nakanishi, T. (eds), The fish immune system. Organism, pathogen, and environment: 63-103. Academic Press, San Diego.

Secombes, C. J., Fletcher, T. C., O'Flynn, J. A., Costello, M. J., Stagg, R. & Houlihan, D. F. 1991a. Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. Comp. Biochem. Physiol. 100C, 133-136.

Secombes, C. J., White, A., Fletcher, T. C. & Houlihan, D. F. 1991b. The development of an ELISPOT assay to quantify total and spesific antibody-secreting cells in dab Limanda limanda (L.). Fish Shellfish Immunol. 1, 87-97.

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

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

Sedgwik, J. D. & Holt, P. G. 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods 57, 301-309.

Siwicki, A. K., Vergnet, C., Charlemagne, J. & Dunier, M. 1994. Monoclonal antibodies against goldfish (Carassius auratus) immunoglobulin: application to the quantification of immunoglobulin and antibody-secreting cells by ELISPOT and seric immunoglobulin and antibody levels by ELISA in carp (Cyprinus carpio). Veterinary Research 25, 458-467.

Sjöström, E. & Alén, R. (eds) 1999. Analytical Methods in Wood Chemistry, Pulping, and Papermaking. 305 p. Springer-Verlag, Heidelberg.

Sjöström, E. & Westermark, U. 1999. Chemical composition of wood and pulps: Basic constituents and their distribution. In: Sjöström, E.& Alén, R. (eds), Analytical methods in wood chemistry, pulping, and papermaking: 1-19. Springer-Verlag, Heidelberg.

Slater, C. H., Fitzpatrick, M. S. & Schreck, C. B. 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen. Comp. Endocrinol. 100, 218-225.

Södergren, A. 1989. Biological effects of bleached kraft mill effluents. Final report from the Environment/Cellulose Project I. In: (eds), 139. Swedish National Environmental Protection Board, Solna.

Södergren, A. 1993. Bleached pulp mill effluents. Composition, Fate and Effects in the Baltic Sea. Final Report from the Environment/Cellulose Project II. In: (eds), 150. Swedish National Environmental Protection Board, Solna.

Soimasuo, R., Aaltonen, T., Nikinmaa, M., Pellinen, J., Ristola, T. & Oikari, A. 1995a. Physiological toxicity of low-chlorine bleached pulp and paper mill effluent on whitefish (Coregonus lavaretus L. s.l.): a laboratory exposure simulating lake pollution. Ecotoxicol. Environ. Saf. 31, 228-237.

Soimasuo, R., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T. & Oikari, A. 1995b. Biomarker responses in a pollution gradient: Effect of pulp and paper mill effluents on whitefish studied by caging technique. Aquat. Toxicol. 31, 329-345.

Soimasuo, M. R., Karels, A. E., Leppänen, H., Santti, R. & Oikari, A. O. J. 1998. Biomarker responses in whitefish (Coregonus lavaretus L. s.l.) experimentally exposed in a large lake receiving effluents from pulp and paper industry. Arch. Environm. Contam. Toxicol. 34, 69 - 80.

Soimasuo, M. R., Karels, A. E. & Oikari, A. O. J. (ed.) 2000. Utility of fish biomarkers to assess effects of process alterations and secondary treatment of BKME by field and laboratory experiment. (In press) p. SETAC press, Pensacola, USA.

Solomon, K., Bergman, H., Hugget, R., Mackay, D. & McKague, B. 1993. A review and assesment of the ecological risks associated with the use of chlorine dioxide for bleaching of pulp. Prepared for the Alliance for the Environmental Technology October 1993, 75.

Spitsbergen, J. M., Schat, K. A., Kleeman, J. M. & Peterson, R. E. 1986. Interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with immune responses of rainbow trout. Vet. Immunol. Immunopathol. 12, 263-280.

Stolen, J., Fletcher, T. C., Anderson, D. P., Robertson, B. S. & van Muiswinkel, W. B. (eds) 1990. Fish Immunology Technical Communications no.1 FITC-1. 196 p. SOS Publications, Fair Haven.

Stolen, J., Fletcher, T. C., Anderson, D. P., Kaattari, S. L. & Rowley, A. F. (eds) 1992. Fish Immunology Technical Communications no.2 FITC-2. 196 p. SOS Publications, Fair Haven.

Stolen, J. S., Fletcher, T. C., Rowley, A. F., Zelikoff, J. T., Kaattari, S. L. & Smith, S. A. (ed.) 1994. Fish Immunology Technical Communications no.3 FITC-3. 190 p. SOS Publications, Fair Haven.

Stolen, J. S., Fletcher, T. C., Smith, S. A., Zelikoff, J. T., Anderson, R. S., Söderhäll, K., Kaattari, S. L. & Weeks-Perkins, B. A. (eds) 1995. Fish Immunology Technical Communications no.4 FITC-4. 258 p. SOS Publications, Fair Haven.

Strömberg, L., Mörck, R., Sousa de, F. & Dahlman, O. 1996. Effects of internal process changes and external treatment on effluent chemistry. In: Servos, M. R., Munkittrick, Carey, J. H.& Van Der Kraak, G. J. (eds), Environmental fate and effects of pulp and paper mill effluents: 3-19. St. Lucie Press, Delray Beach, Florida.

Swanson, S. M., Schyer, R., Shelast, R., Kloepper-Sams, P. J. & Owens, J. W. 1994. Exposure of fish to bleached kraft mill effluent: 3. Fish habitat and population assesment. Environm. Toxicol. Chem. 13, 1497-1507.

Tahir, A. & Secombes, C. J. 1995. The effect of diesel oil-based drilling mud extracts on immune responses of rainbow trout. Arch. Environm. Contam. Toxicol. 29, 27-32.

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.). Aquat. Toxicol. 27, 71-82.

Talal, N. 1992. Sex hormones and immunity. In: Roitt, I. M. & Delves, P. J. (eds), Encyclopedia of Immunology: 1369-1371. Academic Press, Inc., San Diego.

Tana, J. & Lehtinen, K.-J. 1996. The aquatic environmental impact of pulping and bleaching operations : an overview. Finnish Environment Institute Report, pp. 103., Helsinki, Finland.

Taylor, M. & Hoole, D. 1989. Ligula intestinalis (L.) (Cestoda: Pseudophyllidea): an ultrastructural study of the cellular response of roach fry Rutilus rutilus L., to an unusual intramuscular infection. Journal of Fish Disease 12, 523-528.

Taylor, M. J. & Hoole, D. 1993. Ligula intestinalis (L.) (Cestoda: Pseudophyllidea): polarization of cyprinid leucocytes as an indicator of host- and parasite-derived chemoattractants. Parasitology 107, 433-440.

Taylor, M. J. & Hoole, D. 1994. Modulation of fish lymphocyte proliferation by extracts and isolated proteinase inhibitors of Ligula intestinales (Cestoda). Fish Shellfish Immunol. 4, 221-230.

Taylor, M. J. & Hoole, D. 1995. The chemiluminescence of cyprinid leucocytes in response to zymosan and extracts of Ligula intestinalis (Cestoda). Fish Shellfish Immunol. 5, 191-198.

Thomas, P. T. & Sherwood, R. L. 1996. Host resistance models in immunotoxicology. In: Smialowicz, R. J. & Holsapple, M. P. (eds), Experimental immunotoxicology: 29-45. CRC raton, Boca Raton, Florida.

Thuvander, A., Wiss, E. & Norrgren, L. 1993. Sublethal exposure of rainbow trout (Oncorhynchus mykiss) to Clophen A50: effects on cellular immunity. Fish Shellfish Immunol. 3, 107-117.

Tremblay, L. & Van Der Kraak, G. 1998. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of beta -sitosterol in rainbow trout. Aquat. Toxicol. 43, 149-162.

Tucker, E. S. 1994. Consequence of immunodeficiency. In: Dean, J. H., Luster, M. I., Munson, A. E. & Kimber, I. (eds), Immunotoxicology and Immunopharmatology: 1-18. Raven Press, New York.

Turoski, V. (ed.) 1998. Chlorine and chlorine compounds in the paper industry. 361 p. Ann Arbor Press, Chelsea, Mihigan.

Valtonen, E. T. & Koskivaara, M. 1989. Effects of effluents from pulp and paper mill on parasites of the roach in Central Finland. In: Bauer, O. N. (ed.), Parasites of Freshwater Fishes of North-West Europe: 163-168. Petrozavodsk.

Valtonen, E. T. & Koskivaara, M. 1994. Relationships between the parasites of some wild and cultured fishes in the two lakes and a fish farm in Central Finland. International Journal of Parasitology 24, 109-118.

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

Verta, M., Ahtiainen, J., Nakari, T., Langi, A. & Talka, E. 1996. The effect of waste constituents on the toxicity of TCF and ECF pulp bleaching effuents. In: Servos, M. R., Munkittrick, Carey, J. H. & Van Der Kraak, G. J. (eds), Environmental fate and effects of pulp and paper mill effluents: 41-51. St. Lucie Press, Delray Beach, Florida.

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. Mar. Ecol. Prog. Ser. 91, 173-192.

Voccia, I., Krzystyniak, K., Dunier, M., Flipo, D. & Fournier, M. 1994. In vitro mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 29, 37-48.

Vos, J. G. 1980. Immunotoxicity assessment: screening and function studies. Arch. Toxicol. 4, 95-108.

Vos, J. G. & van Loveren, H. 1996. Immunotoxicology: determination of immunotoxic effects and immunotoxicity mechanisms. In: Niesink, R. J. M., de Vries, J. & Hollinger, M. A. (eds), Toxicology. Principles and Applications: 841-868. CRC Press, Boca Raton.

Voss, R. H., Wearing, J. T., Mortimer, R. D., Kovacs, T. & Wong, A. L. 1980. Chlorinated organics in kraft bleachery effluents. Pap. Puu 62, 809-814.

Waterstrat, P. R., Brazil, J. & Ainsworth, A. J. 1991. Use of an ELISA-based assay for the detection of antibody-secreting cells in channel catfish, Ictalurus punctatus (Rafinesque). Journal of Fish Disease 14, 669-675.

Weeks, B. A. & Warinner, J. E. 1984. Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. Mar. Environm. Res. 14, 327-335.

Weeks, B. A., Warinner, J. E. & Rice, C. D. 1990. Recent advances in the assessment of environmentally-induced immunomodulation. Oceans 89, 408-411.

Weeks, B. A., Anderson, D. P., DuFour, A. P., Fairbrother, A., Goven, A. J., Lahvis, G. P. & Peters, G. 1992. Immunological biomarkers to assess environmental stress. In: Hugget, R. J., Kimerle, P. M., Mehrle Jr, P. M. & Bergman, H. L. (eds), Biomarkers, Biochemical, Physiochemical, and Histological Markers of Anthropogenic Stress: 211-234. Lewis Publishers, Chelsea.

Wendelaar Bonga, S. E. 1997. The stress response in fish. Physiological Reviews 77, 591-625.

Wester, P. W., Vethaak, A. D. & van Muiswinkel, W. B. 1994. Fish as biomarkers in immunotoxicology. Toxicology 86, 213-232.

Weyts, F. A. A., Verburg-van Kemenade, B. M. L. & Flikt, G. 1998a. Characterication of corticoid receptors in carp, Cyprinus carpio L., peripheral blood leucocytes. Gen. Comp. Endocrinol. 111, 1-8.

Williams, M. A. & Hoole, D. 1992. Antibody response of the fish Rutilus rutilus to the metacestoda of Ligula Intestinalis. Dis. Aquat. Org. 12, 83-89.

Yano, T. 1996. The nonspecific immune system: humoral defence. In: Iwama, G. & Nakanishi, T. (eds), The fish immune system. Organism, pathogen, and environment: 105-157. Academic Press, San Diego.

Yin, C.-F., Joyce, T. W. & Chang, H.-M. 1989. Characerization and biological treatment of bleach plant effluent. In: Proceedings of the Industrial Waste Water Conference. Lafaytte, Indiana.

Yin, C.-F., Joyce, T. W. & Chang, H.-M. 1990. Reduction of pollution load from bleach plant by applying oxygen bleaching, chlorine dioxide substitution and by ultrafiltration and sequential biological treatment. p. 152-173. In: Proceedings of the 24th EUCEPA Conference. Stocholm, Sweden.

Zapata, A. 1981a. Lymphoid organs of teleost fish. I. Ultrastructure of renal lymphoid tissue of Rutilus rutilus and Gobio gobio. Dev. Comp. Immunol. 5, 685-690.

Zapata, A. 1981b. Lymphoid organs of teleost fish. I. Ultrastructure of the thymus of Rutilus rutilus. Dev. Comp. Immunol. 5, 427-436.

Zapata, A. 1982. Lymphoid organs of teleost fish. III. splenic lymphoid tissue of Rutilus rutilus and Gobio gobio. Dev. Comp. Immunol. 6, 87-94.

Zelikoff, J. 1993. Metal pollution-induced immunomodulation in fish. Annu. Rev. Fish Dis. 2, 305-325.

Zelikoff, J. T. 1994. Fish immunotoxicology. In: Dean, J. H., Luster, M. I., Munson, A. E. & Kimber, I. (eds), Immunotoxicology and immunopharmacology: 71-95. Raven Press. Ltd., New York.

ORIGINAL PAPERS

I

Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay.

by

Aalтonen T.M., Jokinen E.I. & Valtonen E.T.

Fish & Shellfish Immunology 4: 129-140, 1994

https://doi.org/10.1006/**fsim.**1994.1011

Fish & Shellfish Immunology (1994) **4**, 129–140

Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay

T. M. Aaltonen, E. I. Jokinen and E. T. Valtonen

Department of Biology, University of Jyväskylä, SF-40100 Jyväskylä, Finland

(*Received 14 October 1992, accepted in revised form 3 March 1993*)

The roach (*Rutilus rutilus* L.) which is a cyprinid fish, was immunised with bovine γ-globulin (BGG) and the antibody synthesis was studied by counting the number of specific antibody secreting cells (SASC) in the spleen and anterior kidney, and by measuring the antibody concentration in the circulation. SASCs and the total number of immunoglobulin secreting cells (TISC) were counted with the ELISPOT (enzyme-linked immunospot) assay, and anti-BGG antibodies and the concentration of immunoglobulin in sera were assayed by ELISA (enzyme-linked immunosorbent assay). The present modification of the ELISPOT-assay takes advantage of biotin-avidin amplification and yields easily detectable and nonfading spots that may be counted even after several weeks of storage. It is also possible to count the number of SASC and TISC in different organs.

In this study, immunisation of the roach with protein antigen via the intraperitoneal route resulted in a similar response in the spleen and in the kidney. There was no preferential organ distribution of the number of SASC or TISC and no difference in the timing of the response in the organs. The humoral response due to immunisation in the serum correlated with cellular responses in the organs.

Key words: *Rutilus rutilus*, antibody-secreting cells, antibody synthesis, ELISPOT-assay, lymphoid organs.

I. Introduction

Activation of the humoral immune system after contact with antigens results in differentiation of B lymphocytes into antibody secreting cells (ASC) and subsequent secretion of antibodies. Antibodies in the circulation, gut and skin constitute an important part of the resistance against pathogenic microbes. The antibody response may be quantified by measuring the concentration of the antibody in serum which may be performed by immunodiffusion, agglutination, radioimmune assays (RIA) or enzyme-immunoassays (EIA). Among the EIAs, the enzyme-linked immunosorbent assay (ELISA) has been widely used to study the responses against bacterial antigens e.g. against *Vibrio anguillarum* and *Aeromonas salmonicida* in rainbow trout (Chart *et al.*, 1984; Thuvander *et al.*, 1987) and *Edwardisella ictaluri* in catfish (Waterstrat *et al.*, 1989). ELISA against viral piscine antigens have been developed to assay

1050–4648/94/020129+12 $08.00/0

Egtved virus (Cossarini-Dunier, 1985; Lorensen *et al.*, 1988) and penacid baculovirus in salmonids (Lewis, 1986) or IPNV (Rodak *et al.*, 1988). Antibodies against certain parasites and other antigens (Whyte *et al.*, 1990; Arkhoosh & Kaattari, 1991) may also be quantified by specific ELISA in fish. ELISA has also been used in vaccination studies [e.g. *V. anguillarum* in rainbow trout (Thuvander *et al.*, 1987; Thorburn & Jansson, 1988) and cod (Espelid *et al.*, 1991)].

The antibody response can also be followed by monitoring the appearance of ASC in the blood or lymphoid organs. The main method for quantification of ASC in fish has been the haemolytic plaque assay developed by Jerne & Nordin (1963). The plaque assay has been widely used to study the response of injected sheep red blood cells (Rijkers *et al.*, 1980; Ingram, 1985; Nakanishi, 1986; Grondel *et al.*, 1987) and a variety of other antigens in several species of fish (Kaattari & Irwin, 1985; Ruglys, 1985; Georgopoulou & Vernier, 1986). In the present study the enzyme-linked immunospot assay (ELISPOT) was used for quantifying ASC. Although the ELISPOT has been commonly used to study human and rodent immunology the technique has only recently been applied to fish, (Secombes *et al.*, 1991; Waterstrat *et al.*, 1991).

Here we report a study utilising ELISA and ELISPOT to follow the kinetics of immunisation in roach. The procedure for the ELISA and the modified ELISPOT assay with biotin-avidin amplification, resulting in long lasting spots, is described. The response of specific antibody secreting cell (SASC) against injected protein antigen, bovine serum γ-globulin (BGG), and the total number of immunoglobulin secreting cells (TISC) were studied in the spleen and kidney. The antigen specific antibody levels and the total concentration of immunoglobulin in serum were measured by ELISA.

II. Materials and Methods

IMMUNISATION OF FISH

Fifty roach (*Rutilus rutilus*) were caught at the end of June by angling from lake Peurunkajärvi in Central Finland when the water temperature was 14° C. Peurunkajärvi is an oligotrophic lake which is still in a natural state. The average length of the fish was 128 mm (S.D. 15 mm) and the average weight 25 g (S.D. 14 g). After catching the fish, any ectoparasites were killed by administration of a commercial drug, Ichide (N.T. Laboratories Ltd., England) 10 μl l^{-1}. During the experiment the fish were kept in an aquarium with a volume of 300 l in aerated tap water at a constant temperature of 17·5 ± 0·5° C and fed daily with commercial pelleted dry food. The fish were immunised at the end of July with a single intraperitoneal injection of bovine gamma globulin (BGG), emulsified in Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, U.S.A.). Each fish received 200 μl of a solution containing 500 μg BGG. Before immunisation 10 fish were sampled as controls. The immune response was studied by examining 10 fish 14, 21 and 28 days after the immunisation. Blood was collected from the caudal vein for serum separation. The spleen and anterior kidney were removed immediately after killing for lymphocyte isolation. A further sample (10 fish) was taken on day 35 after immunisation for serum samples alone.

BLOOD SAMPLES

Following extraction the blood was allowed to clot for 3 h at room temperature (22° C), stored overnight at 4° C and then centrifuged at 4000 *g* for 5 min to remove the serum. The sera were stored at − 20° C.

ISOLATION OF LYMPHOCYTES

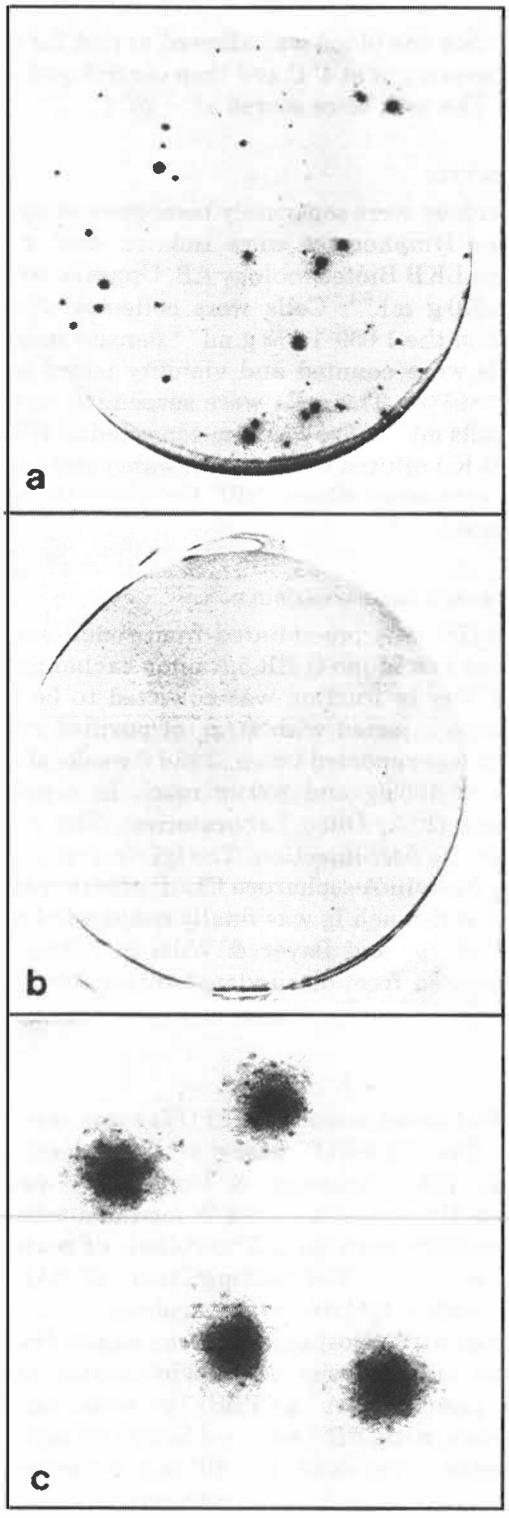
The spleen and kidney were separately homogenised against a nylon net (80 mesh) and then the lymphocytes were isolated with a three step Percoll-gradient (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with densities 1·030, 1·050 and 1·070 g ml^{-1}. Cells were collected after centrifugation at 4000 *g* for 20 min from the 1·050–1·070 g ml^{-1} density interface. After washing the number of cells were counted and viability tested by using trypan blue staining (viability >95%). The cells were suspended in culture medium at a density of 2×10^6 cells ml^{-1}. The medium consisted of RPMI-1640 (Gibco BRL Co. Ltd., Paisley, U.K.) diluted to 80% with water and supplemented with 3% Ultroser G serum substitute (Gibco BRL Co., Ltd.), 10 mM Hepes and 0·5 mg ml^{-1} sodium pyruvate.

PRODUCTION OF ANTI-ROACH IMMUNOGLOBULIN

Immunoglobulin (Ig) was precipitated from roach serum with ammonium sulphate and applied to a Mono Q HR 5/5 anion exchanger (FPLC, Pharmacia, Uppsala, Sweden). The Ig fraction was collected to be used for immunising rabbits. Rabbits were injected with 80 μg of purified roach Ig emulsified in FCA. Immunisation was repeated twice (3 and 6 weeks after the first injection) by administration of 100 μg and 200 μg roach Ig emulsified with Freund's incomplete adjuvant (FIA, Difco Laboratories). The rabbit serum was collected 9 weeks after the first injection. The IgG fraction of the rabbit sera was affinity purified by Protein-A-sepharose CL-4B affinity chromatography (Pharmacia). The rabbit anti-roach Ig was finally conjugated to biotin as described by Guesdon *et al.* (1979) and Bayer & Wilchek (1980). Any unreacted free reagents were separated from biotinylated anti-roach Ig by Sephadex-G25M-chromatography (PD-10 column, Pharmacia).

ELISPOT ASSAY

The enzyme-linked immunospot (ELISPOT) assay was used for quantifying TISC, and SASC. The ELISPOT assay as applied for human lymphocytes (Czerkinsky *et al.*, 1983; Sedgwick & Holt, 1983) was adapted to roach. Flat-bottomed, 96-well microtitre plates (Nunc-Immunoplate Maxi Sorp, Nunc Co., Roskilde, Denmark) were used. The volume of reagent solutions in each step of the assay was 50 μl. For coating (assay of SASC), 10 μg BGG ml^{-1} 50 mM carbonate buffer (pH 9·6) was incubated overnight at 4° C. After washing three times with phosphate buffered saline (PBS) containing 0·05% Tween, wells were masked with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, U.S.A.) in PBS. The wells were washed twice with PBS-Tween and once with PBS before adding the cells to the wells. Three different cell densities were used: 10^5, 10^4 and 10^3 cells/well. Isolated spleen and kidney lymphocytes were allowed to secrete antibodies in the wells for 3 h


a
b
c

at 18° C. The bound antibodies were detected with biotin-conjugated anti-roach Ig. After incubation overnight at 4° C and after washing three times with PBS-Tween, alkaline phosphatase conjugated avidin (Biomakor, Rehovot, Israel) diluted 1:1000 in 1% BSA/PBS was added and the plates incubated at 37° C for 30 min. After three washes the substrate, bromo-chloro-indolyl phosphate (Sigma Chemical Co.) mixed with warm agarose (Agarose type I, Sigma Chemical Co.), final concentration 0·9%, was added to the wells (Sedgwick & Holt, 1983). Finally, the number of spots was counted under a stereomicroscope after storage of the assay plates at 4° C for 1-6 weeks. For counting of the total number of immunoglobulin-secreting cells (TICS), the plates were coated with anti-roach Ig (10 μg ml^{-1} carbonate buffer, pH 9·6). The cell densities were adjusted to 10^3 and 10^2 cells/well. The other steps of the assay were as described above.

QUANTIFICATION OF SERUM ANTIBODIES WITH ELISA

The plates were coated either with 10 μg BGG ml^{-1} 50 mM carbonate buffer, pH 9·6 (for determination of specific anti-BGG antibodies) or with 5 μg ml^{-1} anti-roach Ig (for total Ig concentration). After saturation of the plates with BSA, samples of sera diluted 1:10^5–1:10^7 (for total Ig) or 1:10^2–1:10^4 (for anti-BGG) were incubated in the wells for 30 min at 37° C. The two subsequent steps in these assays (second antibody and avidin alkaline phosphatase conjugate) followed as described for above ELISPOT. *p*-nitro-phenylphosphate (Sigma Chemical Co.) in 1 M diethanolamine buffer (pH 9·8) was used as a substrate. The optical density was read with Titertek plate reader (Flow Laboratories, Herts, U.K.) at 405 nm.

STATISTICAL ANALYSES

The *t*-test and two way ANOVA were used to study the statistical significance. The relationship between continuous associated variables was assessed by Pearson's coefficient of correlation with Bonferroni adjusted probability (Sokal & Rohlf, 1987). The Systat for Macintosh (Systat Inc., U.S.A.) statistical package was used.

III. Results

THE KINETICS OF SASC IN THE ORGANS

The appearance of the wells that contain spots of anti-BGG antibody secreting cells is showing in Fig. 1. The number of SASC isolated both from the kidney and spleen were counted. A small number of SASC could be detected already by day 14 (Fig. 2). The peak SASC response occurred on day 21 post immunisation after which there was a sharp decrease. The peak SASC responses in the spleen and kidney were concurrent. Although the number of

Fig. 1. Anti-BGG secreting cells from the spleen of immunised fish, detected with ELISPOT. (a) BGG-coated well, 10^3 cells 50 μl^{-1}. (b) Control well (not coated), 10^3 cells 50 μl^{-1}. (c) Close up of anti-BGG spots; note the diffuse edge which is characteristic for these spots as a result of secretion by lymphocytes. The edge can be used as a criterion for distinguishing true spots from 'pseudo' spots.

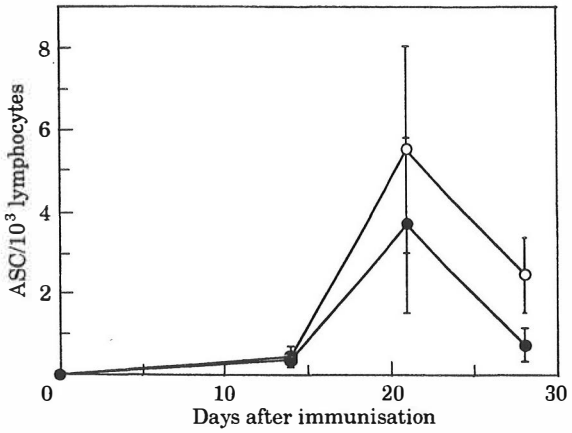


Fig. 2. Specific antibody secreting cells (SASC) in the spleen (●) and the anterior kidney (○) following immunisation of fish with BGG. Each point represents the mean value ± S.E. of 9–10 fish.

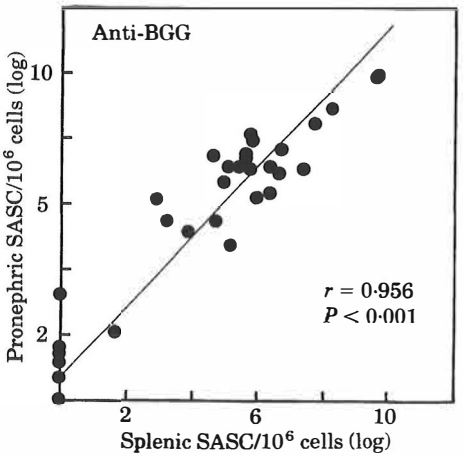


Fig. 3. Number of SASC in spleen *v.* kidney.

SASC/10^6 cells isolated from the kidney was higher than from the spleen, the difference was not statistically significant at any time of sampling. A highly significant correlation was found between the responses in the two organs (r=0·96, P<0·001) (Fig. 3). The data in Fig. 3 include samples collected at all time points. The correlation analysis of samples separately on days 14, 21 and 28 gave similar results and showed that the activation of lymphocytes was parallel in the spleen and kidney (data not shown).

TISC IN LYMPHOID ORGANS

Immunisation was found not to influence the number of TISC in the kidney and spleen during the course of the experiment (Fig. 4). The proportion of TISC of the total number of cells plated in the wells ranged from 16 to 28% in

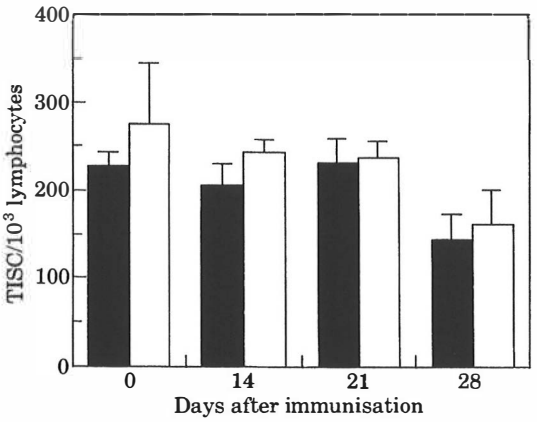


Fig. 4. Total number of immunoglobulin secreting cells (TISC) in the spleen (■) and kidney (□). Each column is the mean ± of 9–10 fish. There were no significant differences between the number of TISC between the two organs.

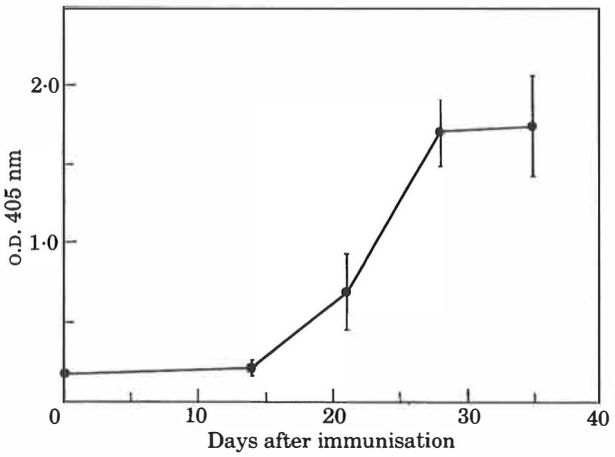


Fig. 5. Anti-BGG antibodies in serum after immunisation of fish with BGG (injection on day 0). Data are expressed as the mean ± S.E. The sera of 10 fish were analysed at each time point.

kidney and 15 to 23% in spleen. The number of TISC in the spleen and kidney were not statistically significantly different at any time. The SASC/TISC ratio showed no significant differences between responses in the spleen and in the kidney (mean ratio SASC/TISC 1·9% at day 21).

SERUM ANTIBODIES

The kinetics of anti-BGG specific antibodies in the sera are shown in Fig. 5. The antibody concentration increased rapidly between day 21 and 28 after immunisation, i.e. after SASC had reached maximum. Between day 28 and day 35 the level of antibodies remained constant. The concentration of total serum immunoglobulin, also analysed by ELISA, varied individually between 22 and

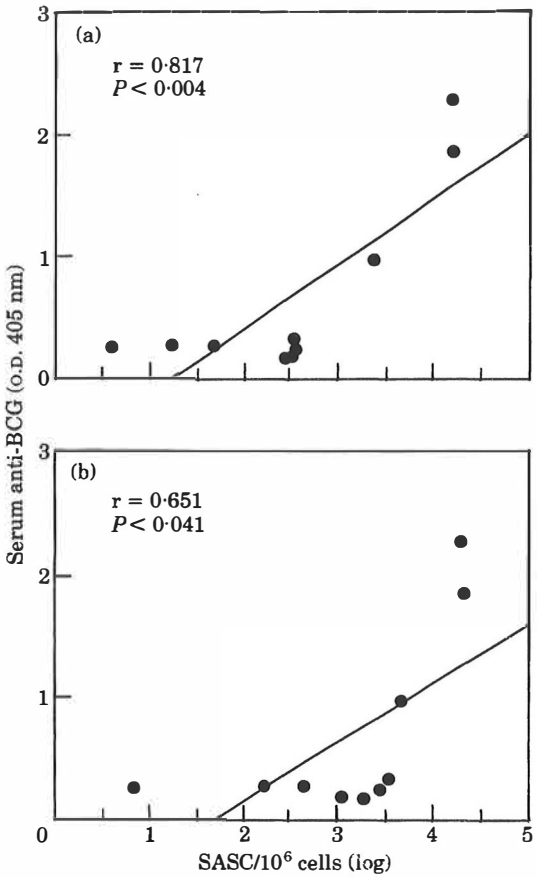


Fig. 6. SASC responses in lymphoid organs and specific antibody in serum collected at the time of the SASC peak (day 21 post immunisation). (a) Spleen, (b) kidney.

42 mg ml^{-1}; there was no trend over time as concerns the total Ig concentration.

It was not possible to calculate the correlation between the anti-BGG specific cellular peak response and the fully developed anti-BGG antibody response in serum of individual fish because the fish had to be killed for the SASC assay. The correlations between SASC and serum antibody response at the day of peak SASC (day 21 post immunisation), when circulatory antibody was significantly elevated but had not yet reached its maximum, are shown in Fig. 6. The cellular responses, both in the spleen and in the kidney, were correlated to serum antibody levels (r=0·82, P<0·004 for the spleen, and r=0·65, P<0·041 for the kidney).

IV. Discussion

The most widespread method for assaying the development of antibodies at the cellular level has been the haemolytic plaque assay developed by Jerne & Nordin (1963). Although the assay may be performed with slight variations the

system relies on haemolysis in a monolayer of intact or antibody coated target red cells in the vicinity of an immunoglobulin-secreting lymphocyte in the presence of complement. The plaque assay suffers from several drawbacks, but the two most important ones are the lability of the haemolytic plaques and the need for active complement.

In this study we have used the ELISPOT assay, which has only recently been applied to fish (Secombes *et al.*, 1991; Waterstrat *et al.*, 1991). The ELISPOT assay may readily be modified for detection of antibodies against a variety of antigens and all the reagents needed in the assay are stable and non-hazardous. In ELISPOT, almost any molecule or microbe that can attach to the solid phase can be used as an antigen. Some intact microbes cannot be used in the plaque assay because of their ability to activate complement which results in total haemolysis of the indicator cells. In this study, we have used biotinylated secondary antibody which increases the sensitivity of the assay because of its capability to react with several molecules of enzyme-labelled avidin. Alkaline phosphatase-labelled reagents with indolyl phosphate as the substrate yield clearly detectable spots which are very stable over several weeks of storage before counting.

In the present study, the maximum SASC response of fish kept at 18° C was recorded on day 21 after immunisation. Kinetic studies of roach using the ELISPOT method have not been published before, but our results are consistent with studies on a related cyprinid fish, the carp (*Cyprinus carpio*). Rijkers (Rijkers *et al.*, 1980; Rijkers, 1982) utilised the haemolytic plaque assay to study the response against injected SRBC and found a peak response on day 10–12 when the fish were kept at 20° C and on day 27 when they were kept at 16° C. Van Muiswinkel *et al.* (1985) detected the peak of PFC after i.p. immunisation with SRBC on day 9–11 when the carp were kept at 23° C. In a recent study by Williams & Hoole (1992) the crude homogenate of a cestode parasite (*Ligula intestinalis*) was used to immunise roach kept at 20° C. They found the peak of PFC on days 4–10 post immunisation. Earlier, several researchers have reported that the kinetics of the response in fish is dependent on temperature as well as on diet, fish species and age (Rijkers, 1982; Kaattari & Irwin, 1985; Pourreau *et al.*, 1986; Nakanishi, 1987). In the present study the timing of the response was similar for the kidney and spleen, which is in accordance with a previous report (Williams & Hoole, 1992).

We found the magnitude of SASC and TISC responses to be similar in the two lymphoid organs that we studied, the spleen and the anterior kidney. The results of Williams & Hoole (1992) also showed only slightly more SASC in the kidney that in the spleen. The study of Nakanishi (1987) in which the crucian carp (*Carassius auratus*) was injected with horse erythrocytes showed that the number of PFC was greater in the spleen than in the kidney. On the other hand, studies in which carp have been immunised with SRBC showed that more PFC are recovered from the kidney than the spleen (Rijkers *et al.*, 1980, 1981). Results from studies of trout injected with LPS (Ingram & Alexander, 1980), rainbow trout (van Ginkel *et al.*, 1985) and catfish (*Saccobranchus fossilis*) (Khangarot & Tripathi, 1991) immunised with SRBC further suggest that the organ distribution of secreting lymphocytes may vary with the species of fish and the antigen.

The method of obtaining cells from the organs may also have an effect on the results. In many studies (e.g. Kaattari & Irwin, 1985; Nakanishi, 1987; Khangarot & Tripathi, 1991) cells for use in the PFC assay have been used after simply disrupting the organ by pressing it through a mesh, without further purification. In some studies leucocytes are obtained with Percoll to remove erythrocytes (Secombes *et al.*, 1991) or the cells are fractioned with density gradient centrifugation (Waterstrat *et al.*, 1991). In addition to the quality of the cells, the type of assay may also be critical. It is obvious that assays vary in their sensitivity for detecting secreting cells. Using the ELISPOT technique, Secombes *et al.* (1991) reported 3–27 TISC/10^3 erythrocyte depleted leucocytes isolated from the kidney of dab (*Limanda limanda*). In the present study we detected 200–300 TISC/10^3 density gradient fractioned cells from the kidney of the roach. Methodological details must clearly be considered when comparing results obtained by different techniques.

The peak of the SASC response was on day 21 after immunisation and at that time the specific serum antibody response was already detectable. The serum response reached its maximum 1–2 weeks after the cellular peak in the spleen and kidney, an observation that is consistent with other results obtained by the plaque technique (Anderson & Dixon, 1980; Nakanishi, 1987).

This study has shown the ELISPOT assay to be a sensitive and reliable method for detecting SASC and TISC in the roach. Presumably, this method will gradually replace the PFC assay because of the many practical advantages it offers.

This work was supported by Academy of Finland. We are indebted to Dr R. Paul for revising the language of this manuscript.

References

Anderson, D. P. & Dixon, O. W. (1980). Immunological memory in rainbow trout to a fish disease bacterin administered by flush exposure. In *Phylogeny of Immunological Memory* (M. J. Manning, ed.) pp. 103–111. Amsterdam: Elsevier/North-Holland Biomedical Press.

Arkhoosh, M. R. & Kaattari, S. L. (1991). Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. *Developmental and Comparative Immunology* **15,** 279–293.

Bayer, E. A. & Wilchek, M. (1980). The use of the avidin-biotin complex as a tool in molecular biology. In *Methods of Biochemical Analysis* (D. Glick, ed.) pp. 2–45. New York: John Wiley & Son.

Chart, H., Pearson, T. W. & Trust, T. J. (1984). Detection of specific fish antibody using an inhibition enzyme-linked immunosorbent assay (Inhibition ELISA). *Journal of Immunological Methods* **68,** 19–25.

Cossarini-Dunier, M. (1985). Indirect enzyme-linked immunosorbent assay (ELISA) to titrate rainbow trout serum antibodies against two pathogens: *Yersinia ruckeri* and Egtved virus. *Aquaculture* **49,** 197–208.

Czerkinsky, C. C., Nilsson, L.-Å., Nygren, H., Ouchterlony, Ö. & Tarkowsky A. (1983). A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *Journal of Immunological Methods* **65,** 109–121.

Espelid, S., Rødseth, O. M. & Jørgensen, T. Ø. (1991). Vaccination experiments and studies of the humoral immune responses in cod, *Gadus morhua* L. to four strains of monoclonal-defined *Vibrio anguillarum*. *Journal of Fish Diseases* **14,** 185–197.

Georgopoulou, U. & Vernier, J.-M. (1986). Local immunological response in the posterior intestinal segment of the rainbow trout after oral administration of macromolecules. *Developmental and Comparative Immunology* **10,** 529–537.

van Ginkel, F. W., van Muiswinkel, W. B., Merchant, B., Lizzio, E. F., Dixon, O. W. & Anderson, D. P. (1985). Temperature comparisons for antibody production *in vitro* by plaque-forming cells from trout, *Salmo gairdneri* (Richardson), and mice. *Journal of Fish Biology* **27,** 265–272.

Grondel, J. L., Nouws, J. F. M. & van Muiswinkel, W. B. (1987). The influence of antibiotics on the immune systems: immuno-pharmokinetic investigations on the primary anti-SRBC response in carp, *Cyprinus carpio* L., after oxytetracycline injection. *Journal of Fish Diseases* **10,** 35–43.

Guesdon, J.-T., Ternyck, T. & Avrameas, S. (1979). The use of avidin-biotin interaction in immunoenzymatic techniques. *The Journal of Histochemistry and Cytochemistry* **27**(8), 1131–1139.

Ingram, G. A. (1985). The immune response of brown trout, *Salmo trutta* to sheep and human 'O' erythrocytes. In *Fish Immunology* (M. J. Manning & M. F. Tatner, eds) pp. 157–169. London: Academic Press.

Ingram, G. A. & Alexander, J. B. (1980). The immune response of the brown trout *Salmo trutta* to lipopolysaccharide. *Journal of Fish Biology* **16,** 181–197.

Jerne, K. & Nordin, A. A. (1963). Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405.

Kaattari, S. L. & Irwin, M. J. (1985). Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. *Developmental and Comparative Immunology* **9,** 433–444.

Khangarot, B. S. & Tripathi, D. M. (1991). Changes in humoral and cell-mediated immune responses and in skin and respiratory surfaces of catfish, *Saccobranchus fossilis*, following copper exposure. *Ecotoxicology and Environmental Safety* **22,** 291–308.

Lewis, D. H. (1986). An enzyme-linked immunosorbent assay (ELISA) for detecting penaeid baculovirus. *Journal of Fish Diseases* **9,** 519–522.

Lorenzen, N., Olesen, N. J. & Vestergård Jørgensen, P. E. (1988). Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. *Diseases of Aquatic Organisms* **4,** 35–42.

van Muiswinkel, W. B., Anderson, D. P., Lamers, C. H. J., Egberts, E., van Loon, J. J. A. & Ijssel, J. P. (1985). In *Fish Immunology* (M. J. Manning & M. F. Tatner, eds) pp. 1–8. London: Academic Press.

Nakanishi, T. (1986). Antibody producing cells in the marine teleost, *Sebastiscus marmoratus*. Organ distribution and morphology. In: *Pathology in Marine Aquaculture* (C. P. Vivares, J. R. Bonami & E. Jaspers, eds) European Aquaculture Society, Special Publication No 9, pp. 333–342. Bredene, Belgium.

Nakanishi, T. (1987). Kinetics of transfer of immunity by immune leucocytes and PFC response to HRBC in isogeneic ginbuna crucian carp. *Journal of Fish Diseases* **30,** 723–729.

Pourreau, C. N., Evenberg, D., de Raadt, W. M., van Mechelen, J. A. N. & van Muiswinkel, W. B. (1986). Does *Aeromonas salmonicida* affect the immune system of carp, *Cyprinus carpio* L.? *Veterinary Immunology and Immunopathology* **12,** 331–338.

Rijkers, G. T. (1982). Kinetics of humoral and cellular immune reactions in fish. In *Immunology and Immunization of Fish* (W. P. van Muiswinkel & E. L. Cooper, eds) pp. 93–100. New York: Pergamon Press.

Rijkers, G. T., Frederix-Wolters, E. M.-H. & van Muiswinkel, W. B. (1980). The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41,** 91–97.

Rijkers, G. T., van Oosterom, R. & van Muiswinkel, W. B. (1981). The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp (*Cyprinus carpio*). *Veterinary Immunology and Immunopathology* **2,** 281–290.

Rodak, L., Pospisil, Z., Tomanek, J., Vesely, T., Obr, T. & Valicek, L. (1988). Enzyme-linked immunosorbent assay (ELISA) detection of infectious pancreatic necrosis virus (IPNV) in culture fluids and tissue homogenates of the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases* **11,** 225–235.

Ruglys, M. P. (1985). The secondary immune response of young carp, *Cyprinus carpio* L., following injection of cortisol. *Journal of Fish Biology* **26,** 429–434.

Secombes, C. J., White, A., Fletcher, T. C. & Houlihan, D. F. (1991). The development of an ELISPOT assay to quantify total and specific antibody-secreting cells in dab *Limanda limanda* (L.). *Fish & Shellfish Immunology* **1,** 87–97.

Sedgwick, J. D. & Holt, P. G. (1983). A solid-phase immunoenzymatic technique of specific antibody-secreting cells. *Journal of Immunological Methods* **57,** 301–309.

Sokal, R. R. & Rohlf, F. J. (1987). *Introduction to Biostatistics.* New York: W. H. Freeman & Co.

Thorburn, M. A. & Jansson, E. L. K. (1988). The effects of booster vaccination and fish size on survival and antibody production following Vibrio infection of bath-vaccinated rainbow trout, *Salmo gairdneri. Aquaculture* **71,** 285–291.

Thuvander, A., Hongslo, T., Jansson, E. & Sundquist, B. (1987). Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. *Journal of Fish Diseases* **10,** 479–486.

Waterstrat, P., Ainsworth, J. & Capley, G. (1989). Use of man indirect enzyme-linked immunosorbent assay (ELISA) in the detection of channel catfish, *Ictalurus punctatus* (Rafinesque), antibodies to *Edwardsiella ictaluri. Journal of Fish Diseases* **12,** 87–94.

Waterstrat, P. R., Brazil, J. & Ainsworth, A. J. (1991). Use of an ELISA-based assay for the detection of antibody-secreting cells in channel catfish, *Ictalurus punctatus* (Rafinesque). *Journal of Fish Diseases* **14,** 669–675.

Whyte, S. K., Chappell, L. H. & Secombes, C. J. (1990). Protection of rainbow trout, *Oncorhynchus mykiss* (Richardson), against *Diplostomum spathaceum* (Digenea): the role of specific antibody and activated macrophages. *Journal of Fish Diseases* **13,** 281–291.

Williams, M. A. & Hoole, D. (1992). Antibody response of the fish *Rutilus rutilus* to the metacestode of *Ligula Intestinalis. Diseases of Aquatic Organisms* **12,** 83–89.

II

Subchronic effects of pulp and paper mill effluents on the immunoglobulin synthesis of roach, *Rutilus rutilus*.

by

Jokinen E.I., Aaltonen T.M. & Valtonen E.T.

Ecotoxicology and Environmental Safety 32: 219-225, 1995

https://doi.org/10.1006/eesa.1995.1107

ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 32, 219–225 (1995)

Subchronic Effects of Pulp and Paper Mill Effluents on the Immunoglobulin Synthesis of Roach, *Rutilus rutilus*

E. I. JOKINEN, T. M. AALTONEN, AND E. T. VALTONEN

Department of Biological and Environmental Science, University of Jyväskylä, FIN-40100 Jyväskylä, Finland

Received October 19, 1994

Roach (*Rutilus rutilus*), from a unpolluted lake, were caged in a lake receiving treated waste waters from a pulp and paper mill 16 km upstream, and simultaneously a group of fish were held in cages in the unpolluted reference lake. The level of serum immunoglobulin of the fish kept in cages in these lakes was quantified. Serum immunoglobulin levels decreased when the fish were transferred to the contaminated lake and remained significantly reduced for the 8-week period fish were monitored. In another experiment fish were immunized with bovine γ-globulin (BGG) after 5 weeks caging in the lakes. The kinetics of specific antibody synthesis were determined by assaying the number of antibody secreting cells in the spleen using enzyme-linked immunospot assay and by measuring the concentration of specific antibody in the serum. The peak response of the effluent exposed fish against BGG was lower than that for fish kept in the unpolluted reference lake, and the antibody titers, due to immunization, increased more slowly in the exposed fish. These results demonstrate that subchronic exposure of fish in a lake receiving treated waste waters from a bleached kraft mill affects the antibody-mediated immunity resulting in, besides lower levels of Ig, weak responsiveness against antigen.

INTRODUCTION

The effluents from pulp and paper mills cause wide environmental contamination in countries with chemical wood processing industry, especially when chlorine bleaching is used. The undiluted, treated effluents may be, occasionally, lethally toxic to fish, but when diluted in recipient waters the toxicity is decreased beyond lethality (Walden, 1976; Hutchins, 1979; Oikari *et al.*, 1984). Bleached kraft mill effluent (BKME) is a complex mixture of hundreds of organic and inorganic compounds of a hydrophilic or hydrophobic nature, e.g., organic halogens, and unsoluble particles and fibers (Oikari and Niittylä, 1985; Dahlman *et al.*, 1991; Paasivirta, 1991). The treatment of waste waters in activated sludge plants reduces the levels of fatty acids and resin acids, but the chlorophenols and related substances remain significant components (McLeay, 1987; Paasivirta *et al.*, 1988). A variety of environmental factors have been demonstrated to affect the immune system and maybe coincident with, for example, infectious diseases (Snieszko, 1974; Brown *et al.*, 1979; O'Neil, 1981). There are extensive toxicological studies concerning with the effect of metals (Cossarini-Dunier *et al.*, 1988; Ghanmi *et al.*, 1989; Thuvander, 1989; Carballo and Munoz, 1991; Khangarot and Tripathi, 1991; Saxena *et al.*, 1992), various pesticides, and other organic chemicals (Bennett and Wolke, 1987; Plumb and Areechon, 1990; Dunier and Siwicki, 1993; Tahir *et al.*, 1993) on the immunity of fish. An altered white blood cell picture in perch (*Perca fluviatilis*) due to exposure to pulp mill effluent has been reported (Andersson *et al.*, 1988), but more detailed studies of the effects of BKME on the immune system of fish are missing.

Several studies indicate that BKME affects fish physiology, pathology, biochemical measures, and reproduction (Owens, 1991; Södergren, 1993). Typical symptoms are liver enlargement, induction of liver EROD (ethoxyresorufin-*O*-deethylase) activity, altered carbohydrate metabolism, disturbed plasma ion balance, and stimulated red blood cell production. The effluent also affects growth (Webb and Brett, 1972; Stoner and Livingston, 1978; Södergren, 1989; Förlin *et al.*, 1991; Owens, 1991). BKME is reported to have effects not only on individual fish but also other levels of biological organization, population, and communities (Adams *et al.*, 1992).

The present study was designed to determine the effects of BKME on the antibody-mediated immunity of roach (*Rutilus rutilus*) subchronically exposed to BKME-related contaminants in a polluted lake.

MATERIALS AND METHODS

Fish and Exposure to Pollutants

The study area is presented in Fig. 1. Roach were caught in the middle of May, promptly after the lake had been released from ice cover, from Lake Peurunka, an oligotrophic lake near a natural state. Lake Peurunka receives no industrial or municipal effluents. The average length and weight of fish was 168 mm and 32.5 g and their age varied from 3 to 5 years. After capture ca. 100 roach were allowed to acclimatize in cages for 3 weeks in Lake Peurunka. After the period of adaptation 50 fish were transferred to Lake Vatia to be exposed to BKME for 5 weeks. Lake Vatia receives secondary-treated waste waters from a pulp and paper mill situated 16 km upstream from the cages (see Fig. 1). The mill used pine (60%) and birch (40%)

0147-6513/95 $12.00

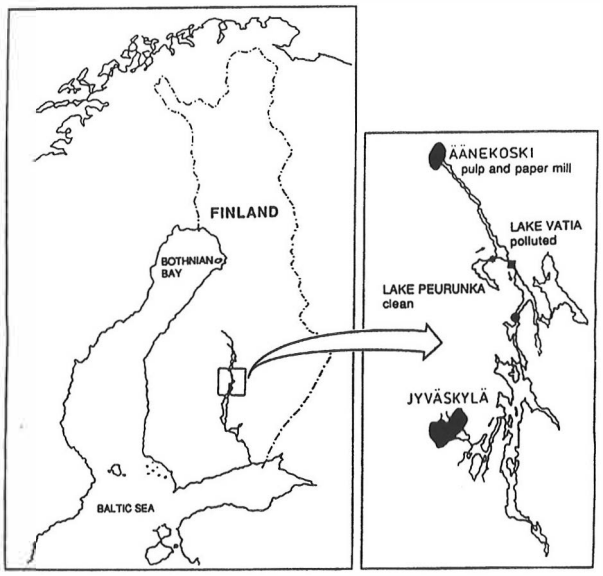


FIG. 1. The field experimental area. Lake Peurunka receives no industrial or municipal effluents and served as a reference caging site. The roach used in the study were captured from Lake Peurunka. Lake Vatia receives secondary-treated effluents from a pulp and paper mill 16 km upstream from the caging site.

as raw material and produced 399,700 tons of bleached pulp and 172,900 tons of paper in the year 1990. The bleaching sequence at the time was for pine C/DE(o)DED (C: 75%, D: 25%) and for birch DE(o)DED (where C stands for chlorine, D for chlorine dioxide, E for alkaline extraction, and o for oxygen). The waste water (72,400 m^3/day) was treated in an activated sludge plant. Another 50 fish, used as controls, were caged in Lake Peurunka. Water quality parameters of the two lakes are given in Table 1. The fish in both lakes were fed daily with commercial pelleted dry food (Finnewos Co., Finland). Water temperature was monitored regularly and the position of the cages was changed in order to keep the fish in both lakes at the same temperature (±1°C). The temperature of the water was 14°C when the exposure was started in June, gradually rising to 18°C at the end of July.

Immunization

In order to study the capability to respond to foreign antigens the fish were immunized, after an exposure period of 5 weeks, with a single intraperitoneal injection of bovine γ-globulin (BGG, Sigma Chemical Co., St. Louis) emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Each fish received 200 μl of antigen solution containing 500 μg BGG. Control fish kept in the clean lake were treated similarly. The immunized fish were kept in the cages until responses were determined. The kinetics of immune response to BGG was followed by examining 10 fish 14, 21, and 28 days after immunization. A group of fish were kept in both lakes as unimmunized controls.

TABLE 1

The Water Quality in the Lakes of the Experimental Area and the Effluent Load at the Time of Caging in June–July 1990

Parameter	Lake Peurunka (reference)[a]	Lake Vatia (contaminated)[b,c]	Waste water[b,d]
pH	7.0	6.9	
Conductivity (mS/m)	4.9	8.3	
Color (mg Pt/liter)	20	65	
Alkalinity (mmol/liter)	0.19	0.21	
Chlorophenols (ng/liter)		890	
AOX (μg/liter)		420	1.67 t/day
COD_{Mn} (mg/liter)	6.1	10.4	45.1 t/day
BOD_7 (mg/liter)		1.6	2.8 t/day
Total phosphorus (mg/liter)	0.012	0.045	84 kg/day
Total nitrogen (mg/liter)	0.43	0.38	534 kg/day
Suspended solids (mg/liter)	1.3	4.5	8.5 t/day

[a] Data from Finnish Game and Fisheries Research Institute, Laukaa State Aquaculture Research Institute, Finland.
[b] Priha and Paavilainen, 1991.
[c] Granberg et al., 1994.
[d] Granberg, 1992.

Quantification of Serum Immunoglobulin

The blood was collected from the caudal vein of each fish for serum separation. The amount of total IgM and specific anti-BGG antibody in roach serum was measured by enzyme-linked immunosorbent assay. Antibody against purified roach IgM was raised in rabbits and prepared as described earlier (Aaltonen *et al.,* 1994). The specificity of antibody was tested with immunoelectrophoresis of roach serum. Only a single precipitation arch appeared when reacted with rabbit anti-roach IgM antibody. First, the plates were coated either with BGG, for the determination of specific antibodies, or with rabbit anti-roach immunoglobulin, for total immunoglobulin concentration. After masking the plate with bovine serum albumin (BSA, Sigma Chemical Co.) diluted samples of sera were incubated in the wells. The trapped roach IgM was detected with biotin-conjugated anti-roach Ig. Next, alkaline phosphatase-conjugated avidin (Biomakor, Rehovot, Israel) was added. Washing was performed between each step with phosphate-buffered saline–Tween 20 (0.05%), pH 7.4 (PBS-Tween). *p*-Nitrophenylphosphate (Sigma Chemical Co.) was used as a substrate and the optical density was read with a Titertek plate reader (Flow laboratories) at 405 nm. In case of specific antibody, a calibration curve was constructed using a pool of high titer sera obtained from another experiment in which fish were immunized with several injections of BGG. The concentration of anti-BGG of the pool serum was given 1000 artificial units per milliliter (U/ml). The concentrations in the samples were then expressed as U/ml. The assay of total serum immunoglobulin was standardized with known concentrations of purified roach IgM. Some sera were analyzed also with single radial immunodiffusion in order to confirm the concentration of IgM obtained with ELISA. These two assays were found to give identical results.

Enumeration of Secreting Lymphocytes with ELISPOT Assay

The enzyme-linked immunospot (ELISPOT) assay (Czerkinsky *et al.,* 1983; Sedgwik and Holt, 1983) was used for enumeration antigen (BGG) specific antibody-secreting cells (ASC) in the spleen. The ELISPOT assay for roach has previously been described in detail (Aaltonen *et al.,* 1994). The spleen of each roach was removed and disrupted against a nylon mesh. Cells were suspended in RPMI 1640 cell culture medium (Gibco Co., Paisley, UK) osmolarity adjusted by adding 20% water and supplemented with 3% of serum substitute (Ultroser G, Gibco Co.). Cells were separated on stepwise Percoll (Pharmacia, Uppsala, Sweden) density gradient and lymphocytes in the interphase between densities 1.050 and 1.070 g/ml were collected. Flat-bottomed 96-well microtiter plates (Nunc-Immunoplate Maxisorb) were used. For coating, BGG was incubated in the wells followed by saturation with BSA. Cells were pipetted onto plates, at 10^5, 10^4, and 10^3 cells per well, and allowed to secrete antibodies for 3 hr at 18°C. Trapped antibodies were detected with biotin-conjugated anti-roach Ig followed with alkaline phosphatase-conjugated avidin (Biomakor). Between each step the plates were washed three times with PBS-Tween. The substrate, bromo-chloro-indolyl phosphate (Sigma Chemical Co.) was mixed with warm agarose (Agarose type I, Sigma Chemical Co.) and added to the wells. Finally, spots were counted using a stereo microscope.

Statistical Analysis

An analysis of variance was carried out to compare the results from various groups of fish.

RESULTS

Serum Immunoglobulin

The concentration of immunoglobulin in the serum of wild roach in Lake Peurunka was determined at the time of fish caught for the exposure experiments. The concentration was 15.5 mg ± 6.6 IgM/ml (mean ± SD, $n = 22$). Groups of fish were then transferred to cages in the two lakes, Lake Peurunka (reference lake) and Lake Vatia (BKME), and sera were analyzed again after 5 and 8 weeks. A transient decrease (22%) in the IgM level, revealed only at 5 weeks, was noted in the reference lake. In contrast, a significantly decreased (64%) IgM concentration was found in the exposed group as compared with the unexposed group ($P < 0.01$) at 5 weeks. The IgM levels of the exposed fish remained lower (29%) than that of the fish kept in the reference lake also after 8 weeks of caging (Fig. 2).

Antibody Secreting Cell Response to Immunization

After immunization with BGG, no anti-BGG antibody secreting cells were detected before Day 14 post immunization (p.i.). The peak of ASC response was noted at Day 21 p.i. and

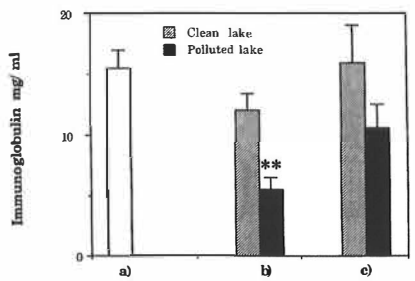


FIG. 2. The immunoglobulin concentration in the serum of roach. (a) Free living fish in the unpolluted reference lake, Lake Peurunka. The sera were collected at the time of capture. The results are shown as the mean + SE, $n = 22$. (b) Nonimmunized fish kept in cages for 5 weeks in Lake Peurunka (shaded bar), $n = 6$, and Lake Vatia (black bar), $n = 6$. $^{**}P < 0.01$ compared to the clean lake. (c) Nonimmunized fish caged for 8 weeks in Lake Peurunka (shaded bar), $n = 14$, and Lake Vatia (black bar), $n = 7$.

at Day 28 the number of secreting cells had decreased to levels found at Day 14 (Fig. 3). The pattern of appearance of ASC was similar in both groups of fish. Importantly, however, the fish exposed in the lake contaminated with BKME responded weakly to immunization. This was reflected as a reduced number of anti-BGG ASC in the spleen at Days 14 and 21 p.i., when compared with the responses to fish held in the reference lake. At the time of peak response (Day 21 p.i.) the mean number of anti-BGG secreting cells of unexposed and exposed fish were 6976 ± 7174 and 2540 ± 3147 (mean ± SD) ASC/10^6 spleen cells, respectively ($P < 0.05$). After the peak in the response, on Day 28 p.i., the number of ASC in the spleen of fish exposed to BKME was not different from that of fish kept in the unpolluted reference lake.

Specific Antibody Response in Immunization in Serum

The concentration antibodies against BGG in the serum had increased considerably at 21 days p.i., and the level of anti-BGG in the sera of unexposed fish remained about at the same level also on Day 28 (Fig. 4). In constrast, at Day 21 p.i. the average level of anti-BGG antibody in fish exposed to BKME contamination was lower than that of the fish kept in the reference lake. However, at Day 28 p.i., the levels of anti-BGG in the fish exposed to contaminants had reached the value of reference fish. The specific serum antibody responses varied considerably between individuals in both groups, reference and exposed fish, but one can say that exposure to effluents does appear to slow down the synthesis of serum antibodies.

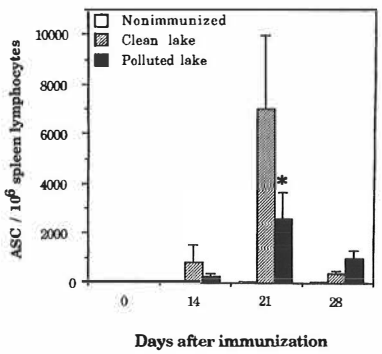


FIG. 3. The kinetics of anti-BGG antibody secreting cell (ASC) response in the spleen of fish held in cages in the unpolluted Lake Peurunka (shaded bar) and the polluted Lake Vatia (black bar). The ASC were assayed at Days 0, 14, 21, and 28 post immunization. Before injection the fish were caged in the unpolluted reference lake or in the BKME-contaminated lake for 5 weeks. The results are expressed as the number of ASC/10^6 spleen lymphocytes. Each bar represents the mean + SE of eight fish. *$P < 0.05$ compared to the clean lake.

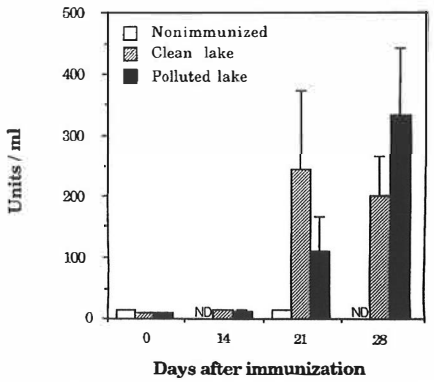


FIG. 4. The development of anti-BGG antibodies in the serum of fish preexposed in cages in the reference and contaminated lake (see Fig. 3). The antibodies were assayed at Days 0, 14, 21, and 28 post injection. The results are expressed as the mean concentration of anti-BGG antibody Units/ml serum + SE, $n = 8$ at each point in time. ND, not determined.

DISCUSSION

The impact of pollutants on fish can be studied using fish caught from contaminated areas. These studies suffer from certain difficulties concerning length of exposure and dose. Further, fish living in polluted areas may not be representative to the species studied because only those able to adapt to polluted environment are selected. When fish are exposed to pollutants in aquarium the exposure time and concentration are easily controlled but in laboratory experiments many components of a polluted ecosystem are missing, for example, the influence of sedimenting of particles and soluble compounds which may modify the bioavailability of impacting pollutants. Here an on-site type of exposure was chosen because of close resemblance to exposure of natural populations present in polluted ecosystem.

In this study roach were exposed to BKME from a mill using chlorine/chlorine dioxide bleaching by keeping them in cages in a polluted lake for 8 weeks. In the polluted lake the level of IgM decreased distinctly well below normal and did not return to reference values during the experiment. This suggests that the contaminants in the lake receiving effluents from the pulp and paper mill have impairing effects on the circulating level of serum antibodies. A recent study on the effect of BKME on white fish demonstrated similar decreased serum contents of IgM as in the present study (Soimasuo *et al.*, 1994).

Further evidence on the immunotoxic effect of BKME was obtained in experiments during which exposed fish were immunized. The assay of the number of ASC in the spleen revealed that the effluent suppressed the responsiveness of the immune system of fish: the maximum number of ASC was

significantly lower in the spleen of exposed roach as compared to those kept in the unpolluted lake. In addition, the antibodies against BGG in the scrum raised more slowly in the exposed fish compared to the unexposed fish, although the concentration finally reached the same level.

The specific mechanisms involved in impaired immune responses due to exposure to BKME remains open in the present study. Exposure of fish to aquatic contaminants has resulted in contradictory results as both immune suppressive and stimulatory effects have been noted (Dunier and Siwicki, 1993; Tahir *et al.,* 1993). Complex regulation of immunological events and insufficient knowledge on immunological mechanisms in fish make the interpretation of the results difficult. A suppressive impact of pollutants, as reported here, may be a consequence of the stress that pollutants exert on fish and compromise the function of their defense mechanisms (Anderson, 1990; Bucke, 1991). Stress contributes lymphocyte functions in an indirect way by increasing serum cortisol which may depress cytokine-mediated functions (Tripp *et al.,* 1987) and change the affinity and number of leukocyte receptors (Maule and Schreck, 1991). Stress in mammals is known to also affect production of growth hormone, gonadotrophins, and thyroid hormones (Khansari *et al.,* 1990). On the other hand, direct toxic effects on lymphoid cells or on the generation and maturation of cells in lymphoid organs of fish are potential candidates to explain the suppressive effects of the effluent.

In another study, roach caught from the polluted Lake Vatia demonstrated lower numbers of lymphocytes and higher numbers of granulocytes than fish from the unpolluted Lake Peurunka (Jeney *et al.,* 1995). Furthermore, decreased numbers of blood lymphocytes and other abnormalities in blood white cell counts of perch living in water polluted by effluents from a pulp mill has been reported in an earlier study (Andersson *et al.,* 1988). The changes in hematological counts may reflect more profound effect of BKME on lymphocyte production and maturation, being in parallel to the finding on decreased immunoglobulin synthesis. All this gives support to the concept of suppressed lymphocyte-related functions due to chlorine-based bleaching process. Roach is a fish species rather well tolerating BKME and in other, more sensitive species the disturbance of responses may be even more serious.

Despite extensive studies, the substances in BKME responsible for the effects on physiology and the effects on immune system of fish are still not well known. Concerning physiological parameters, resin acids, fatty acids, and organic chlorine compounds are suspected to be the major harmful constituents (Oikari and Niittylä, 1985; Lehtinen *et al.,* 1990; Dahlman *et al.,* 1991). However, also nonchlorinated compounds of BKME are reported to be toxic (Oikari and Nakari, 1982). As one approach, the fractionation of effluent and further screening *in vitro* is required to determine which are the effective compounds and what are the mechanisms of their action on lymphoid cells. This will not be easy, because although more than 200 different compounds have been found in BKME, its composition in not fully known yet.

One of the main aims of immunotoxicology is to study relationship between increased susceptibility to disease related to xenobiotics. The causal relationship between a pollutant and biological responses is seldom clear, and whether or not the lowered immune competence due to BKME affects the health of fish cannot be definitely determined on the basis of this study. The present work implies, however, that compounds present in secondary-treated BKME, at concentrations found in the recipient lake, are injurious to the immune system of fish and have the potential to increase the susceptibility to, for instance, microbial diseases and parasite infections. Indeed, in the present study area a ciliate protozoan, *Ichthyophthirius multifiliis,* has a significantly increased prevalence on roach in the polluted lake as compared to unpolluted lake (Valtonen and Koskivaara, 1994). Furthermore, in the two eutrophic lakes downstream of the polluted Lake Vatia, a mass infection of a bucephalid digenean, *Rhipidocotyle fennica,* in the fins of roach in sustained populations has been reported (Valtonen and Koskivaara, 1989). In both cases the changes are interpreted as being due to the impaired immunity of fish.

In addition to antibodies, the resistance of fish to pathogens includes other protective mechanisms, such as humoral agents, cell-mediated immunity, and phagocytes. Studies on these parts of immunity are also needed to evaluate the integrity of immune system and the immunological status of fish. Experiments focused on immunomodulatory effects of BKME on nonspecific parameters of immunity are underway in this laboratory.

CONCLUSIONS

Roach were exposed subchronically (total time 8 weeks) in cages in a lake polluted by treated effluents from a pulp and paper mill using chlorine bleaching. The decreased serum IgM concentration and poor responsiveness to immunization were found in fish exposed to BKME. The changes in the immune system may be reflected as increased parasite infections of fish as supported by the ecological and parasitological data from the contaminated area studied.

ACKNOWLEDGMENTS

This study was supported by the Board of Environmental Science of Academy of Finland. Professor A. Oikari is acknowledged for critical reading of the manuscript and Dr. D. I. Gibson for checking the language of the manuscript. The authors thank fishermen Mr. O. Järvinen and Mr. M. Tappinen for their help.

REFERENCES

Aaltonen, T. M., Jokinen, E. I., and Valtonen, E. T. (1994). Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. *Fish Shellfish Immunol.* **4,** 129–140.

Adams, S. M., Crumby, W. D., Greeley, M. S., Shugart, L. R., and Saylor,

C. F. (1992). Responses of fish populations and communities to pulp mill effluents: A holistic assessment. *Ecotoxicol. Environm. Safety* **24,** 347–360.

Anderson, D. P. (1990). Immunological indicators: Effects of environmental stress on immune protection and disease outbreaks. *Am. Fish. Soc. Symp.* **8,** 38–50.

Andersson, T., Förling, L., Härding, J., and Larsson, Å. (1988). Physiological disturbances in fish living in coastal waters polluted with bleached kraft pulp mill effluents. *Can. J. Fish Aquat. Sci.* **45,** 1525–1536.

Bennett, R. O., and Wolke, R. E. (1987). The effect of sublethal endrin exposure on rainbow trout, *Salmo gairdneri* Richardson. I. Evaluation of serum cortisol consentrations and immune responsiveness. *J. Fish Biol.* **31,** 375–385.

Brown, E. R., Koch, E., Sinclair, T. F., Spitzer, R., and Callaghan, O. (1979). Water pollution and diseases in fish (An epizootiologic survey). *J. Env. Path. Toxicol.* **2,** 917–925.

Bucke, D. (1991). Current approaches to the study of pollution-related diseases in fish. *Bull. Eur. Ass. Fish. Pathol.* **11,** 46–51.

Carballo, M. and Munoz, J. (1991). Effect of sublethal concentration of four chemicals on susceptibility of juvenile rainbow trout (*Oncorhynchus mykiss*) to saprolegniosis. *Appl. Environm. Microbiol.* **57,** 1813–1816.

Cossarini-Dunier, M., Demael, A., Lepot, D., and Guerin, V. (1988). Effects of manganese ions on the immune response of carp (*Cyprinus carpio*) against *Yersinia ruckeri. Dev. Comp. Immunol.* **12,** 573–579.

Czerkinsky, C. C., Nilsson, L.-Å., Nygren, H., Ouchterlony, Ö., and Tarkowsky, A. (1983). A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J. Immunol. Methods* **65,** 109–121.

Dahlman, O., Mörck, R., Johansson, L., and De Sousa, F. (1991). Chemical composition of modern bleach kraft mill effluents. In *Environmental Fate and Effects of Bleached Pulp Mill Effluents* (A. Södergren, Ed.), pp. 47–56. Swedish Environmental Agency, Stockholm.

Dunier, M., and Siwicki, A. (1993). Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: A review. *Fish Shellfish Immunol.* **3,** 423–438.

Förlin, L., Andersson, T., Balk. L., and Larsson, Å. (1991). Biochemical and physiological effects of pulp mill effluents on fish. In *Environmental Fate and Effects of Bleached Pulp Mill Effluents* (A. Södergren, Ed.), pp. 235–243. Swedish Environmental Agency, Stockholm.

Ghanmi, Z., Rouabhia, M., Othmane, O., and Deschaux, P. A. (1989). Effects of metal ions cyprinid fish immune response: *In Vitro* effects of Zn^{2+} and Mn^{2+} on the mitogenic response of carp pronephros lymphocytes. *Ecotoxicol. Environm. Safety* **17,** 183–189.

Granberg, K. (1992). *Äänekosken reitin velvoitetarkkailu 1991. Fysikaalis kemialliset tutkimukset ja kasviplankton.* Institute for Environmental Research, University of Jyväskylä, Jyväskylä, Finland.

Granberg, K., Palomäki, A., Hynynen, J., Meriläinen, J., and Salo. H. (1994). *Äänekoski-Vaajakoski -vesireitin velvoitetarkkailu vuonna 1993.* Institute for Environmental Research, University of Jyväskylä, Jyväskylä, Finland.

Hutchins, F. E. (1979). Toxicity of pulp and paper mill effluent: A literature review. In *Report of Corvallis Environmental Research Laboratory,* pp. 1–43. Office of Research and Development, U.S. Environmental Protection Agency.

Jeney, Z., Valtonen, E. T., Jeney, G., and Jokinen, I. (1995). Sublethal effects of pulp and paper mill effluent (BKME) on physiological and biochemical parameters of roach (*Rutilus rutilus* L.). Submitted for publication

Khangarot. B. S., and Tripathi, D. M. (1991). Changes in humoral and cell-mediated immune response and in skin and respiratory surfaces of catfish, *Saccoobranchus fossilis,* following copper exposure. *Ecotoxicol. Environm. Safety* **22,** 291–308.

Khansari, D. N., Murgo, A. J., and Faith, R. E. (1990). Effects of stress on the immune system. *Immunol. Today* **11,** 170–175.

Lehtinen, K.-J., Kierkegaard, A., Jakobsson, E., and Wändell, A. (1990). Physiological effects in fish exposed to effluents from mills with six different bleaching processes. *Ecotoxicol. Environm. Safety* **19,** 33–46.

Maule, A. G., and Schreck, C. B. (1991). Stress and cortisol treatment changed affinity and number of glucocorticoid receptors in leukocytes and gill of Coho Salmon. *Gen. Comp. Endocrinol.* **84,** 83–93.

McLeay, D. J. (1987). Aquatic toxicity of pulp and paper mill effluents: a review. *Environm. Can. Reports EPS 4 /pf/1.* 191.

O'Neil, J. G. (1981). The humoral immune response of *Salmo trutta* L. and *Cyprinus carpio* L. exposed to heavy metals. *J. Fish. Biol.* **19,** 297–306.

Oikari, A., and Nakari, T. (1982). Kraft pulp mill effluents cause liver dysfunction in trout. *Bull. Environm. Contam. Toxicol.* **28,** 266–270.

Oikari, A., Nakari, T., and Holmbom, B. (1984). Sublethal actions of simulated kraft mill effluent (KME) in *Salmo gairdneri* Richardson, in fresh water. *Water Res.* **17,** 81–89.

Oikari, A., and Niittylä, J. (1985). Subacute physiological effects of bleached kraft mill effluent (BKME) on the liver of trout, *Salmo gaidneriT. Ecotoxicol. Environm. Safety* **10,** 159–172.

Owens, J. W. (1991). The hazard assessment of pulp and paper effluents in the aquatic environment: A review. *Environm. Toxicol. Chem.* **10,** 1511–1540.

Paasivirta, J. (1991). Organochlorine compounds in pulp mill effluents and in sediments. In *Environmental Fate and Effects of Bleached Pulp Mill Effluents* (A. Södergren, Ed.), pp. 168–179. Swedish Environmental Protection Agency, Stockholm.

Paasivirta, J., Knuutinen, J., Knuuttila, M., Maatela, P., Pastinen, O., Virkki, L., Paukku, R., and Herve, S. (1988). Lignin and organic chlorine compounds in lake water and the role of the chlorobleaching effluents. *Chemosphere* **17,** 147–158.

Plumb, J. A., and Areechon, N. (1990). Effect of malathion on humoral immune response of channel catfish. *Dev. Comp. Immunol.* **14,** 355–358.

Priha, M., and Paavilainen, K. (1991). *Äänekoski-Vaajakoski -vesialueen velvoitetarkkailu vuonna 1990.* Oy Keskulaboratorio, Espoo, Finland.

Saxena, M. P., Gopal, K., Jones, W., and Ray, P. K. (1992). Immune responses to *Aeromonas hydrophila* in Cat fish (*Heteropneustis fossilis*) exposed to cadmium and hexachlorocyclohexane. *Bull. Environm. Contam. Toxicol.* **48,** 194–201.

Sedgwik, J. D. and Holt, P. G. (1983). A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J. Immunol. Methods* **57,** 301–309.

Snieszko, S. F. (1974). The effects of environmental stress on outbreaks of infectious disease of fishes. *J. Fish. Biol.* **6,** 374–383.

Soimasuo, R., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T., and Oikari, A. (1994). Biomarker responses in a pollution gradient: Effects of pulp and paper mill effluents on whitefish studied by caging technique. *Aquat. Toxicol.,* **31,** 329–345.

Stoner, A. W., and Livingston, R. J. (1978). Respiration, growth and food conversion efficiency of pinfish *Lagodon rhomboides* exposed to sublethal concentrations of bleached kraft mill effluent. *Environ. Pollut.* **17,** 207–217.

Södergren, A. (1989). *Biological Effects of Bleached Kraft Mill Effluents.* Final report from the Environment/Cellulose Project I. Swedish National Environmental Protection Board, Solna.

Södergren, A. (1993). *Bleached Pulp Mill Effluents. Composition, Fate and Effects in the Baltic Sea.* Final report from the Environment/Cellulose Project II. Swedish National Environmental Protection Board, Solna.

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

Thuvander, A. (1989). Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: Effects on immune functions. *J. Fish. Biol.* **35,** 521–529.

Tripp, R. A., Maule, A. G., Schreck, C. B., and Kaattari, S. L. (1987). Cortisol mediated suppression of salmonid lymphocyte responses *in vitro*. *Dev. Comp. Immunol.* **11,** 565–576.

Valtonen, E. T., and Koskivaara, M. (1989). Effects of effluents from pulp and paper mill on parasites of the roach in Central Finland. In *Parasites of Freshwaterfishes of North-West Europe* (O. N. Bauer, Ed.), pp. 163–168. Petrozavodsk.

Valtonen, E. T., and Koskivaara, M. (1994). Relationships between the parasites of some wild and cultured fishes in the two lakes and a fish farm in Central Finland. *Int. J. Parasitol.* **24,** 109–118.

Walden, C. C. (1976). The toxicity of pulp and paper mill effluents and corresponding measurement procedures. *Water Res.* **10,** 639–664.

Webb, D. M., and Brett, J. R. (1972). The effects of sublethal concentrations of whole bleached kraft mill effluents on the growth and food conversion efficiency of underyearling sockey salmon *Oncorhyncus nerka*. *J. Fish. Res. Board Can.* **30,** 499–507.

III

Immunoreactivity of roach, *Rutilus rutilus*, following laboratory exposure to bleached pulp and paper mill effluents.

by

Aaltonen, T.M., Valtonen, E.T. & Jokinen, E.I.

Ecotoxicology and Environmental Safety 38: 266-271, 1997

https://doi.org/10.1006/eesa.1997.1588

ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 38, 266–271 (1997)
ARTICLE NO. ES971588

Immunoreactivity of Roach, *Rutilus rutilus,* Following Laboratory Exposure to Bleached Pulp and Paper Mill Effluents

T. M. Aaltonen,[1] E. T. Valtonen, and E. I. Jokinen

Department of Biological and Environmental Science, University of Jyväskylä, FIN-40100 Jyväskylä, Finland

Received May 15, 1997

In order to study immunomodulation, controlled laboratory experiments were carried out with roach (*Rutilus rutilus*) exposed to bleached kraft mill effluent (BKME) or spent bleach liquor (SBL) from two pulp and paper mills using elementary chlorine and chlorine dioxide for bleaching. The total number of immunoglobulin-secreting cells (ISC) and the number of specific antibody-secreting cells (ASC) were determined by ELISPOT assay. Exposure to BKME resulted in decreased numbers of ISC in the spleen. To study the capability of response against foreign antigens the fish were immunized with bovine γ-globulin. Exposure to BKME or SBL reduced antigen-specific ASC response in fish. Moreover, the suppression of ASC response did not require exposure prior to immunization. Depuration of fish in clean water reveals that the immunosuppression caused by BKME is reversible. The ASC response in the BKME-pretreated fish returned to the same or even a higher level compared to untreated immunized fish and, in addition, the number of ISC increased greatly. The results of this study verify an earlier finding on poor antibody response due to exposure in a lake contaminated with BKME. Exposure to SBL alone suppresses antibody-mediated immunity, suggesting that compounds formed in bleaching are at least partly responsible for immunotoxic effects. © 1997 Academic Press

Key Words: **bleached kraft mill effluents; pollution; immunoglobulin synthesis; ELISPOT assay.**

INTRODUCTION

A wide range of environmental contaminants are potentially immunotoxic to fish. Immunological effects of single defined chemicals, for example, heavy metals (Zelikoff, 1993; Voccia *et al.,* 1994), organochlorine pesticides (Spitsbergen *et al.,* 1986; Bennett and Wolke, 1987), and polychlorinated biphenyls (Thuvander and Carlstein, 1991; Thuvander *et al.,* 1993), have been studied. However, only a few studies have been carried out on fish exposed to complex mixtures of chemicals such as polluted sediments and sludges (Secombes *et al.,* 1991, 1995) or bleached kraft mill effluents (BKME) (Jokinen *et al.,* 1995). In countries with a wood-processing industry, pulp mill effluents are one of the major contaminants in lakes. BKME is a complex mixture of extractives, organic acids, a range of sulfur-containing compounds, chlorinated phenolics, neutral compounds, and organic acids (see Södergren, 1993).

Several physiological and biochemical studies, but only a few immunological studies, have been published on the effect of BKME on fish. The altered white blood cell picture in perch (*Perca fluviatilis*) due to exposure to pulp mill effluent has been reported (Andersson *et al.,* 1988). In a previous study the authors reported that subchronic exposure of fish in a lake receiving treated waste waters from a pulp and paper mill affects the antibody-mediated immunity of roach (*Rutilus rutilus*) resulting in weak responsiveness against antigen, in addition to lower levels of IgM in serum (Jokinen *et al.,* 1995). Depressed concentration of plasma IgM has been reported also in whitefish (*Coregonus lavaretus* L.) exposed to BKME (Soimasuo *et al.,* 1995a,b).

Even the on-site type of exposure resembling natural exposure suffers from a lack of properly controlled conditions. The present study evaluates, in aquarium circumstances, the effect of BKME and spent bleach liquor (SBL) on the antibody response of roach. The results confirm previous findings of impaired immunoreactivity and, in addition, describe the recovery of immune functions due to depuration of fish after returning to clean water.

MATERIALS AND METHODS

Fish

Roach (*R. rutilus*) were caught early in June 1991 for experiment I (Exp. I) and in July 1993 for experiment II (Exp. II) from Lake Peurunka, an uncontaminated oligotrophic lake in Central Finland. After the fish had been caught, ectoparasites were killed by administration of a commercial drug, Ichide (N.T. Laboratories Ltd., England), at 10 μl/liter. The average length and weight of the fish were 147 mm (SD = 16) and 23 g (SD = 11), and 158 mm (SD = 22) and 34 g (SD = 16) in Exp. I and Exp. II, respectively. During the experiments the fish were kept in aquaria with a volume of 300 liters in aerated tap water at a constant temperature of 17.5 ± 0.5°C and fed daily with commercial pelleted dry food (FinnEwos Aqua Co., Finland). Half of the water was changed weekly. A photoperiod of 12 h light (300–500 lux, Delta OHM, the photometric

[1] To whom correspondence should be addressed at Department of Biological and Environmental Science, University of Jyväskylä, Vapaudenkatu 4, FIN-40100 Finland. Fax: +358 14 602 221. E-mail: turniaa@jyu.fi.

0147-6513/97 $25.00

probe HD 9221/S1, Italy) and 12 h dark was used. The experiments were performed between July and August (Exp. I) and between August and November (Exp. II).

Experiment I

Roach (n = 80) were exposed to 3.5% BKME, from the same mill studied earlier (Jokinen *et al.,* 1995). At the time of the study (1991) elementary chlorine and chlorine dioxide were used for the bleaching of softwood pulp, the main product of the mill (Table I). Activated sludge-treated waste water from the softwood process was stored frozen until used. After 3 weeks exposure the fish were immunized. At the time of immunization 40 of the exposed roach were transferred to clean water (preexposed group) and the remaining 40 roach were kept in BKME (exposed group). Control roach (n = 40) were also immunized and kept the whole time in tap water. The fish were kept in the aquaria until sampled on days 0, 14, 21, and 28 postimmunization (p.i.).

Experiment II

The untreated SBL came from the elementary chlorine/chlorine dioxide bleaching sequence of the softwood process (Table 1) of a mill situated on the western coast of Finland. Alkaline and acid liquors were sampled separately straight from the bleaching process, stored cold, and then mixed together in the ratio in which they were produced at the mill. The pH of mixed effluent was adjusted to neutral with NaOH and the mixture was stored frozen until used. A total of 100 roach were immunized after adaptation to laboratory conditions for 1 month. After immunization 50 fish were kept in clean water (control group) and the remaining 50 fish were exposed to 25% SBL (exposed group) until sampled 0, 7, 14, 21, 28, and 35 days p.i.

TABLE 1

The Production of Cellulose and Paper, Pulp Bleaching Sequence, and the Effluent Discharge of the Two Mills Studied

	Mill 1 (Exp. I)	Mill 2 (Exp. II)
Production		
Cellulose bleached (t/a)		
Softwood	247,000	242,000
Hardwood	151,000	200,000
Cellulose unbleached (t/a)		
Paper (t/a)	176,000	138,400
Bleaching sequence		
Softwood process	C/D Eo(p) D E(p) D	D/C Eo D E D
Hardwood	C/D Eo(p) D E(p) D	O D Eo D E D
Effluent		
Solids (t/a)	2,246	1,136
BOD_7 (t/a)	1,024	1,643
COD_7 (t/a)	15,251	26,739
P (t/a)	26	27
N (t/a)	168	188
AOX (kg/tc)	1.3	1.2

Note. C, elementary chlorine; D, chlorine dioxide; E, alkaline extraction; Eo, alkaline extraction with oxygen treatment; E(p), alkaline extraction with peroxide treatment; Eo(p), alkaline extraction with oxygen and peroxide treatment; O, oxygen.

Immunization of Fish

In order to study the capability of roach to respond to foreign antigens the fish in both experiments were immunized with a single intraperitoneal injection of bovine γ-globulin (BGG, Sigma Chemical Co., St. Louis, MO) emulsified in Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, MI). Each fish received 200 μl of antigen solution containing 500 μg BGG.

Enumeration of Secreting Lymphocytes with ELISPOT Assay

The enzyme-linked immunospot (ELISPOT) assay (Czerkinsky *et al.,* 1983; Sedgwik and Holt, 1983) was used for the enumeration of total immunoglobulin-secreting cells (ISC) and antigen (BGG)-specific antibody-secreting cells (ASC) in the spleen (Exp. I and Exp. II.) and the head kidney (Exp. II). The ELISPOT assay for roach has been described in detail previously (Aaltonen *et al.,* 1994). The spleen and kidney of each roach were removed and disrupted separately against a nylon mesh. Cells were suspended in modified RPMI 1640 cell culture medium (Gibco Co., Paisley, UK) supplemented with 3% serum substitute (Ultroser G, Gibco Co.). Cells were separated on a stepwise Percoll (Pharmacia, Uppsala, Sweden) density gradient and lymphocytes at the 1.050–1.070 g/ml interphase were collected. Flat-bottomed 96-well microtiter plates (Nunc-Immunoplate, Maxi Sorp) were coated either with BGG, for the determination of specific ASC, or with rabbit anti-roach immunoglobulin antibody, for total ISC. Cells (at 10^5, 10^4, and 10^3 per well for ASC, and 10^3 and 10^2 per well for ISC) were pipetted into wells after saturating the plate with 1% BSA and allowed to secrete antibodies for 3 h at 18°C. Trapped antibodies were detected with biotin-conjugated anti-roach IgM antibody followed by alkaline phosphatase-conjugated avidin (Biomakor, Rehovot, Israel). Between each step the plates were washed three times with PBS–Tween. The substrate, bromo-chloro-indolyl phosphate (Sigma Chemical Co.), was mixed with warm agarose (Agarose type I, Sigma Chemical Co.) and added to the wells. Finally, blue spots were counted using a stereo microscope.

Statistical Analysis

The data were analyzed for statistically significant differences by the Mann–Whitney U test.

RESULTS

Immunoglobulin Secretion of Exposed Fish

The total number of ISC in the spleen was studied in Exp. I. The 3-week exposure to BKME before immunization diminished the number of ISC by 53% in exposed fish compared to the controls (Fig. 1, day 0). The number of ISC of control fish remained at the same level during the study except on day 28

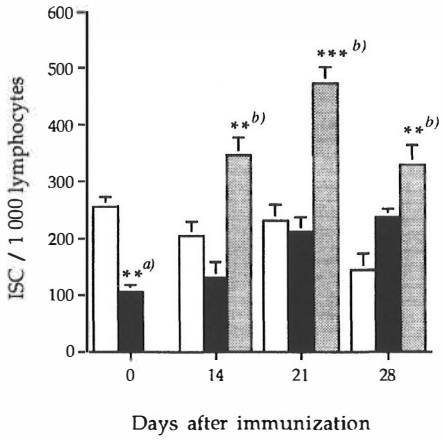


FIG. 1. Total number of immunoglobulin secreting cells (ISC) in the spleen of the fish in the control group (open bar), exposed group (black bar), and preexposed group (shaded bar) in Exp. I. The results are expressed as the number of ISC/1000 splenic lymphocytes. Each bar represents the mean + SE of 10 fish. Statistical significance [a]between exposed and control groups on day 0 and [b]between preexposed and control groups at each time point (**$P < 0.01$, ***$P < 0.001$).

when the number of ISC was smaller than earlier. The number of ISC in the spleen of exposed fish increased gradually after immunization and reached the level of the controls on day 21 p.i. (Fig. 1).

Reactivity against Immunogen Following Exposure to BKME or SBL

The number of antigen-specific ASC in the spleen after immunization with BGG was studied in both Exp. I (Table 2) and Exp. II (Fig. 2A). Only a few ASC were detected on days 7 and 14 p.i., and the peak ASC response occurred on day 21 p.i. The number of anti-BGG ASC was reduced in fish exposed to effluents compared to controls in both experiments and the decrease was most prominent at the time of the peak response. In Exp. I (Table 2) the ASC response in the spleen decreased in exposed fish by 33% and in Exp. II by 57% (Fig. 2A) compared to the controls.

ASC response was studied also in the head kidney in Exp. II (Fig. 2B). The response in the kidney was similar to that in the spleen concerning both the effect of the effluent and the response kinetics. At the time of peak response (day 21 p.i.) the mean numbers of pronephric anti-BGG-secreting lymphocytes of exposed and control fish were 4300 and 6320/10^6 lymphocytes, respectively (32% decrease).

Depuration and the Recovery of Immunoreactivity

A group of exposed fish in Exp. I was transferred to clean water at the time of immunization (preexposed fish). The number of splenic ISC in preexposed fish increased markedly compared to fish exposed to BKME or to the controls (Fig. 1), and the increase was found at every sampling. Higher numbers of anti-BGG ASC were found in the preexposed fish than in fish exposed also after immunization, or even in unexposed control fish (Table 2).

The ratio of ASC/ISC, given in Table 2, was calculated to indicate the proportion of lymphocytes producing antigen-specific antibodies. In the preexposed fish 0.9% of all ISC secreted anti-BGG antibodies, while the percentage was 1.7% in the exposed group and 1.5% in the control group.

DISCUSSION

Enumeration of ASC revealed that laboratory exposure to effluents from pulp and paper mills affects the immunoreactivity of fish. The maximum number of ASC in the spleen of exposed roach was reduced in both experiments and also in the head kidney in Exp. II. The results documented here parallel those of a previous study in a contaminated lake and verify the finding of poor humoral response due to BKME exposure (Jokinen *et al.*, 1995). Studies concerning the influence of pollutants on antibody formation in fish have given contradictory results. Anderson *et al.* (1990) found reduced numbers of an-

TABLE 2
The Antibody Response in the Spleen of Fish in Experiment I

				Antibody secretion in the spleen			
		Treatment		Days p.i.			
Group	*n*	Before immun.	After immun.	0	14	21	28
Control	40	None	None	0 ± 0	366 ± 152	4080 ± 2360	707 ± 400[a]
				0 ± 0	0.3 ± 0.18	1.5 ± 0.88	0.4 ± 0.14[b]
Exposed	40	BKME	BKME	0 ± 0	440 ± 283	2720 ± 700	343 ± 130
				0 ± 0	0.4 ± 0.17	1.7 ± 0.49	0.1 ± 0.04
Preexposed	40	BKME	None	0 ± 0	712 ± 505	4420 ± 2790	758 ± 249
				0 ± 0	0.2 ± 0.11	0.9 ± 0.59	0.3 ± 0.11

[a] ASC/10^6 lymphocytes, mean ± SE.
[b] % ASC/ISC × 100, mean ± SE.

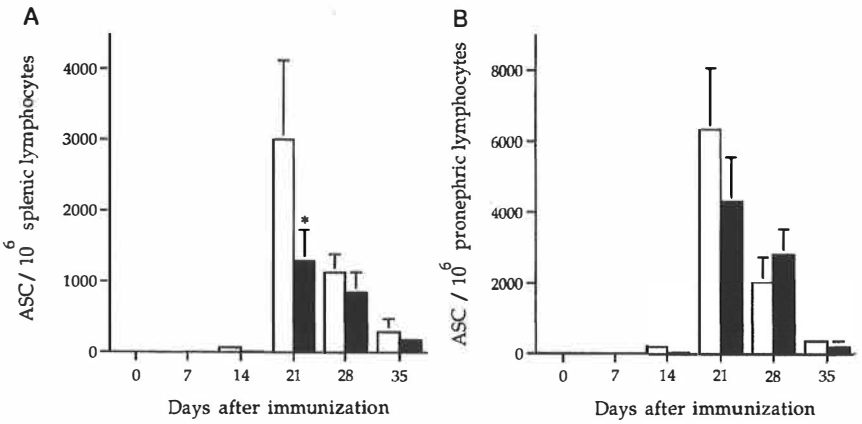


FIG. 2. Anti-BGG antibody secreting cell (ASC) response in Exp. II. Untreated fish were immunized with BGG. The fish were then transferred to clean water (control, open bar) or to 25% of SBL (exposed, black bar) and sampled at 7-day intervals. Each bar represents the mean + SE of 10 fish sampled on days 14, 21, and 28 p.i. and 8 fish on days 0, 7, and 36 p.i. Significantly different from control group ($*P < 0.05$). (A) The number of ASC/10^6 lymphocytes from the spleen. (B) The number of ASC/10^6 lymphocytes from the kidney.

tibody-producing cells in phenol-pretreated rainbow trout in immunization by bath with bacterin antigen. On the other hand, the plaque-forming cell (PFC) response was not significantly different in trout receiving halogenated aromatic hydrocarbons or PCB (Cleland *et al.*, 1988) and an increase in the number of PFC is reported after exposure to dioxin (Spitsbergen *et al.*, 1986). The complexity of the immune system makes it difficult to find an explanation for the inconsistent effects of pollutants in immunoglobulin production. Theoretically, toxicants can have direct effects on lymphocytes and lymphoid organs, or indirect effects mediated via the neuroendocrine system (Dunier and Siwicki, 1993; Sanders, 1996; Vos and van Loveren, 1996).

The effluents in the current experiments came from two mills which both use elemental chlorine and chlorine dioxide for the bleaching of softwood pulp (see Table 1). Similar chemical substances can be assumed to be formed in these basically alike processes of pulping and bleaching. In Exp. I secondary-treated BKME was used which contained waste waters from pulping, bleaching, and debarking, and also from making paper. Spent bleach liquor in Exp. II was a mixture of nontreated acid and alkaline liquors containing high concentrations of compounds formed in bleaching. Noteworthy is the fact that chemicals in SBL alone, with no other effluents from the mill, induced immune suppression, suggesting that compounds responsible for the effects originate from the bleaching process. Of course it cannot be stated that in the making of cellulose bleaching is the only step that produces immunosuppressive compounds.

Further evidence of the harmful effect of BKME on antibody synthesis was seen in the total number of ISC in the spleen. Exposure to BKME for 3 weeks reduced the number of ISC by 53%. Afterwards, postimmunization, the number of ISC increased to the level of the control fish, but no such increase was seen in unexposed fish. The increase of secreting lymphocytes is probably due to the adjuvant used in immunization. General stimulation of the immune system by mycobacteria in Freund's complete adjuvant (Anderson, 1992) can be reflected as an increase of ISC in fish with suppressed immunity.

This study indicates that suppression of antibody-mediated immunity after subchronic exposure to BKME is reversible. When the fish were taken back to clean water after 3 weeks exposure to BKME the ASC response returned to levels of the unexposed controls, or was even higher (Table 2). Alterations in physiological parameters in fish after exposure to BKME are reversible (Södergren, 1993) but there are no previous data concerning the recovery of immunological parameters. During depuration, the recovery of lymphocyte response to Con A and LPS (Faisal *et al.*, 1991) and macrophage functions (Weeks and Warinner, 1984) have been reported after exposure to aromatic hydrocarbons. Interestingly, in the present study after 2 weeks depuration the number of ISC was significantly higher than that in the exposed or control fish (Fig. 1).

The exposure to BKME had no marked effect on the proportion of ASC among ISC on day 21, although the number of ASC was reduced (Table 2). The unchanged proportion indicates that exposure affects both the ASC response and the number of ISC to the same degree and suggests that the pro-

duction of immunoglobulin is suppressed in general. On the other hand, even though there was no difference in the number of ASC, the percentage of ASC in preexposed fish was smaller than that of the controls due to the huge increase in ISC. It is probable that ASC response in control and preexposed fish has reached its maximum due to immunization. An increased number of ISC during depuration may be a consequence of two events: an overcorrective compensatory response after exposure and immunostimulation caused by the adjuvant mediated via stimulated cytokine formation.

On the grounds of this study it is not possible to say whether the afferent limb (antigen presentation by macrophages) or the efferent limb of the immune system (lymphocytes), or both, is harmed. The compromised function of macrophages after exposure to BKME might contribute to poor responsiveness in immunization. Indeed, the exposure to BKME of fish treated with immunostimulants (*Vibrio anguillarum* O-antigen or FCA) or left unstimulated resulted in reduced production of reactive oxygen mediates (authors' unpublished results).

BKME increases the prevalence of fin erosion and causes deformities (see Södergren, 1993). The present study demonstrates that pulp mill effluents, and also spent liquor from chlorine bleaching, affect antibody production and thus have potential to increase susceptibility to diseases. Indeed, an increasing amount of evidence has accumulated linking malignant and infectious diseases with pollutant discharges (Anderson, 1990; Waterman and Kranz, 1992; Zelikoff, 1993). In addition, marked changes in fish parasites and parasite communities in the water system beneath the present mill (mill 1) have been documented and increases of infection are suggested to be due to lowered resistance against parasites (Valtonen and Koskivaara, 1989; Valtonen *et al.,* 1997). New bleaching technologies, including total chlorine-free bleaching using hydrogen peroxide, ozone, and enzymes, are now entering production and will be used on a broad scale in the immediate future. It remains to be seen whether the effects of effluents from the new processes on fish defense system are less harmful, as hoped.

CONCLUSIONS

Roach were exposed in aquaria to BKME or SPL from two mills using elementary chlorine and chlorine dioxide for bleaching. Antibody-mediated immunity was studied after immunization of fish. Exposure to BKME resulted in a reduced number of antigen-specific antibody secreting cells and immunoglobulin secreting cells in the spleen. After exposure to SBL the number of antibody-secreting cells decreased, suggesting that compounds formed in bleaching contribute to immunosuppression. Depuration of fish in clean water after exposure to effluents indicates that the suppression caused by BKME is reversible.

ACKNOWLEDGMENTS

This study was supported by the Board of Environmental Science of the Academy of Finland, by the University of Jyväskylä, and by the Graduate School on Fish Biology and Fisheries in Finland. Leigh Plester is acknowledged for checking the language of the manuscript. The authors thank Mr. O. Järvinen for catching the roaches.

REFERENCES

Aaltonen, T. M., Jokinen, E. I., and Valtonen, E. T. (1994). Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. *Fish Shellfish Immunol.* **4,** 129–140.

Anderson, D. P. (1990). Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. *Am. Fish. Soc. Symp.* **8,** 38–50.

Anderson, D. P. (1992). Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. *Ann. Rev. Fish Dis.* **2,** 281–307.

Anderson, D. P., Dixon, O. W., and van Muiswinkel, W. B. (1990). Reduction in the numbers of antibody-producing cells in rainbow trout, *Oncorhynchus mykiss,* exposed to sublethal doses of phenol before bath immunization. In *Aquatic Toxicology and Risk Assessment* (W. G. Landis and W. H. van der Schalie, Eds.) pp. 331–337. American Society for Testing and Materials, Philadelphia.

Andersson, T., Förling, L., Härdig, J., and Larsson, Å. (1988). Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. *Can. J. Fish Aquat. Sci.* **45,** 1525–1536.

Bennett, R. O., and Wolke, R. E. (1987). The effect of sublethal endrin exposure on rainbow trout, *Salmo gairdneri* Richardson. I. Evaluation of serum cortisol concentrations and immune responsiveness. *J. Fish. Biol.* **31,** 375–385.

Cleland, G. B., McElroy, P. J., and Sonstegard, R. A. (1988). The effect of dietary exposure to Aroclor 1254 and/or mirex in humoral immune expression of rainbow trout (*Salmo gairdneri*). *Aquat. Toxicol.* **2,** 141–146.

Czerkinsky, C. C., Nilsson, L-Å., Nygren, H., Ouchterlony, Ö., and Tarkowsky, A. (1983). A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J. Immunol. Methods* **65,** 109–121.

Dunier, M., and Siwicki, A. K. (1993). Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: A review. *Fish Shellfish Immunol.* **3,** 423–438.

Faisal, M., Marzouk, M. S. M., Smith, C. L., and Huggett, R. J. (1991). Mitogen induced proliferative responses of lymphocytes from spot (*Leiostomus xanthurus*) exposed to polycyclic aromatic hydrocarbon contaminated environments. *Immunopharmacol. Immunotoxicol.* **13,** 311–327.

Jokinen, E. I., Aaltonen, T. M., and Valtonen, E. T. (1995). Subchronic effects of pulp and paper mill effluents on the immunoglobulin synthesis of roach, *Rutilus rutilus. Ecotoxicol. Environm. Saf.* **32,** 219–225.

Sanders, V. M. (1996). Neurotransmitters, neuropeptides, and Immune Function: Implications for Immunotoxicology. In *Experimental Immunotoxicology* (R. J. Smialowicz and M. P. Holsapple, Eds.), pp. 169–185. CRC Press, Boca Raton.

Secombes, C. J., Fletcher, T. C., O'Flynn, F. A., Costello, M. J., Stagg, R., and Houlihan, D. F. (1991). Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. *Comp. Biochem. Physiol.* **100C,** 133–136.

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

Sedgwik, J. D., and Holt, P. G. (1983). A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J. Immunol. Methods* **57,** 301–309.

Soimasuo, R., Aaltonen, T., Nikinmaa, M., Pellinen, J., Ristola, T., and Oikari, A. (1995a). Physiological toxicity of low-chlorine bleached pulp and paper

mill effluent on whitefish (*Coregonus laveretus* L. s.l.): A laboratory exposure simulating lake pollution. *Ecotoxicol. Environm. Saf.* **31,** 228–237.

Soimasuo, R., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T., and Oikari, A. (1995b). Biomarker responses in a pollution gradient: Effect of pulp and paper mill effluents on whitefish studied by caging technique. *Aquat. Toxicol.* **31,** 329–345.

Södergren, A. (1993). In *Bleached pulp mill effluents. Composition, Fate and Effects in the Baltic Sea. Final Report from the Environment/Cellulose Project II.* Swedish National Environmental Protection Board, Solna.

Spitsbergen, J. M., Schat. K. A., Kleeman, J. M., and Peterson, R. E. (1986). Interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with immune response of rainbow trout. *Vet. Immunol. Immunopathol.* **12,** 263–280.

Thuvander, A., and Carlstein, M. (1991). Sublethal exposure of rainbow trout (*Oncorhynchus mykiss*) to polychlorinated biphenyls: Effect on the humoral immune response to *Vibrio anguillarum. Fish Shellfish Immunol.* **1,** 77–86.

Thuvander, A., Wiss, E., and Norrgren, L. (1993). Sublethal exposure of rainbow trout (*Oncorhynchus mykiss*) to Clophen A50: effects on cellular immunity. *Fish Shellfish Immunol.* **3,** 107–117.

Valtonen, E. T., Holmes, J. C., and Koskivaara, M. (1997). Eutrophication, pollution and fragmentation: Effects on parasite communities on roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) in four lakes in central Finland. *Can. J. Fish. Aquat. Sci.* **54,** 572–585.

Valtonen, E. T., and Koskivaara, M. (1989). Effects of effluents from pulp and paper mill on parasites of the roach in Central Finland. In *Parasites of Freshwater Fishes of North-West Europe* (O. N. Bauer, Ed.), pp. 163–168. Petrozavodsk.

Voccia, I., Krzystyniak, K., Dunier, M., Flipo, D., and Fournier, M. (1994). In vitro mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **29,** 37–48.

Vos, J. G., and van Loveren, H. (1996). Immunotoxicology: Determination of immunotoxic effects and immunotoxicity mechanisms. In *Toxicology: Principles and Applications* (R. J. M. Niesink, J. de Vries, and M. A. Hollinger, Eds.), pp. 841–868. CRC Press, Boca Raton.

Waterman, B., and Kranz, H. (1992). Pollution and fish diseases in the North Sea. Some historical aspects. *Mar. Pollut. Bull.* **24,** 131–137.

Weeks, B. A., and Warinner, J.E. (1984). Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. *Mar. Environ. Res.* **14,** 327–335.

Zelikoff, J. (1993). Metal pollution-induced immunomodulation in fish. *Ann. Rev. Fish. Dis.* **2,** 305–325.

IV

Modulation of immune parameters of roach, *Rutilus rutilus*, exposed to untreated ECF and TCF bleached pulp effluents.

by

Aaltonen, T.M., Jokinen E.I., Salo, H.M., Markkula, S.E. & Lammi, R.

Aquatic Toxicology 47: 277-289, 2000

https://doi.org/10.1016/S0166-445X(99)00014-4

Reprinted from

aquatic
toxicology

Aquatic Toxicology 47 (2000) 277–289

www.elsevier.com/locate/aquatox

Modulation of immune parameters of roach, *Rutilus rutilus*, exposed to untreated ECF and TCF bleached pulp effluents

T.M. Aaltonen [a,*], E.I. Jokinen [a], H.M. Salo [a], S.E. Markkula [a], R. Lammi [b]

[a] *Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FIN-40100 Jyväskylä, Finland*
[b] *UPM-Kymmene Corporation, Pietarsaari Mills, FIN-68601 Pietarsaari, Finland*

Received 4 June 1998; received in revised form 24 February 1999; accepted 4 March 1999

ELSEVIER

ELSEVIER

Aquatic Toxicology 47 (2000) 277–289

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Modulation of immune parameters of roach, *Rutilus rutilus*, exposed to untreated ECF and TCF bleached pulp effluents

T.M. Aaltonen [a,*], E.I. Jokinen [a], H.M. Salo [a], S.E. Markkula [a], R. Lammi [b]

[a] *Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FIN-40100 Jyväskylä, Finland*
[b] *UPM-Kymmene Corporation, Pietarsaari Mills, FIN-68601 Pietarsaari, Finland*

Received 4 June 1998; received in revised form 24 February 1999; accepted 4 March 1999

Abstract

The present study was designed to assess the effects of elemental chlorine free (ECF) and totally chlorine free (TCF) pulp and paper mill effluents on the immune defence of the roach (*Rutilus rutilus*). Fish were exposed for 5 weeks to concentrations of 0, 0.6, 2, 6 and 20% of untreated effluents from ECF or TCF bleaching processes. In order to study the capability to respond to foreign antigens the fish were immunised with bovine γ-globulin (BGG) 3 weeks before sampling. The numbers of anti-BGG antibody-secreting cells and immunoglobulin-secreting cells in the spleen and blood, as well as the levels of the anti-BGG antibodies and concentrations of immunoglobulin in plasma and the proliferation of lymphocytes after mitogenic activation were determined. In addition, phagocytosis-related functions, respiratory burst activity and migration of leukocytes, were measured. Formation of specific anti-BGG antibody-secreting cells in the blood and immunoglobulin-secreting cells in the spleen and blood were suppressed in the fish exposed to a high concentration (20%) of ECF effluent. Similarly, the level of anti-BGG antibody and the stimulation indices of Concanavalin A-stimulated proliferation responses in vitro were lower in these fish. A decreased migration of granulocytes was observed in the fish exposed to 0.6–2% of ECF and TCF effluents. These results demonstrate that untreated ECF and TCF effluents significantly affected the immune parameters of the roach. Taken as a whole, fish exposed to TCF effluent showed less immunomodulation than those exposed to EFC effluent.

Keywords: BKME; Bleached kraft mill effluents; ECF; Immune system of fish; Pollution; TCF

1. Introduction

Increased production of pulp and paper during the last few decades has not led to a correspond-

* Corresponding author. Tel.: +358-14-602224; fax: +358-14-602221.
E-mail address: tumiaa@jyu.fi (T.M. Aaltonen)

0166-445X/00/$ - see front matter
PII: S0166-445X(99)00014-4

ing increase in the effects of discharges owing to changes in pulping and bleaching processes and the treatment of waste waters. More attention is now being paid to chlorinated organic compounds formed during bleaching, and the primary importance has been placed on studying the effects of these compounds. Today, discharges of chlorinated organic compounds in effluents, e.g. the amount of absorbed organic chlorine (AOX), have decreased markedly due to the tendency to use less elemental chlorine in the bleachery. Most of the pulp in Western Europe is produced without the use of elemental chlorine, and several bleacheries in the Nordic countries are today equipped with oxygen pre-delignification. The main types of bleaching methods are elemental chlorine-free (ECF) bleaching and totally chlorine free (TCF) bleaching. The chemicals used in the ECF process are chlorine dioxide, alkali (extraction of dissolved lignin), peroxide and oxygen (reinforcement of the extraction stages). The bleaching chemicals used in the TCF process are oxygen, ozone, peroxide and alkali (reviewed by Axegård et al., 1993; Anonymous, 1997).

Many physiological and biochemical (reviewed by Södergren, 1993; Tana and Lehtinen, 1996) but only few immunological studies (Jokinen et al., 1995; Aaltonen et al., 1997) involving the effect of bleached kraft mill effluents (BKME) on fish have been published. Most of the former studies has been done with effluents from mills using elemental chlorine in bleaching, but currently interest has been shifted towards the ECF and TCF bleaching techniques. Many studies demonstrate that ECF and TCF effluents are toxic, but there is no clear relation between the bleaching process and the outcome of exposure to these effluents on the physiological parameters (Kovacs et al., 1994; Priha, 1996; Verta et al., 1996). The effects of the ECF and TCF effluents on the immune defence of fish has not been studied.

We have previously established that subchronic exposure of fish to secondary treated effluents from a mill using chlorine/chlorine dioxide bleaching affects the antibody-mediated immunity of the roach (*Rutilus rutilus*) in lake (Jokinen et al., 1995) and in aquarium conditions (Aaltonen et al., 1997). Depressed levels of plasma IgM have also been reported in whitefish (*Coregonus lavaretus* L.) exposed to treated BKME (Soimasuo et al., 1995a,b). Exposure to diluted untreated bleaching effluents alone from chlorine/chlorine dioxide processes suppresses the antibody-mediated immunity in roach, suggesting that compounds formed in bleaching may be at least partially responsible for immunotoxic effects (Aaltonen et al., 1997). In the present study the effects of exposure to untreated ECF and TCF bleaching effluents on fish immune parameters were investigated.

2. Materials and methods

2.1. Fish and exposure to effluents

Roach (*Rutilus rutilus*) were caught in Lake Peurunka, an uncontaminated oligotrophic lake in Central Finland in September 1994 and the experiments were performed during the following December and January. After the fish were caught, ectoparasites were killed by administration of a commercial drug, Ichide (N.T. Laboratories, UK) at 10 μl/l. The average weight and length of the fish were 43.4 g (S.D. = 15.1) and 176 mm (S.D. = 17.8). After adaptation (3 months) in laboratory conditions the fish were transferred from 300-l flow-through tanks into 60-l aquaria filled with aerated and filtered untreated bleaching effluent diluted with dechlorinated tap water. Roach, ten fish in each aquarium, were exposed for 5 weeks to untreated ECF or TCF effluents in concentrations of 0.6, 2, 6 and 20% at a constant temperature of 17.4–17.9°C and control fish were kept in identical aquaria in aerated tap water (Experiment 1). Half of the water was changed weekly and the oxygen concentration (6–8 mg/ml) was monitored by an oxygen probe (YSI 55, YSI Incorporated, Yellow Springs, OH, USA). The fish were fed daily with commercial pelleted dry food (FinnEwos Aqua, Finland) and kept on a regime of 12 h light and 12 h dark. During exposure to 20% TCF effluent the fish were lost for some unknown reason. This part of the study was repeated later in October using fish ($n = 7$ for exposed, $n = 7$ for controls)

from the same lake and TCF effluent from the same frozen batch (Experiment 2). The results from Experiments 1 and 2 were analysed as originating from two separate experiments.

After 2 weeks' exposure both exposed and control fish were immunised with a single intraperitoneal injection of bovine γ-globulin (BGG, Sigma, St. Louis, USA) emulsified in Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, MI). Each fish received 200 μl of antigen solution containing 500 μg BGG. The fish were exposed in the aquaria until sampled on day 21 post immunisation (p.i.) at the time of peak response against the antigen. The kinetics and the time of the peak response are based on several experiments with controlled temperatures both in aquaria and in natural waters (Aaltonen et al., 1994, 1997; Jokinen et al., 1995).

2.2. Effluents

The untreated effluents came from the ECF sequence of the softwood process and the TCF sequence of the hardwood process from a mill situated on the western coast of Finland. Alkaline and acid liquors from each process were sampled separately straight from the bleaching process, stored cold (+4°C) and then mixed together in the ratio in which they were produced at the mill. The pH of the mixed effluent was then adjusted to neutral either with NaOH (TCF effluent) or with HCl (ECF effluent) and the mixtures were stored frozen (−20°C) until used. Free fatty acids (decanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, oleic acid and octadecanoic acid), resin acids (pimaric acid, isopimaric acid, palustric acid, levopimaric acid, dehydroabietic acid, abietic acid and neoabietic acid) and sterols (stigmasterol, β-sitosterol and campesterol) of untreated and non-filtered effluents were determined with the method described by Örså and Holmbom (1994). Other chemical analysis were done in UPM-Kymmene Pulp Center Research Laboratory, Pietarsaari, Finland. The contents of the effluents are given in detail in Table 1.

2.3. Sampling

Feeding was stopped 2 days before the sampling of the fish. The fish were anaesthetised with MS-222 (Sigma) and their weight and length were recorded. The weight and length of the fish were used to calculate the condition factor (CF = [weight (g)/length (cm)^3] × 100). A blood sample was taken from the caudal vein of each fish with

Table 1
The bleaching sequences in the mill and characteristics of the untreated effluents from elemental chlorine free (ECF) and totally chlorine free (TCF) bleaching processes[a]

Parameter	ECF	TCF
Wood	Softwood	Hardwood
Bleaching sequence	DO-EO-D-EP-D	Q-Z-EOP-Z-EP-E
Waste water amount (l/s)	213	123
Waste water amount (m^3/tc)	20	12
pH	11.9	5.5
Conductivity (mS/m)	662	306
Suspended solids (mg/l)	23	15
COD_{cr} (mg/l)	2748	1438
BOD_7 (mg/l)	634	581
Evaporation residue (mg/l)	5137	3117
Ignition residue (mg/l)	3566	2166
Loss on ignition (mg/l)	1571	951
Organic carbon, TOC (mg C/l)	922	502
AOX (mg/l)	25	0.40
EOX (mg/l)	0.51	0.05
Fatty acids (mg/l)	3.2	2.5
Resin acids (mg/l)	9.8	0.3
Sterols (mg/l)	0.7	0.8
Colour (mg Pt/l)	4870	620

[a] Q, chelating agents under alkaline conditions; Z, ozone; EOP, alkaline extraction with oxygen and peroxide; EP, alkaline extraction with peroxide; E, alkaline extraction; DO, chlorine dioxide with oxygen; EO, alkaline extraction with oxygen; D, chlorine dioxide; COD, chemical oxygen demand; BOD, biological oxygen demand; TOC, total organic carbon; AOX, adsorbable organic halogen; EOX, extractable organic halogen.

a 1-ml heparinised syringe and 25-gauge needle. The hematocrits were measured in heparinised hematocrit tubes. The blood was centrifuged ($400 \times g$, 5 min) for the separation of plasma. Plasma was stored frozen (-20°C) for the determination of antibody levels.

After plasma separation the cells were resuspended in heparinized roach-modified Hank's balanced salt solution (rHBSS) containing HBSS diluted to 80% with water and supplemented with 0.5 mg/ml sodium pyruvate. Suspended cells were layered on the top of a Percoll density gradient medium (1.077 g/ml, Pharmacia, Uppsala, Sweden) and after centrifugation ($400 \times g$, 30 min) the lymphocytes from the interphase were collected, resuspended in rHBSS and, if necessary, run again in an identical Percoll gradient for further removal of contaminating red blood cells. The cells collected were washed twice ($400 \times g$, 10 min) with rHBSS and resuspended in roach culture medium: RPMI-1640 (Gibco, Paisley, UK) diluted to 80% with water, supplemented with 5% foetal calf serum (FCS), 2% own plasma from each fish, 0.5 mg/ml sodium pyruvate, 50 μM mercaptoethanol, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin and 10 mM Hepes, pH 7.4.

The spleen and head kidney of each roach were removed and disrupted separately against a nylon net (80 mesh). Leukocytes were separated on a two-step Percoll density gradient ($400 \times g$, 30 min). Lymphocytes from the spleen were collected at the 1.040–1.070 g/ml interphase and neutrophils from the head kidney at the 1.070–1.090 g/ml interphase. The collected cells were washed twice ($400 \times g$, 10 min) with rHBSS. Splenic lymphocytes were resuspended in roach medium (rRPMI): RPMI-1640 diluted to 80% with water, supplemented with 3% of serum substitute Ultroser G (Gibco), 0.5 mg/ml sodium pyruvate and 10 mM Hepes, pH 7.4. Cells from the head kidney were resuspended in phenol red-free rRPMI. Cells were counted by trypan blue exclusion in a haemocytometer (viability $> 95\%$). The number of lymphocytes from blood and spleen were adjusted to 2×10^6/ml.

2.4. Immune function assays

2.4.1. Quantification of serum immunoglobulin

The amount of total immunoglobulin M (IgM) and specific anti-BGG antibodies in roach plasma was measured by an enzyme-linked immunosorbent assay (ELISA) as described earlier (Aaltonen et al., 1994). First, flat-bottomed 96-well microtiter plates (Nunc-Immunoplate, Maxisorp) were coated either with BGG, for the determination of specific antibodies, or with rabbit anti-roach immunoglobulin for total IgM concentration. The procedures and the testing of cross-reactivities of anti-roach IgM antibody are described elsewhere (Aaltonen et al., 1994; Jokinen et al., 1995). After masking the plate with bovine serum albumin (BSA, Sigma), diluted samples of plasma were incubated in the wells. The trapped roach IgM was detected with biotin-conjugated anti-roach IgM. Next, alkaline phosphatase-conjugated avidin (Biomakor, Rehovot, Israel) was added. Washing was performed between each step with phosphate-buffered saline–Tween 20 (0.05%), pH 7.4 (PBS–Tween). *p*-Nitrophenylphosphate (Sigma) was used as a substrate and the optical density was read with a Titertek plate reader (Flow laboratories) at 405 nm. In the case of anti-BGG specific antibody, a calibration curve was constructed using a pool of high titer plasmas obtained from fish immunised with several injections of BGG. The concentration of anti-BGG antibody in the pool plasma was given as 1000 artificial units per ml. The concentrations of the samples were then expressed as units/ml (U/ml). The assay of plasma IgM was standardised with known concentrations of purified roach IgM.

2.4.2. Enumeration of secreting lymphocytes with ELISPOT assay

The enzyme-linked immunospot (ELISPOT) assay (Czerkinsky et al., 1983; Sedgwik and Holt, 1983) was used for the enumeration of immunoglobulin-secreting cells (ISC) and antigen (BGG)-specific antibody-secreting cells (ASC) in the spleen and blood from fish exposed to 0, 2, 6 or 20% effluent. The ELISPOT assay for roach has been described in detail previously (Aaltonen et al., 1994). The plates were coated, saturated

and washed as in the ELISA assay. Cells diluted in rRPMI (at 10^5, 10^4 and 10^3 per well for ASC, and 10^3 and 10^2 per well for ISC) were pipetted into antibody/BGG-coated and BSA-saturated wells and allowed to secrete antibodies for 3 h at 18°C. Trapped antibodies were detected with biotinylated anti-roach IgM antibody followed by alkaline phosphatase-conjugated avidin. The substrate, bromo-chloro-indolyl phosphate (Sigma), was mixed with warm agarose (Agarose type I, Sigma) and added to the wells. Finally, blue spots were counted using a stereo microscope.

2.4.3. Proliferation assay

To determine the proliferative responses, lymphocytes from the blood of fish exposed to 0, 2, 6 or 20% effluent were added in triplicate in a volume of 200 μl (4×10^5 cells /well) to 96-well plates (Nunc Microwell 96U, Roskilde, Denmark), and the cultures were activated with 20 μl Concanavalin A (ConA, Sigma) to give the final concentration of 100 μg/ml. The concentration of ConA had been tested earlier to ensure that it was in the optimum range for activation of roach lymphocytes. The same volume of culture medium was added to the unstimulated control wells. After a 5-day incubation at 26°C, 10 μl [^{3}H]thymidine (1 μCi/culture, Amersham International, Buckingshamshire, UK) was added and the cultures were incubated for 18 h and harvested (Cell Harvester, Nunc, Denmark) by water lysis and adherence to glass fibre filters (Whatman Grade 934 AH). The results were expressed as cpm and as a stimulation index (SI). SI was calculated for each individual fish according to the formula: SI = Mean cpm of triplicate stimulated cultures/Mean cpm of triplicate non-stimulated cultures.

2.4.4. Respiratory burst

Phagocytes were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) and the respiratory burst activity was determined by the luminol-enhanced chemiluminescence (CL) method (Scott and Klesius, 1981). Stock solutions of luminol (Sigma) and PMA (Sigma) were prepared in dimethyl sulfoxide (DMSO; Fluka Chemie AG, Switzerland) and stored in small aliquots at −20°C. A model 1250 LKB-Wallac luminometer with temperature control was used to monitor the chemiluminescence at 25°C. Cells (5×10^5) resuspended in phenol red-free rRPMI were pipetted in a volume of 250 μl into a polypropylene vial and 500 μl luminol, final concentration 5×10^{-5} M, diluted in rHBSS was added. The assay was initiated by adding 250 μl PMA diluted in rHBSS (the final concentration of PMA 2 μg/ml). The reaction was followed until the peak was passed (mean peak time 8 min). The peak value in millivolts (mV) was determined from each reaction with the use of a recorder (2210 Recorder, LKB, Bromma, Sweden).

2.4.5. Migration

The capability of head kidney neutrophils to move was assayed by a migration-under-agarose technique modified from Nelson et al. (1975). A total of 6 ml of a mixture containing 1% agarose (Type I: Low EEO; Sigma) and 0.25% gelatine (Difco, MI) in rRPMI at 56°C was dispensed onto each microscope slide which had been previously cleaned with 1 + 1 mixture of 3 M HCl and ethanol. The gel was allowed to set and the slides were stored in humidified boxes at 4°C until used. Just prior to the assay, a row of three wells, 2.5 mm in diameter and 2.0 mm apart, were punched out of the gel. The middle wells were filled with 10 μl casein (100 mg/ml, from bovine milk, Sigma) and duplicate samples, 3×10^5 neutrophils in a volume of 10 μl, were pipetted into outer wells. The cells were allowed to migrate in a humidified environment at 25°C for 3 h, then fixed overnight with methanol. The agarose was carefully removed and the slides were stained with a haematological stain (Diff-Quick, Baxter Diagnostic, Germany). The patterns of migration were examined under the microscope. The distance of cellular migration in each sample were quantified by the leading-front method using an ocular micrometer. The distance the cells had migrated from the margin of the well towards the well containing casein represents the directed migration, and the distance in the opposite direction represents the random migration.

Table 2
The length, weight, condition factor and hematocrit (mean ± S.E.) of the fish in Experiments 1 and 2

Experiment/treatment	*n*	Length (mm)	Weight (g)	Condition factor	Hematocrit (%)
Exp. 1:					
Control	15	177 ± 4	44.9 ± 2.8	0.79 ± 0.02	45 ± 1
ECF (%)					
0.6	10	176 ± 5	43.6 ± 4.7	0.78 ± 0.03	46 ± 2
2	10	170 ± 5	39.1 ± 3.9	0.78 ± 0.03	38 ± 2**
6	9	180 ± 11	48.6 ± 9.1	0.75 ± 0.03	33 ± 1**
20	10	168 ± 6	36.9 ± 3.9	0.75 ± 0.02	38 ± 2**
TCF (%)					
0.6	10	177 ± 6	46.8 ± 5.7	0.80 ± 0.03	47 ± 1
2	11	179 ± 4	45.5 ± 4.1	0.77 ± 0.02	44 ± 1
6	10	181 ± 3	45.9 ± 3.1	0.76 ± 0.02	44 ± 2
Exp. 2:					
Control	7	179 ± 9	50.9 ± 6.2	0.86 ± 0.04	33 ± 1
TCF (%)					
20	7	170 ± 7	45.2 ± 5.6	0.88 ± 0.02	34 ± 1

** $P \leq 0.001$ statistical significance compared to control.

2.5. Statistical analysis

The data were analysed for statistically significant differences by the Mann–Whitney *U*-test. A statistically significant difference from controls is expressed as $*P \leq 0.05$, $**P \leq 0.01$ or $***P \leq 0.001$.

3. Results

3.1. Mortality, growth and condition of roach

During the 5-week exposure to ECF or TCF effluents there was no occurrence of mortality except among the fish exposed to 20% TCF effluent; these fish all died during the first week. The fish in all the other groups (Exp. 1) exhibited normal behaviour. The exposure to 20% TCF effluent was later repeated (Exp. 2) and no mortality or signs of diseases were noted on that occasion. There were no differences in the weight, length or CF of the exposed fish compared to the control fish (Table 2), indicating that no severe loss of body weight occurred owing to the treatments in either experiment.

3.2. Hematocrit values

A significant decrease in hematocrit ($P \leq 0.001$) was seen in fish exposed to 2, 6 and 20% ECF effluent, whereas exposure to TCF effluent had no effect in any concentration tested (0.6, 2, 6 or 20%) (Table 2).

3.3. Specific antibody-secreting cells

The number of ASC in the blood of fish exposed to ECF effluent, irrespective of concentration, decreased compared to the controls and reached statistical significance ($P \leq 0.05$) in the group exposed to 20% ECF (Fig. 1A). However, no statistically significant differences were found in the number of ASC in the spleen between controls and the fish exposed to any of the concentrations of ECF effluent.

Exposure to TCF effluent had no clear effect on the antibody secretion of lymphocytes in the spleen (Fig. 1B, Table 4). Contradictory results were seen in the blood: exposure to 2% TCF effluent caused a decreased, and exposure to 6% TCF effluent an increased ASC response, but neither of these changes were statistically significant. There was a decrease in the number of both

the splenic and the blood ASC after exposure to 20% TCF effluent compared to the controls, but the difference did not reach statistical significance (Table 4).

3.4. Immunoglobulin-secreting cells

The number of ISC both in the spleen and in the blood decreased after exposure to 20% ECF effluent. The number of ISC in the fish exposed to concentrations of 2 and 6% ECF effluent was not different from that of the controls (Fig. 2A).

After exposure to TCF effluent no statistically significant changes in the number of ISC were noted in the spleen (Fig. 2B, Table 4). The number of ISC in the blood showed contradictory results. Exposure to 6% TCF effluent suppressed the number of ISC in the blood ($P = 0.049$); but when exposed to 2 and 20% TCF effluent the number of ISC increased but did not reach statistical significance.

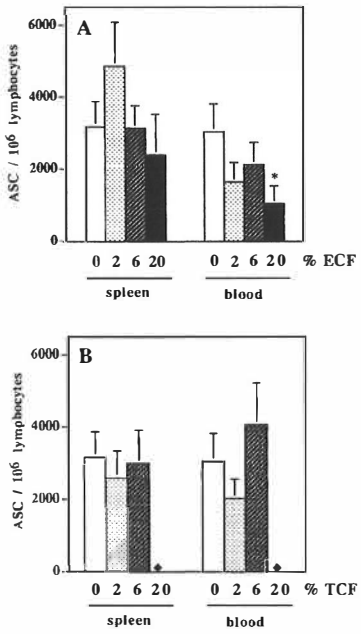


Fig. 1. Anti-BGG antibody-secreting cell (ASC) response after exposure to untreated effluent from (A) ECF bleaching or (B) TCF bleaching. (◆) For the results after exposure to 20% TCF effluent, see Table 4. Each bar represents the mean ± S.E. of eight to 11 fish sampled on day 21 after immunisation.

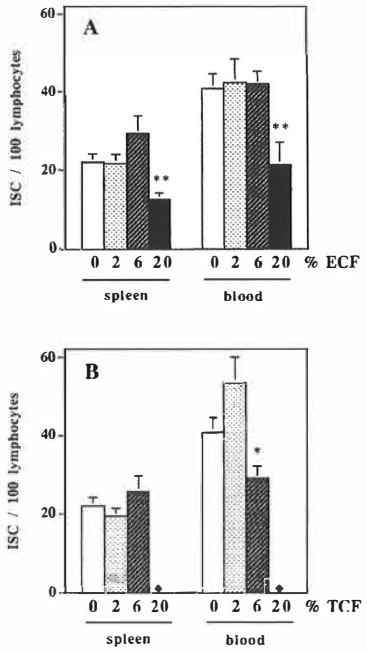


Fig. 2. Total numbers of immunoglobulin-secreting cells (ISC) after exposure to untreated effluent from (A) ECF bleaching or (B) TCF bleaching. (◆) For the results after exposure to 20% TCF effluent, see Table 4. Each bar represents the mean ± S.E. of eight to 11 fish.

3.5. Plasma antibodies

Exposure of fish to either ECF or TCF effluent had no effect on the plasma total IgM concentration (Fig. 3, Table 4). Exposure to 20% ECF effluent decreased the level of anti-BGG antibodies in the plasma ($P \leq 0.05$) (Fig. 4). There were no statistically significant differences after exposure to TCF effluent at any of the concentrations tested (Fig. 4, Table 4).

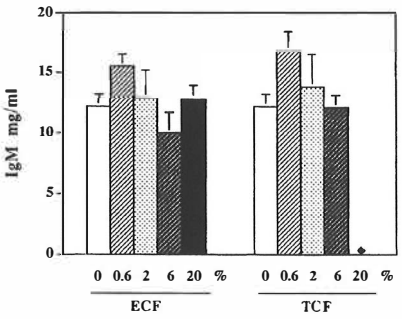


Fig. 3. The immunoglobulin (IgM) concentration in the plasma of roach after exposure to untreated ECF and TCF bleaching effluents. Each bar represents the mean ± S.E. of nine to 15 fish. (◆) For the results after exposure to 20% TCF effluent, see Table 4.

Table 3
The proliferation of fish blood lymphocytes in unstimulated and ConA-activated cultures expressed as incorporated radioactivity (counts per minute, cpm) and stimulation index (SI)

Treatment	Unstimulated (cpm)	ConA (cpm)	SI
Control	6591 ± 3871	22 702 ± 7613	22.4 ± 9.2
ECF (%)			
2	2275 ± 1658	15 807 ± 8827	16.5 ± 9.0
6	4018 ± 2982	12 789 ± 6029	8.6 ± 3.7
20	10 185 ± 6837	33 688 ± 11454	4.9 ± 1.9*
TCF (%)			
2	1924 ± 1304	14 434 ± 5375	16.6 ± 21.6
6	5838 ± 4444	15 496 ± 5653	21.6 ± 11.1
20	ND[a]	ND	ND

[a] ND, not done.
* $P \leq 0.05$.

3.6. Lymphocyte proliferation

ConA-activated proliferative responses of lymphocytes in vitro, expressed as the incorporated radioactivity (cpm), were decreased by 30–44% after exposure of fish to low concentrations (2 and 6%) of either ECF or TCF effluent (Table 3). But, by contrast, when exposed to 20% ECF effluent the lymphocyte proliferation was increased by 48%. Similar results were obtained in non-stimulated cultures as well. However, because of the wide variation of blastogenic responses, statistically significant differences were not found. A downward trend in proliferation, when expressed as a stimulatory index (SI), occurred after exposure to ECF effluent, reaching statistical significance in fish exposed to the highest concentration of ECF effluent ($P \leq 0.05$).

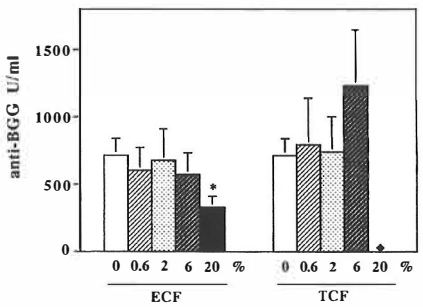


Fig. 4. The levels of anti-BGG antibodies in the plasma of fish exposed to untreated ECF and TCF bleaching effluents. Each bar represents the mean ± S.E. of nine to 15 fish. (◆) For the results after exposure to 20% TCF effluent, see Table 4.

3.7. Effects of exposure on neutrophils

In general exposure to ECF effluent suppressed the respiratory burst by head kidney neutrophils, but the difference between the exposed fish and controls did not reach statistical significance (Fig. 5). Exposure of fish to 0.6–6% TCF effluent had no clear effect on the respiratory burst but there was a decrease of 24% in after exposure to 20% effluent ($P = 0.092$, Table 4).

Exposure of fish to small concentrations of effluents from both ECF and TCF bleaching inhibited random migration, whereas high concentrations had no such effect (Fig. 6, Table 4). Exposure to 2% ECF and 2% TCT effluents ($P \leq 0.05$) also decreased directed migration.

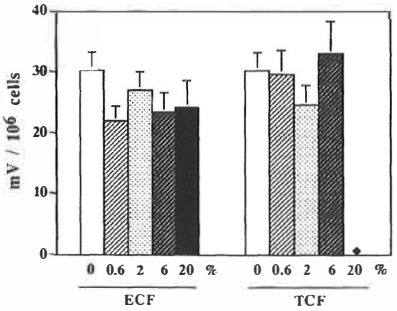


Fig. 5. The respiratory burst activity of pronephric granulocytes after exposure of fish to untreated ECF and TCF bleaching effluents. Each bar represents the mean ± S.E. of PMA-induced chemiluminescence of seven to 15 fish. (◆) For the results after exposure to 20% TCF effluent, see Table 4.

4. Discussion

Exposure of fish to contaminants in a lake receiving effluents from chlorine/chlorine dioxide bleaching is reported to impair the immunoreactivity of fish (Jokinen et al., 1995), as is also laboratory exposure of fish to BKME or to untreated bleaching effluents from chlorine/chlorine dioxide bleaching (Aaltonen et al., 1997). The processes of pulping and bleaching have gone through considerable changes in recent years, mainly with the objective of decreasing the discharge of chlorinated organic matter. In the present study immune parameters were investigated after exposure of fish to untreated effluents from two main bleaching processes, ECF and TCF.

Table 4
The immunological parameters (mean ± S.E.) of control fish ($n = 7$) and of fish exposed to 20% TCF effluent ($n = 7$) in Exp. 2

Parameter	Control	TCF 20%
ASC/10^6 cells from		
Spleen	3002 ± 1024	2403 ± 911
Blood	2830 ± 1024	912 ± 347
ISC/100 cells from		
Spleen	10.5 ± 1.9	9.1 ± 2.3
Blood	11.6 ± 2.2	22.4 ± 4.8
Plasma IgM (mg/ml)	11.8 ± 0.9	13.8 ± 2.5
Plasma anti-BGG (Units/ml)	868 ± 380	707 ± 188
Respiratory burst activity (mV)/10^6 pronephric granulocytes	110.8 ± 10.2	83.7 ± 12.7
Migration of pronephric granulocytes (μm)		
Random	247 ± 52	236 ± 40
Directed	541 ± 64	508 ± 48

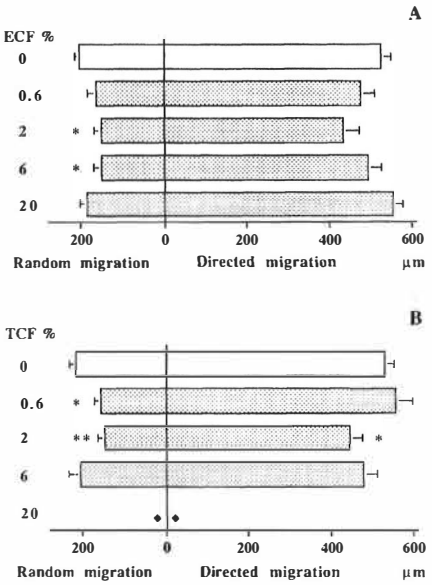


Fig. 6. The random and directed migration of pronephric granulocytes after exposure of fish untreated effluent from (A) ECF bleaching or (B) TCF bleaching. (◆) For the results after exposure to 20% TCF effluent, see Table 4. Each bar represents the mean ± S.E. migration distance of granulocytes from seven to 15 fish.

The specific ASC response in the spleen of fish exposed to ECF effluent, even at a concentration as high as 20%, did not differ significantly from the unexposed control values. In our earlier study (Aaltonen et al., 1997) roach were exposed in aquaria to 25% effluent from a bleaching process employing elemental chlorine/chlorine dioxide. Comparison of the two studies reveal that the

decrease in the ASC response in the present study was less prominent (decrease 24%, NS, Fig. 1) than in the earlier study (decrease 57%, $P \leq 0.05$, Aaltonen et al., 1997). This suggests that the untreated effluents from the ECF and TCF processes affect the immune responsiveness of fish less than untreated effluents from the chlorine and chlorine/chlorine dioxide bleaching. The number of ASC in the blood after exposure to 20% ECF and TCF effluents decreased, suggesting that the blood ASC response could serve as a more sensitive indicator of immunotoxicity than the response in the spleen.

The decrease in the total number of ISC in the spleen and blood of fish exposed to 20% ECF effluent was distinct, but no such effect occurred after exposure to lower concentrations of ECF effluent, or to any concentration of TCF (0.6–20%) effluent. The fish were injected with the immunogen mixed with Freund's complete adjuvant, which acts as a general immunostimulant (Anderson, 1992) and may restore, to a certain extent, the suppressed responses (Aaltonen et al., 1997). It is probably due to the stimulation by the adjuvant that decreased responses were seen only in the fish which received high doses of toxicants.

Exposure to ECF or TCF effluents caused no significant change in plasma IgM concentrations. Plasma levels of IgM in the fish exposed to BKME in cages in a polluted lake decreased even though the effluent concentration in the contaminated water area was below 1% (Jokinen et al., 1995). In the present study, exposure to a low concentration (0.6%) of effluents increased IgM levels rather than suppressed them. Indeed, both favourable and inhibitory modulatory responses to an increasing level of toxicants are common in studies on the immunological parameters of fish (Robohm, 1986; Rice and Weeks, 1989; Thuvander, 1989).

Plant-derived lectins induce activation and maturation of lymphocytes in vitro and serve as indicators of lymphocyte functionality (Maluish and Strong, 1986). Controversial results on ConA-stimulated proliferative responses, as well as on non-stimulated proliferation, were obtained in the present study: decreased responses in the fish exposed to low effluent concentrations but increased responses in the fish exposed to high concentrations. Several pollutants impair mitogen-stimulated proliferation (Spitsbergen et al., 1986; Faisal et al., 1991), but stimulation of proliferative responses has been described, e.g. after exposure to polychlorinated biphenyls (Thuvander et al., 1993).

The successful killing of pathogenic organisms demands a good level of mobility by the phagocytes in order to trap the pathogen, and the production of toxic substances is necessary to destroy the invader. In cyprinid fish the head kidney is the major phagocytic organ (Lamers and Parmentier, 1985). Although we did not find a statistically significant reduction in the production of reactive oxygen species by head kidney granulocytes, exposure of fish to any concentration of ECF effluent suppressed respiratory burst activity. Interestingly, the migration of neutrophils was inhibited in fish exposed to low concentrations, but normal migration was noted after exposure to a high (20%) concentration of ECF and TCF effluents. An explanation for this controversial observation is difficult to find, but nonlinear responses of immunological parameters to toxic agents are not uncommon (Koller, 1996). Tahir et al. (1993), for example, found low migratory indices in fish exposed to small concentrations of oil drilling mud, but the indices were equal to the control values when the fish were exposed to higher concentrations.

Inconsistent results have been obtained concerning the hematocrit of the white sucker near North American pulp mills: decreased hematocrit values (McMaster, 1991), increased hematocrit values (Hodson et al., 1992) or no change at all (Servos et al., 1992). In the present study the exposure of fish to ECF effluent at concentrations of 2–20% induced a significant decrease in the hematocrit values. By contrast, exposure of fish to equal concentrations of TFC effluent had no effect, suggesting a difference in the action of ECF and TCF effluents on haematopoiesis.

The clearest immunomodulatory effects were seen after exposure of fish to ECF effluent. The compounds responsible for immunomodulation were not determined here, but there are differences in the composition of the effluents that may

be relevant in regard to immunotoxicity. First, the concentration of resin acids in the ECF effluent was 30 times higher than in the TCF effluent. This is probably due to the different raw material in each case (softwood/hardwood, Table 1). Untreated bleaching effluents contain organic substances originating from wood: polysaccharides, lignin and extractives, e.g. resin acids, fatty acids and sterols (LaFleur, 1996; Strömberg et al., 1996), which are known to be harmful to fish (Verta et al., 1996). Secondly, ECF effluent contained 50 times the level of halogenated organic compounds compared to TCF effluent (see Table 1). The importance of halogenated compounds as a reason for decreased responsiveness is supported by an earlier study (Aaltonen et al., 1997). A far clearer suppression of the ASC response was found after exposure of fish to the effluent from chlorine-based bleaching (AOX 53 mg/l, softwood) than to ECF effluent (AOX 25 mg/l, softwood), although the levels of resin acids were essentially the same in both effluents. In addition, the compounds formed in the chlorine-based process are highly chlorinated and more toxic to fish than the compounds of a much lower degree of chlorination produced in the ECF process (reviewed by Axegård et al., 1993).

In terms of relevance for the organism, a test that monitors the net result from a cascade of reactions, e.g. the antibody response to T-cell-dependent antigen, is a predictive indicator of immunotoxicity (Wester et al., 1994). The outcome of immunisation is also dependent, in addition to lymphocytes, on the function of macrophages which act as antigen presenting-cells. Although, definite conclusions about the mechanisms behind the altered functions cannot be drawn on the basis of the present results, there are indications of altered lymphoid cell-related functions which may be relevant to decreased responsiveness: decreased numbers of IgM-secreting cells and reduced stimulation indices to the mitogen ConA. There is a close connection between the immune system and the endocrine system, and it is possible that the changes in the immunological parameters are a consequence of endocrine modulation of the immune system following the stress due to contaminants.

Is the suppression of immune functions noted in the present study of importance when considering the resistance of fish to pathogens? We found significant suppression of immunological parameters only after exposure to relatively high concentrations of untreated effluents. Activated sludge treatment is an effective technique for reducing the load of organic material in pulp mill effluents (Strömberg et al., 1996), and at present the effluents from pulp and paper mills, e.g. in Western Europe, almost never enter recipient waters without secondary treatment. Further, the effluents are highly diluted when released into recipient waters. In view of the magnitude of immunomodulation after exposure to high concentrations of untreated effluents, it seems quite improbable that exposure of fish to treated and highly diluted effluents could result in marked increased susceptibility to diseases in natural waters. Thomas and Sherwood (1996) estimate that suppression of immune parameters by 40–50% or more is required before enhanced susceptibility to infection in laboratory mammals is seen. However, apart from the fact that the structure of the immune system in teleost fish is similar to that in mammals, there is no evidence that this applies as such to fish. Moreover, it is difficult to extrapolate the results from a laboratory exposure to conditions in contaminated water areas where the exposure is permanent and the fish are also exposed to toxicants via contaminated food, sediment and particles. Significance of the results presented here on the resistance to pathogens needs to be verified with host resistance assays.

5. Conclusions

Experimental exposure of fish in the laboratory to untreated effluents from EFC and TCF bleaching processes had modulatory effects on immune parameters. The most distinct effect was a suppressed response to immunisation after exposure of fish to the highest (20%) concentration of ECF effluent. The effects of exposure to TCF effluent were slighter. These results suggest that untreated effluents from TCF and, especially, ECF bleaching contain components which are potentially immunotoxic.

The effluents from pulp and paper mills also contain, besides bleach liquors studied here, debarking waste waters, brown-stock washing waters and condensates. These contain components known to be harmful to fish. For this reason the immunotoxicity of total effluents from mills using ECF and TCF bleaching merit further study.

Acknowledgements

This study was supported by the Board of Environmental Science of the Academy of Finland, by the University of Jyväskylä and by the Graduate School on Fish Biology and Fisheries in Finland. Harri Leppänen is acknowledged for the chemical analysis of sterols, fatty acids and resin acids and Olavi Järvinen for catching the fish. The authors thank Aarno Karels for critical comments and Michael Freeman for checking the language of the manuscript.

References

Aaltonen, T.M., Jokinen, E.I., Valtonen, E.T., 1994. Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. Fish Shellfish Immunol. 4, 129–140.

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. Ecotoxicol. Environ. Safety 38, 266–271.

Anderson, D.P., 1992. Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. Annu. Rev. Fish Dis. 2, 281–307.

Anonymous, 1997. The Finnish background report for the EC documentation of best available techniques for pulp and paper industry. Ministry of the Environment Report 96, Helsinki, Finland, pp. 138.

Axegård, P., Dahlman, O., Haglind, I., Jacobsen, B., Mörck, R., Strömberg, L., 1993. Pulp bleaching and the environment—the situation 1993. Nord. Pulp Pap. Res. J. 4, 365–378.

Czerkinsky, C.C., Nilsson, L.-Å., Nygren, H., Ouchterlony, Ö., Tarkowsky, A., 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65, 109–121.

Faisal, M., Marzouk, M.S.M., Smith, C.L., Huggett, R.J., 1991. Mitogen induced proliferative responses of lymphocytes from spot (*Leiostomus xanthurus*) exposed to polycyclic aromatic hydrocarbon contaminated environments. Immunopharmacol. Immunotoxicol. 13, 311–327.

Hodson, P.V., McWhirter, M., Ralph, K., Gray, B., Thivierge, D., Carey, J.H., van der Kraak, G.J., Whittle, D.M., Levesque, M.-C., 1992. Effects of bleached kraft mill effluent on fish in the St.Maurice river, Quebec. Environ. Toxicol. Chem. 11, 1635–1651.

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*. Ecotoxicol. Environ. Safety 32, 219–225.

Koller, L.D., 1996. Profiling Immunotoxicology: Past, Present, and Future. In: Stolen, J.S., Fletcher, T.C., Bayne, C.J., Secombes, C.J., Zelikoff, J.T., Twerdok, L.E., Anderson, D.P. (Eds.), Modulators of Immune Responses: The Evolutionary Trail. SOS Publications, Fair Haven, NJ, pp. 301–310.

Kovacs, T.G., Tana, J., Lehtinen, K.J., Sangfårs, O., 1994. A comparison of the environmental quality of elemental chlorine-free (ECF) and totally chlorine-free (TCF) hardwood bleach plant effluents, Paper No. 5-3. In Proceedings, 1994 International Non-Bleaching Conference, Miller Freeman Inc., San Francisco, CA.

LaFleur, L.E., 1996. Sources of pulping and bleaching derived chemicals in effluents. In: Servos, M.R., Munkittrick, Carey, J.H., van der Kraak, G.J. (Eds.), Environmental Fate and Effects of Pulp and Paper Mill Effluents. St. Lucie Press, Delray Beach, FL, pp. 21–31.

Lamers, C.H.J., Parmentier, H.K., 1985. The fate of intraperitoneally injected carbon particles in cyprinid fish. Cell Tissue Res. 242, 499–503.

Maluish, A.E., Strong, D.M., 1986. Lymphocyte proliferation. In: Rose, N.R., Friedman, H., Fahey, J.L. (Eds.), Manual of clinical laboratory immunology. American Society for Microbiology, Washington, DC, pp. 274–281.

McMaster, M.E., 1991. Impact of bleached kraft mill effluent (BKME) on fish populations in Jackfish Bay, Lake Superior. M.Sc. Thesis, University of Waterloo, Waterloo, Ontario, Canada. 131 pp.

Nelson, R.D., Quie, P.G., Simmons, R.L., 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115, 1650–1656.

Örså, F., Holmbom, B., 1994. A conventional method for the determination of wood extractives in paper making process waters and effluents. J. Pulp Pap. Sci. 20, J361–J365.

Priha, M.H., 1996. Ecotoxicological impacts of pulp mill effluents in Finland. In: Servos, M.R., Munkittrick, Carey, J.H., van der Kraak, G.J. (Eds.), Environmental Fate and Effects of Pulp and Paper Mill Effluents. St. Lucie Press, Delray Beach, FL, pp. 637–650.

Rice, C.D., Weeks, B.A., 1989. Influence of tributyltin on in vitro activation of oyster toadfish macrophages. J. Aquat. Animal Health 1, 62–68.

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*). Vet. Immunol. Immunopathol. 12, 251–262.

Scott, A.L., Klesius, P.H., 1981. Chemiluminescence: a novel analysis of phagocytosis in fish. Dev. Biol. Stand. 49, 243–254.

Sedgwik, J.D., Holt, P.G., 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods 57, 301–309.

Servos, M.R., Carey, J.H., Ferguson, G.J., van der Kraak, G.J., Parrot, H., Gorman, K., Cowling, R., 1992. Impact of modern bleached kraft mill on white sucker populations in the Spanish River, Ontario. Water Pollut. Res. J. Can. 27, 423–437.

Södergren, A., 1993. Bleached pulp mill effluents. Composition, Fate and Effects in the Baltic Sea. Final Report from the Environment/Cellulose Project II, Swedish National Environmental Protection Board, Solna. 150 pp.

Soimasuo, R., Aaltonen, T., Nikinmaa, M., Pellinen, J., Ristola, T., Oikari, A., 1995a. Physiological toxicity of low-chlorine bleached pulp and paper mill effluent on whitefish (*Coregonus lavaretus* L. s.l.): a laboratory exposure simulating lake pollution. Ecotoxicol. Environ. Safety 31, 228–237.

Soimasuo, R., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T., Oikari, A., 1995b. Biomarker responses in a pollution gradient: Effect of pulp and paper mill effluents on whitefish studied by caging technique. Aquat. Toxicol. 31, 329–345.

Spitsbergen, J.M., Schat, K.A., Kleeman, J.M., Peterson, R.E., 1986. Interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with immune responses of rainbow trout. Vet. Immunol. Immunopathol. 12, 263–280.

Strömberg, L., Mörck, R., de Sousa, F., Dahlman, O., 1996. Effects of internal process changes and external treatment on effluent chemistry. In: Servos, M.R., Munkittrick, Carey, J.H., van der Kraak, G.J. (Eds.), Environmental Fate and Effects of Pulp and Paper Mill Effluents. St. Lucie Press, Delray Beach, FL, pp. 3–19.

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.). Aquat. Toxicol. 27, 71–82.

Tana, J., Lehtinen, K.-J., 1996. The Aquatic Environmental Impact of Pulping and Bleaching Operations: An Overview. Finnish Environment Institute Report 17, Helsinki, Finland. 103 pp.

Thomas, P.T., Sherwood, R.L., 1996. Host resistance models in immunotoxicology. In: Smialowicz, R.J., Holsapple, M.P. (Eds.), Experimental Immunotoxicology. CRC Press, Boca Raton, FL, pp. 29–45.

Thuvander, A., 1989. Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. J. Fish. Biol. 35, 521–529.

Thuvander, A., Wiss, E., Norrgren, L., 1993. Sublethal exposure of rainbow trout (*Oncorhynchus mykiss*) to Clophen A50: effects on cellular immunity. Fish Shellfish Immunol. 3, 107–117.

Verta, M., Ahtiainen, J., Nakari, T., Langi, A., Talka, E., 1996. The effect of waste constituents on the toxicity of TCF and ECF pulp bleaching effluents. In: Servos, M.R., Munkittrick, Carey, J.H., van der Kraak, G.J. (Eds.), Environmental fate and effects of pulp and paper mill effluents. St. Lucie Press, Delray Beach, FL, pp. 41–51.

Wester, P.W., Vethaak, A.D., van Muiswinkel, W.B., 1994. Fish as biomarkers in immunotoxicology. Toxicology 86, 213–232.

V

Effects of primary- and secondary-treated bleached kraft mill effluents on the immune system and physiological parameters of roach.

by

Aaltonen, T.M., Jokinen, E.I., Lappivaara, J., Markkula, S.E.,
Salo, H.M., Leppänen, H. & Lammi R.

Aquatic Toxicology 50(4): (in press), 2000

https://doi.org/10.1016/S0166-445X(00)00101-6

ELSEVIER

Aquatic Toxicology 000 (2000) 000–000

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Effects of primary- and secondary-treated bleached kraft mill effluents on the immune system and physiological parameters of roach

T.M. Aaltonen [a,*], E.I. Jokinen [a], J. Lappivaara [a], S.E. Markkula [a], H.M. Salo [a], H. Leppänen [a], R. Lammi [b]

[a] *Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, FIN-40100 Jyväskylä, Finland*
[b] *UPM-Kymmene Corporation, Pietarsaari Mills, FIN-68601 Pietarsaari, Finland*

Received 23 November 1999; received in revised form 21 February 2000; accepted 29 February 2000

Abstract

The present study was designed to examine, whether, effluents from a modern pulp and paper mill using elemental chlorine-free/total chlorine-free (ECF/TCF) bleaching, exert effects on the immune system of fish and, in addition, to relate these findings to physiological parameters known to be affected by bleached kraft-mill effluents (BKME). Roach (*Rutilus rutilus*) were exposed in laboratory conditions to primary- or secondary-treated effluent from a pulp and paper mill. In order to study their capability to respond to foreign antigens they were immunised with bovine γ-globulin (BGG) prior to exposure. The number of anti-BGG antibody-secreting cells (ASC) and the number of immunoglobulin-secreting cells (ISC) in the spleen and blood as well as the level of anti-BGG specific antibodies and concentration of plasma immunoglobulin (IgM) were studied. Phagocytosis and migration of granulocytes of the head kidney were also determined. In addition to the immunological parameters, the activity of hepatic biotransformation enzymes, the carbohydrate metabolism and osmoregulation were examined. Exposure of roach for 21 days to BKME affected several immunological parameters. Both effluents, primary- and secondary-treated, impaired the immunoreactivity of the fish. Sex-related differences in the immune responses were evident in many parameters e.g. in the number of blood ISC and splenic ASC. Sex also had effects on cortisol levels and in the induction of 7-ethoxyresorufin *O*-deethylase (EROD). These results demonstrate that both primary- and secondary-treated effluent from a pulp and paper mill using ECF/TCF bleaching have effects on fish immune functions. Further, these findings suggest that steroids may contribute to immunomodulation in fish.

Keywords: Bleached kraft mill effluents; Primary- and secondary-treated effluents; Pulp and paper mill; Aquatic pollution; Immunotoxicity; Fish immune system

1. Introduction

The production of pulp and paper causes the discharge of a complex mixture of compounds

* Corresponding author. Tel.: +358-14-2602224; fax: +358-14-2602221.
E-mail address: tumiaa@jyu.fi (T.M. Aaltonen)

0166-445X/00/$ - see front matter
PII: S0166-445X(00)00101-6

into recipient waters. The main component of the effluents from kraft pulp processes are filtrates from bleaching, waste waters from debarking, condensates from cooking and evaporation, black liquor residues and spills occurring during different processing stages. In modern pulp mills, most of the water used in the production processes is recycled resulting in a diminished amount of waste water. Before discharge to recipient waters, effluents are always treated. The separation of suspended solids is generally carried out mechanically in primary waste-water treatment. After that effluents undergo biological treatment by means of either activated sludge or, more seldom, an aerated lagoon. A well operated activated sludge treatment system is highly effective in reducing the amount of discharge, for example, biological oxygen demand (BOD) is diminished by over 95% and chemical oxygen demand (COD) by 70–80%. Resin and fatty acid removal efficiencies are generally greater than 85% (Anonymous, 1997; LaFleur and Barton, 1997). Secondary treatment has also been found necessary in reducing acute and chronic toxicity of effluents (Munkittrick and Sandström, 1997). Some compounds, however, are less responsive to treatment (e.g. phytosterols) and, in addition, some hazardous novel metabolites may be formed that may be also toxic (e.g. retene) (Bright et al., 1997; Billiard et al., 1999).

A variety of biological effects, including structural, physiological and biochemical responses in fish, have been associated with subchronic exposures to effluents from the pulp and paper industry (Lindström-Seppä and Oikari, 1989; Hodson et al., 1992; Södergren, 1993). The effects of pulp-mill effluents on the immunological responses have not been studied extensively. Our earlier studies have established the effects of subchronic exposure to secondary-treated effluents and untreated bleaching effluents on immunoreactivity of fish (Jokinen et al., 1995; Aaltonen et al., 1997, 2000). The antibody-mediated immunity of roach (*Rutilus rutilus*) was affected when the fishes were kept in cages in a lake receiving bleached kraft-mill effluents (BKME) (Jokinen et al., 1995) or exposed in an aquarium (Aaltonen et al., 1997) to secondary-treated effluents from a mill using chlorine/chlorine dioxide for bleaching. Exposure to the untreated bleaching effluent from the chlorine/chlorine dioxide bleaching alone suppresses antibody synthesis suggesting that compounds derived from bleaching are involved in the occurrence of these immunotoxic effects (Aaltonen et al., 1997). Reduced plasma IgM have been reported in whitefish (*Coregonus lavaretus* L.) exposed to effluent from a mill using chlorine/chlorine dioxide bleaching (Soimasuo et al., 1995a,b). Changes in the bleaching process together with the installation of the secondary-treatment system abolished the decrease in plasma IgM (Soimasuo et al., 2000). The macrophage activity of mummichog (*Fundulus heteroclitus*) from waters contaminated by BKME is also affected (Fournier et al., 1998). Our previous study of untreated bleaching effluents from the elemental chlorine-free (ECF) and total chlorine-free (TCF) processes indicate impacts on fish immune parameters (Aaltonen et al., 2000). The main objects of the present study were to examine immunological effects of exposure of fish to whole mill effluents from a modern pulp and paper mill using ECF/TCF bleaching, whether the secondary treatment (activate sludge) reduces these effects, and further, to relate these findings to physiological parameters known to be affected by BKME.

2. Materials and methods

2.1. Fish and exposure regime

Roach (*R. rutilus*) were caught with a fish trap in Lake Peurunka, an uncontaminated oligotrophic lake in central Finland, in November, 1997. After transfer the fish were allowed to adapt to laboratory conditions for 2.5 months before the experiment in February. Ectoparasites were killed by bathing the fish for 1 h in water containing malachite green (0.1 mg/l) and formaldehyde (10 mg/l) every 2 days for 8 days. The average weight and length of the fish were 35 g (S.D. = 12) and 155 mm (S.D. = 16), respectively. The fish were 3–6 years old and sexually mature. A total of 56 roach were immunised with a single intraperitoneal injection (200 μl) of bovine γ-globulin (BGG, 500 μg per fish, Sigma Chemical Co., St

Louis, USA) emulsified in Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, MI) and transferred from 300-l flow-through tanks to 60-l aquaria. After immunisation the fish were kept in clean dechlorinated water (controls, $n = 24$) or exposed to aerated and filtered effluents diluted with dechlorinated tap water, eight roach in each aquarium. Roach were exposed for 3 weeks either to 20% primary-treated ($n = 16$) or to 20% secondary-treated ($n = 16$) effluent from the bleached kraft mill. The aquaria, both control and exposure, were immersed in large tanks with thermostat-controlled water circulation in order to maintain a constant temperature throughout the exposure period. The temperature of the water was 17.5–19.0°C with the maximum difference of 0.5°C between aquaria. Half of the water in each aquarium was changed weekly and the oxygen concentration (6–8 mg/ml) was monitored by an oxygen probe (YSI 55, YSI Inc., Yellow Springs, OH, USA). Fish were fed daily with commercial pelleted dry food (FinnEwos Aqua Co., Finland) and kept on a regime of 12 h light and 12 h dark. The fish were sampled on day 21 post immunisation (p.i.) at the time of peak response to the immunogen (see Aaltonen et al., 1994).

Table 1
Characteristics of primary- and secondary-treated effluents from pulp and paper mill using ECF and TCF bleaching

Parameter	Primary treated	Secondary treated
Waste water amount (l/s)	1280	1890
pH	6.8	7.5
Conductivity (mS/m)	226	173
COD_{Cr} (mg O_2/l)	1338	471
Evaporation residue (mg/l)	1940	1270
Ignition residue (mg/l)	1500	1385
Total phosphorus (mg P/l)	2.2	0.56
Total nitrogen mg (mg N/l)	12.2	6.6
Colour (mg Pt/l)	2088	1385
AOX (mgCl/l)	5.0	3.2
EOX (mg Cl/l)	0.13	0.03
Resin acids (mg/l)	2.5	0.03

2.2. Effluents

The effluents (Table 1) came from a modern integrated pulp and paper mill (UPM-Kymmene, Pietarsaari, Finland) using the ECF sequence for softwood, and the TCF sequence for hardwood bleaching. Primary-treated (clarified) effluent entering the biological treatment and secondary-treated total effluent (clarified and activated sludge-treated) prior to discharge to the recipient water were collected in polyethylene containers and kept in a refrigerator until used for the experiments. Chemical analyses of the effluents were performed at the UPM-Kymmene Research Center, Pietarsaari, Finland, according to Finnish (SFS), Scandinavian (SCAN) and ISO standards. Resin acids (pimaric acid, isopimaric acid, dehydroabietic acid, abietic acid and sandaracopimaric acid) were determined using the method described by Örså and Holmbom (1994).

2.3. Sampling

Feeding was stopped 2 days before sampling. On sampling the fish were anaesthetised with MS-222 (200 mg/l, Sigma Chemical Co.) and their weight and length were recorded for the calculation of the condition factor ($CF = [\text{weight (g)}/\text{length}^3 \text{ (cm)}] \times 100$). A blood sample was taken from the caudal vein of each fish with a 1 ml heparinized syringe and 25-gauge needle. The hematocrits were measured in heparinized hematocrit tubes. The blood was centrifuged ($400 \times g$, 5 min) for the separation of plasma. Plasma was stored frozen (−70°C) for determinations of antibody, glucose, lactate, sodium, potassium and cortisol levels. The gills for determination of AT-Pase activity and liver tissue for analyses of 7-ethoxyresorufin *O*-deethylase (EROD) activity and glycogen concentration were excaved immediately after the blood sample, freezed in liquid nitrogen and stored at −70°C.

After the separation of plasma the blood cells were suspended in heparinized Hank's balanced salt solution modified for roach (rHBSS) containing HBSS diluted to 80% with water and supplemented with 0.5 mg/ml sodium pyruvate. The suspended cells were layered on the top of a

Percoll density gradient medium (1.080 g/cm^3. Pharmacia, Uppsala, Sweden) and after centrifugation (400 × *g*, 30 min) the lymphocytes from the interface were collected, resuspended in rHBSS and run again in an identical Percoll gradient for further removal of contaminated red blood cells. The collected cells were washed twice (400 × *g*, 10 min) with rHBSS and resuspended in roach incubation medium (rRPMI); RPMI-1640 diluted to 80% with water, and supplemented with 3% of serum substitute Ultroser G, (Gibco Co. Pasley. UK), 0.5 mg/ml sodium pyruvate, 50 mM mercaptoethanol, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10 mM Hepes, pH 7.4.

The spleens were removed and homogenised individually through a nylon net (80 mesh). For isolation of lymphocytes, the tissue homogenate was layered on a two-step Percoll density gradient and centrifuged for 30 min at 400 × *g*. The lymphocytes were collected at the 1.040–1.080 g/cm^3 interface, washed twice (400 × *g*, 10 min) with rHBSS, and resuspended in 2 ml rRPMI. Cells were counted by trypan blue exclusion in a haemocytometer (viability >95%) and the numbers of lymphocytes from the blood and spleen were adjusted to 2×10^6/ml.

The head kidney was used as a source of phagocytes for chemiluminescence (CL) and migration assays. Cells from the homogenised head kidney were separated with a two-step Percoll gradient. Granulocytes were collected at the 1.070–1.090 g/cm^3 interface and after washing with rHBSS resuspended in phenol red-free rRPMI.

2.4. Immune function assays

2.4.1. Quantification of serum immunoglobulin

The amount of total IgM and specific anti bovine γ-globulin (BGG) antibody in the roach plasma was measured by an enzyme-linked immunosorbent assay (ELISA), as described earlier (Aaltonen et al., 1994). First, the flat-bottomed 96-well microtiter plates (Nunc-Immunoplate, Maxisorp) were coated either with BGG, for the determination of anti-BGG specific antibodies, or with rabbit anti-roach immunoglobulin M (anti-roach IgM, made in our laboratory), for total IgM concentration. After masking the plate with bovine serum albumin (BSA, Sigma Chemical Co.), diluted samples of plasmas from individual fish were incubated in the wells. The trapped roach IgM was detected with biotin-conjugated rabbit anti-roach IgM. Next, alkaline phosphatase-conjugated avidin (Sigma Chemical Co.) was added. Washing was performed between each step with phosphate buffered saline-Tween 20 (0.05%), pH 7.4 (PBS-Tween). *p*-Nitrophenylphosphate (Sigma Chemical Co.) was used as a substrate and the optical density was read with a Titertek plate reader (Flow laboratories) at 405 nm. In the case of the anti-BGG specific antibodies, a calibration curve was constructed using a pool of high titer plasmas obtained from fish immunised with several injections of BGG. The concentration of anti-BGG antibody of the pooled plasma was given 1000 artificial units per ml (U/ml) and the concentrations of the samples were then expressed as U/ml. The assay of total plasma immunoglobulin was standardised with known concentrations of purified roach IgM.

2.4.2. Enumeration of secreting lymphocytes with ELISPOT assay

The enzyme-linked immunospot (ELISPOT) assay was used for the enumeration of total immunoglobulin-secreting cells (ISC) and antigen (BGG)-specific antibody-secreting cells (ASC) in the spleen and the blood. The ELISPOT assay for roach has been described in detail previously (Aaltonen et al., 1994). Briefly, the plates were coated, saturated and washed as in the ELISA assay. Cells diluted in rRPMI (at 105, 104 and 103 per well for ASC, and 102 per well for ISC) were pipetted into antibody/BGG-coated and BSA-saturated wells (five wells per dilution) and allowed to secrete antibodies for 3 h at 18°C. The trapped antibodies were detected with biotin-conjugated anti-roach IgM antibody followed by alkaline phosphatase-conjugated avidin. The substrate, bromo-chloro-indolyl phosphate (Sigma Chemical Co.), was mixed with warm agarose (Agarose type I, Sigma Chemical Co.) and added to the wells. Finally, blue spots were counted using a stereo microscope.

2.4.3. Respiratory burst

Phagocytes from the head kidney were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co.) and the respiratory burst was determined by the luminol-enhanced CL method (Scott and Klesius, 1981) modified for roach (Salo et al., 1998). Briefly, stock solutions of luminol (Sigma Chemical Co.) and PMA were prepared in dimethyl sulfoxide (DMSO; Fluka Chemie AG, Switzerland) and stored in small aliquots at −20°C. A microplate luminometer (Victor^2, 1420 Multilabel counter, Wallac, Finland) was used to monitor the CL. The wells of a microplate contained 50 μl cell suspension in phenol red-free rRPMI and 100 μl luminol (final concentration, 5×10^{-5} M) diluted in rHBSS. The assay was initiated by adding 50 μl PMA (final concentration, 2 μg/ml) diluted in rHBSS. The peak value in counts per minutes (cpm) was determined for each reaction.

2.4.4. Migration

The capability of head kidney neutrophils to move was assayed by a migration-under-agarose-technique modified from Nelson et al. (1975). The method for roach has been described in more detail elsewhere (Salo et al., 1998). The mixture of agarose (1%, type I, low EEO; Sigma Chemical Co.) and gelatin (0.25%, Difco, MI) in rRPMI at 56°C was dispensed onto microscope slides. Slides were stored in humidified boxes at 4°C until used, and just prior to the assay a row of three wells 2.5 mm in diameter and 2.0 mm apart were punched out of the gel. The middle wells were filled (10 μl) with 100 mg/ml casein (from bovine milk, Sigma Chemical Co.) and the outer wells received 3×10^5 cells in a volume of 10 μl. The cells were allowed to migrate in a humidified environment at 25°C for 3 h, then fixed overnight with methanol. The agarose was carefully removed and slides with migrated cells were stained with a haematological stain (Diff-Quik, Baxter Diagnostic AG, Germany). The distance, the cells had migrated from the margin of the well towards the well containing casein (directed migration) and in the opposite direction (random migration) were measured under the microscope.

2.5. Cortisol assay

Plasma cortisol was measured with a commercial kit Gamma Coat [^{125}I] Cortisol (Incstar Co., Stillwater, MN) using a gammacounter (1270 Rackgamma II, LKB Wallac, Sweden).

2.6. Carbohydrate metabolism and osmoregulation

Plasma (30 μl) was mixed with 0.6 M $HClO_4$ (150 μl) before measuring the concentration of ions, glucose and lactate. Glucose was determined by the GOD-Period method (Boehringer Mannheim, Germany, Cat. no. 124036) and lactate by a modification of the method of Noll (1974) (Boehringer Mannheim, Germany, Cat. no. 256773) by using an iEMS spectrophotometer (Labsystems Inc., Finland). Plasma sodium and potassium concentrations were measured with a Corning 435-flame photometer using lithium (15 mM) as an internal standard.

Glycogen was extracted and purified using a method modified from Lim and Ip (1989); duplicated liver samples (68.2 ± 1.3 mg) were weighed, homogenised in 30% KOH (700 μl) in a bath of boiling water, and precipitated in an oven (60°C, 5 min) after adding ethanol (final ethanol concentration, 60%). After centrifugation (2 min at 15 000 × *g*), the supernatant was removed and the pellet was washed once with 60% ethanol. After centrifugation, the pellet was dissolved in 1 M HCl (1 ml) in a bath of boiling water (2 h) and, after cooling, the amount of glucose released from the original glycogen was measured using the GOD-Period method.

Total and Na^+-K^+-ATPase analyses were carried out by the method described by Schrock et al. (1994) consisting of a careful manual separation of filaments, blood cell and extract removal performed by centrifugation, followed by ultra-homogenisation and incubation (Na^+, 155 mM; K^+, 75 mM; Mg^{2+}, 25 mM; pH 7.0) of each aliquot in the presence of Na_2ATP with or without ouabain. Inorganic phosphate liberated in the reaction was measured in a malachite green solution using an iEMS platereader (Labsystems, Finland). For each crude homogenate, the incubation and measurement steps were performed twice, the

Table 2
Length, weight and condition factor (mean ± S.E.) of roach after exposure to primary- or secondary-treated BKME

Parameter	Control	Exposed	
		Secondary treated	Primary treated
Number of fish (male/female)	24 (18/6)	16 (12/4)	16 (10/6)
Length (mm)	158 ± 3.9	154 ± 3.9	154 ± 2.7
Weight (g)	35.7 ± 2.7	33.4 ± 3.3	36.4 ± 3.9
Condition factor	1.52 ± 0.04	1.56 ± 0.03	1.65 ± 0.06

first sets of gill homogenates (~ 20 pieces per set), consisting of one sample from each fish, were incubated and inorganic phosphate measured in a random order, while the remaining sets of measurement for the same homogenates were carried out in reverse order. The concentration of total proteins in the gill homogenates was measured using a Bio-Rad DC Protein Assay Kit.

2.7. The activity of EROD

The preparation of microsomal fractions of liver tissue has been described in more detail earlier (Soimasuo et al., 1995a,b). Briefly, frozen livers were thawed in an ice-cold Tris–HCl buffer (0.1 M, pH 7.6) containing 1 mM K_2EDTA and 0.25 M sucrose (pH 7.6), and homogenised in a Potter-Elvehjem-type homogeniser. The homogenate was centrifuged (13 000 × g, 20 min). The supernatant was further spun at 105 000 × g for 60 min at 4°C. The pelleted microsomes were resuspended in 50 mM Tris–HCl (pH 7.6) buffer containing 20% (v/v) glycerol, 1 mM EDTA and 1 mM dithiothreitol in a 1:1 ratio of buffer to liver weight. The microsomal fractions were stored in liquid nitrogen until assayed.

Activities of hepatic biotransformation enzymes as quantified by EROD activity were measured fluorometrically in the microsomal liver-fractions using resorufin as internal standard according to the method of Burke et al. (1985) adapted for the microplate method (Soimasuo et al., 1998). Microsomes (protein 0.2–0.9 mg per well) were incubated with 2.5 mM ethoxyresorufin (Sigma Chemical Co.) in 100 mM potassium phosphate buffer (pH 8.0). The reaction was initiated with NADPH (5 mM). Fluorescence (excitation 530 nm, emission 585 nm) was recorded at 30-s intervals for 4 min at 20°C (Shimadzu RF-500 fluorometer). Microsomal protein concentrations were determined using a Bio-Rad DC Protein Assay Kit, with BSA as the standard.

2.8. Statistical analysis

The data were analysed for statistically significant differences by the Mann–Whitney U-test. A statistically significant difference from controls is expressed as *, $P \leq 0.05$; **, $P \leq 0.01$; or ***, $P \leq 0.001$.

3. Results

3.1. Growth and condition of roach

There were no differences in the weight, length or CF of the exposed fish compared with the controls (Table 2). The fish in all groups exhibited normal behaviour and no signs of diseases were noted. In the present experiment, 71% of the roach were males. The relation between sex and size was seen in the weight and the length of the fish, females being bigger than males.

3.2. Antibody secretion by lymphocytes

Exposure to effluents decreased the number of anti-BGG-specific ASC both in the blood and in the spleen (Fig. 1). The decrease was most evident after exposure to the primary-treated effluent. There was only one exception; no effect on splenic

ASC in female roach was noted after exposure to the primary-treated effluent.

There was a significant difference in the blood ISC count between male and female controls (Fig. 2) but no such an effect was found in the spleen. Exposure to either effluent, primary-or secondary-treated, had a significant contrastive effect on the blood ISC count. The number of ISC in the blood after exposure to both effluents decreased in the male and increased in the female roach. Exposure to effluents had no clear effect on the number of ISC in the spleen.

Exposure to primary- or secondary-treated effluent had no significant effect on the plasma concentration of anti-BGG specific antibodies or total IgM (Fig. 3) in either sex.

3.3. *Effects of exposure on granulocyte respiratory burst activity and migration*

Decreased respiratory burst by head kidney granulocytes was noted after exposure to primary-treated effluent but the difference did not reach statistical significance (Table 3). Exposure of fish to effluents had no effect on either the random or directed (chemotactic) migration of head kidney

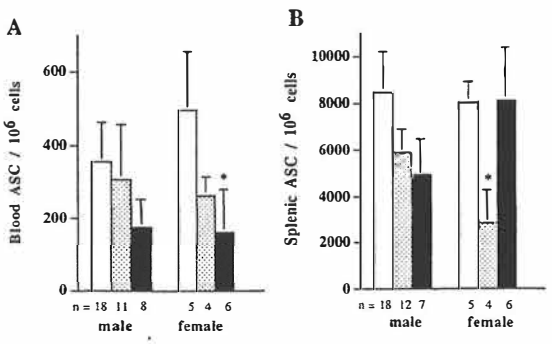


Fig. 1. Anti-BGG antibody-secreting cell (ASC) response (1a) in the blood and (1b) in the spleen after exposure to secondary-treated (shaded bar) or primary-treated (black bar) effluents. The white bar stands for the controls. Each bar represents the mean + S.E. of male or female roach sampled on day 21 after immunisation. Significant difference when compared with controls of the same sex, *, $P \leq 0.05$.

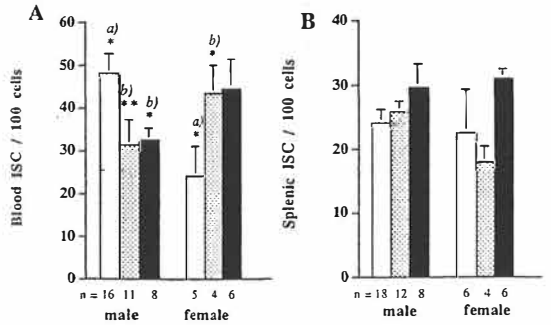


Fig. 2. Total numbers of immunoglobulin-secreting cells (ISC) (2a) in the blood and (2b) in the spleen are expressed as the number of ISC/100 lymphocytes. Each bar represents the mean + S.E. (as Fig. 1). Statistical significance between controls and between exposed and control group of the same sex, *, $P \leq 0.05$; **, $P \leq 0.01$.

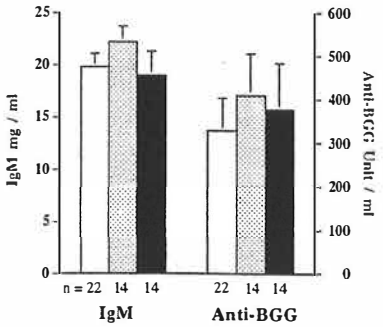


Fig. 3. Concentrations of immunoglobulin (IgM) and anti-BGG specific antibodies in the plasma of roach. Bars as Fig. 1. The results are expressed as mean + S.E.

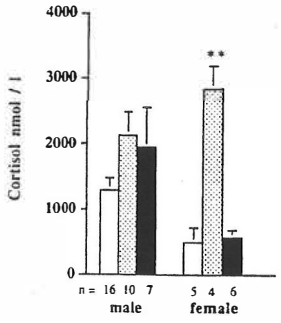


Fig. 4. Concentration of cortisol in the plasma of male and female roach after exposure to secondary-treated (shaded bar) or primary-treated (black bar) effluents. Each bar represents mean + S.E. Significant difference when compared with controls of the same sex. **, $P \leq 0.01$.

granulocytes (Table 3). No differences between the sexes were observed in either respiratory burst or the migration of granulocytes.

3.4. Hematological parameters

Sex-related differences in plasma cortisol were evident in the present study. The female control fish exhibited a lower plasma cortisol level than the male controls, although statistical significance was not reached (Fig. 4). Differences between sexes were also noted after exposure to effluents; only the secondary-treated effluent increased cortisol concentration in female roach, whereas both effluents increased cortisol levels in male roach. The increase in cortisol after exposure to the secondary-treated effluent was more distinct among the females than the males.

The hematocrit value remained unchanged in both the exposed groups (Table 4). The plasma glucose concentrations decreased in both the exposed groups, but not to statistical significance. Similarly, there was no change in the concentration of plasma lactate or in the concentrations of sodium or potassium in the fish exposed to effluents (Table 4). No differences between the sexes were observed.

3.5. The concentration of liver glycogen and the hepatic microsomal EROD activity

The female controls had lower glycogen levels in the liver than the male controls (Fig. 5, $P = 0.058$). Exposure to either of the effluents decreased the concentration of glycogen in male roach. However, a similar decreasing trend was

Table 3
Respiratory burst activity and migration (mean ±S.E.) of pronephric granulocytes from roach exposed to primary- or secondary-treated BKME

Parameter	Control	Exposed	
		Secondary treated	Primary treated
Respiratory burst activity (cpm × 10^3)	107 ± 19	111 ± 14	85 ± 8
Migration (μm)			
Random	328 ± 34	295 ± 39	336 ± 39
Directed	652 ± 37	651 ± 45	632 ± 42

not found in the female roach. The decrease after exposure to primary-treated effluent reached statistical significance ($P \leq 0.05$), when data of both the sexes were combined.

EROD activity in the liver were on the same level in the control roach irrespective of the sex. Exposure to the primary-treated effluent resulted in a twofold increase in EROD activity in the male roach (Fig. 6). On the contrary, in the female roach, increased EROD activity was noted after exposure to the secondary-treated effluent, but the change was not statistically significant.

Table 4
Hematological and blood chemistry parameters (mean ± S.E.) of control and roach exposed to primary- and secondary-treated BKME

Parameter	Control	Exposed	
		Secondary treated	Primary treated
Hematocrit (%)	42.7 ± 1.9	44.8 ± 1.5	42.4 ± 1.1
Glucose (mmol/l)	4.5 ± 0.4	3.8 ± 0.3	3.5 ± 0.3
Lactate (mmol/l)	3.2 ± 0.2	2.8 ± 0.1	3.2 ± 0.2
Sodium (mmol/l)	267 ± 9.6	258 ± 7.1	268 ± 12.3
Potassium (mmol/l)	9.3 ± 1.5	8.2 ± 2.0	8.3 ± 1.5

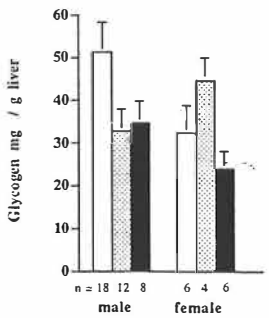


Fig. 5. The concentration of liver glycogen (mean + S.E.) of male and female roach after exposure to effluents. Bars as Fig. 1.

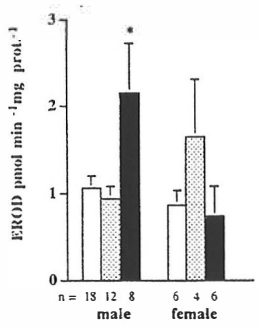


Fig. 6. The activity of hepatic microsomal 7-ethoxyresorufin *O*-deethylase (EROD) in male and female roach. Bars as Fig. 1. Significantly different when compared with controls of the same sex. *, $P \leq 0.05$.

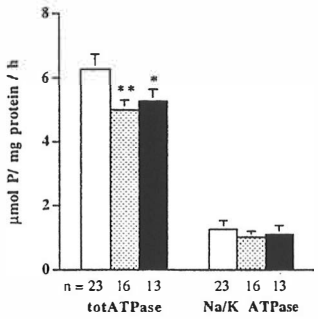


Fig. 7. The ATPase activity of gills. Bars as Fig. 1. Significantly different when compared with controls, *, $P \leq 0.05$; **, $P \leq 0.01$.

3.6. Gill ATPase activity

Exposure of the roach to either of the effluents resulted in a statistically significant decrease in gill total ATPase activity (Fig. 7). Also gill Na^+-K^+-ATPase activity was lower in both the exposed groups compared with controls but the changes did not reach statistical significance.

4. Discussion

A large number of parameters related to the function of the piscine immune system were deter-

mined in the present study. Three weeks exposure of roach to effluents from a modern pulp and paper mill using ECF/TCF bleaching induced liver EROD activity which indicated that the fish had been exposed to xenobiotics. Several immunological functions were affected and, interestingly, differences in responsiveness between the sexes were observed. In males, these effects were mainly inhibitory and were very clear after exposure to the primary-treated effluent. In females, both decreased and increased responses were observed. Further, differences in cortisol responses and EROD induction between sexes as well as between the effluents were evident.

In male roach, both the effluents, primary- and secondary-treated, impaired the immune responsiveness of the exposed fish. Even though statistical significance was not reached when compared with controls, parallel decreases in the ASC count from two sources of lymphoid cells, the blood and the spleen, strengthen the finding of suppressed immune reactivity. The results also indicated that exposure to primary-treated effluent decreases the ASC response more than exposure to secondary-treated effluent. Impaired immunoreactivity has been demonstrated earlier following exposure to secondary-treated effluent and untreated bleaching effluents (Aaltonen et al., 1997, 2000). Also the blood ISC count of males decreased after exposure to the secondary- as well as primary-treated effluents. The ISC enumeration is rarely used in immunotoxicology, probably because species-specific antibodies against immunoglobulin are required. However, the number of ISC has proved to be sensitive e.g. to the influence of BKMEs (Aaltonen et al., 1997, 2000). In the female roach, the effects of BKME exposures were not as explicit as in the case of the males. The number of ASC in the spleen decreased after exposure to secondary-treated effluent but not primary-treated effluent. Further, the number of blood ISC after exposure to the effluents increased, contrary to the decreased values in males. The small number of females in the present study hampered the comparison of the results between sexes but it is well documented in mammals that females and males have their own characteristic immunological features (Ansar Ahmed and Talal, 1990; Talal, 1992; Deschaux and Khan, 1995). Knowledge of sex-related differences in basic immunological parameters in fishes is scarce and even less is known about the effects of xenobiotics on female and male immune functions. Fournier et al. (1998) reported stronger suppression of phagocytic function in female mumminchogs (*F. heteroclitus*) than in males after exposure to BKME. BKME contains hundreds of different substances including wood-derived estrogen-like compounds (Bright et al., 1997). The mechanisms of the sex hormone regulation of the immune system are poorly known. However, receptors for estrogens and androgens on lymphocytes and macrophages (Bhalla, 1989; Savino and Dardenne, 1995; Slater et al., 1995) offer a possible route for immunomodulation.

The suppressed immunity of fish with increased plasma cortisol is well demonstrated and a high cortisol level is widely used as an indicator of stress (reviewed by Wendelaar Bonga, 1997). Moderately increased plasma cortisol levels in the male roach were noted after exposure to both effluents but in females, cortisol increased only after exposure to secondary-treated effluent. In case of a chronic stressor, cortisol levels may remain elevated, although well below acute peak-values. Such cortisol responses have been reported after prolonged exposures to variety of pollutants (reviewed by Donaldson, 1981; Barton and Iwama, 1991; Brown, 1993) and in salmonids, even a slight chronic elevation in cortisol level has been implicated in suppression of immune functions (Maule and Schreck, 1987; Maule et al., 1993; Pickering and Pottering, 1989). Indeed, in the present study, high cortisol levels appear to be associated with a low splenic ASC count (Pearson correlation test, $r = -0.295$, $P = 0.047$). The results following exposure to the primary-treated effluent in the female roach are difficult to interpret and the lack of cortisol response in these fishes does not necessarily indicate the absence of stressors (Wendelaar Bonga, 1997). The impaired cortisol response may be related to enhanced cortisol clearance or downregulation of the inter-renal response to ACTH in chronically stressed fish. For example, Hontela et al. (1992, 1997) concluded that exposure e.g. to BKMEs may lead to

exhaustion of the pituitary-interrenal axis as a result of prolonged hyperactivity of the system.

The immunological effects of the effluents in the present study were also correlated with physiological parameters often applied in studies of the effects of pulp and paper mill effluents on fish. The induction of liver mixed-function oxidase (MFO) enzyme activity has been widely used as an indicator of exposure to pulp and paper mill effluents (Owens, 1991; Stegeman et al., 1992). Recent changes in the processes and the treatment of effluents at pulp mills have decreased, but not eliminated, the induction of EROD activity in the liver of fish (Munkittrick and Sandström, 1997; Soimasuo et al., 2000). Our present results are in line with the finding that the primary-treated effluents are more potent inducers of EROD activity than secondary-treated effluents (Martel and Kovacs, 1997) and, further, with the observation that EROD induction is more prominent in male than in female fish (Lindström-Seppä, 1985; Munkittrick et al., 1994; Karels et al., 1998). In agreement with the present study, altered liver glycogen concentration has also been noted in juvenile white fish, caged in discharge areas of bleached kraft mills applying an activated sludge system in waste water treatment (Lappivaara and Oikari, 1999). An increase in plasma cortisol level has been shown to be connected with a subsequent increase in the number of branchial chloride cells and Na^+-K^+-ATPase activity (Epstein et al., 1971; Langdon et al., 1984; Laurent and Perry, 1990; Franklin et al., 1992). The present decrease in ATPase activity in roach, exhibiting high plasma cortisol levels, may be due to direct toxic actions of compounds, such as resin acids, to the gill epithelium (Tuurala and Soivio, 1982). Alternatively, the decrease in exposed fish may reflect diminished ionic gradients and reduced water and ion transfers in a slightly more salty environment in exposure aquaria compared with control aquaria.

Despite changes in pulping and bleaching process and more effective treatment of waste waters, it is obvious that fish are still exposed to toxic substances in recipient waters. The present study provides evidence that pulp and paper mill effluents, including secondary-treated ones, induce immune modulation in fish. The difference in the capability of primary- and secondary-treated effluents to affect the immune system is clearly demonstrated in the number of ASC post immunisation. Immune responsiveness is a nested assessment of the function of the immune system and the suppression of the cellular events of antibody production, i.e. the blood ASC response proved to be a sensitive indicator of exposure. The exposure of fish to effluents on-site in contaminated waters for a longer period is needed to answer the question as to whether the immunomodulation observed in the present study is manifested in lowered resistance to diseases. The present findings suggest that sex hormones are involved in immunomodulation after exposure to BKME and thus emphasise that sex-related factors should not be ignored in the assessment of immunotoxicity.

Acknowledgements

This study was supported by the Board of Environmental Science of the Academy of Finland, by the University of Jyväskylä and by the Graduate School on Fish Biology and Fisheries in Finland. The authors thank O. Järvinen for catching the fish and Elina Virtanen for the enumeration of ASC and ISC.

References

Aaltonen, T.M., Jokinen, E.I., Valtonen, E.T., 1994. Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. Fish Shellfish Immunol. 4, 129–140.

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. Ecotoxicol. Environ. Saf. 38, 266–271.

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

Anonymous, 1997. The Finnish background report for the EC documentation of best available techniques for pulp and paper industry. Ministry of the Environment, Report 96, Helsinki, Finland, 138 pp.

Ansar Ahmed, S., Talal, N., 1990. Sex hormones and the immune system — part 2. Animal data. Bailliere's Clin. Rheumatol. 4, 13–31.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the responses and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3–26.

Bhalla, A.K., 1989. Hormones and the immune response. Ann. Rheum. Dis. 48, 1–6.

Billiard, S.M., Querbach, K., Hodson, P.V., 1999. Toxicity of retene to early life stages of two freshwater fish species. Environ. Toxicol. Chem. 18, 2070–2077.

Bright, D., Hodson, P.V., Lehtinen, K.-J., McKague, B., Rodgers, J., Solomon, K., 1997. Evaluation of ecological risks associated with the use of chlorine dioxide for the bleaching of pulp. Scientific progress since 1993. In: Proceedings of the Third International conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents. Millenium, Rotarua, New Zealand, November 9–13, 1997. pp. 11–20.

Brown, J.A., 1993. Endocrine responses to environmental pollutants. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman and Hall, London, pp. 276–296.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer, T.T., 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem. Pharmacol. 34, 3337–3345.

Deschaux, P., Khan, N.A., 1995. Immunophysiology: the immune system as a multifunctional physiological unit. Cell. Mol. Biol. 41, 1–10.

Donaldson, E.M., 1981. The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering, A.D. (Ed.), Stress in Fish. Academic Press, London, pp. 11–47.

Epstein, F.H., Cynamon, M., MacKay, W., 1971. Endocrine control of Na-K-ATPase and saltwater adaptation in *Anguilla rostrata*. Gen. Comp. Endocrinol. 16, 323–328.

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. Ecotoxicol. Environ. Saf. 40, 177–183.

Franklin, C.E., Forster, M.E., Davison, W., 1992. Plasma cortisol and osmoregulatory changes in sockeye salmon transferred to sea water: comparison between successful and unsuccessful adaptation. J. Fish. Biol. 41, 113–122.

Hodson, P.V., McWhirter, M., Ralph, K., Gray, B., Thivierge, D., Carey, J.H., van der Kraak, G.J., Whittle, D.M., Levesque, M.-C., 1992. Effects of bleached kraft mill effluent on fish in the St. Maurice river, Quebec. Environ. Toxicol. Chem. 11, 1635–1651.

Hontela, A., Rasmussen, J.B., Audet, C., Chevalier, G., 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch. Environ. Contam. Toxicol. 22, 278–283.

Hontela, A., Daniel, C., Rasmussen, J.B., 1997. Structural and functional impairment of the hypothalamo-pituitary-interrenal axis in fish exposed to bleached kraft mill effluent in the St Maurice River, Quebec. Ecotoxicology 6, 1–12.

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*. Ecotoxicol. Environ. Saf. 32, 219–225.

Karels, A., Soimasuo, R., Lappivaara, H., Leppänen, H., Aaltonen, T., Mellanen, P., Oikari, A., 1998. Effects of ECF-bleached kraft mill effluent on reproductive steroids and liver MFO activity in populations of perch and roach. Ecotoxicology 7, 123–132.

LaFleur, L.E., Barton, D.A., 1997. Pulp mill effluent characterization and treatment: an update. In: Proceedings of the Third International Conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents, Millenium, Rotarua, New Zealand, November 9–13, 1997, pp. 1–10.

Langdon, J.S., Thorpe, J.E., Roberts, R.J., 1984. Effects of cortisol and ACTH on gill Na^+/K^+-ATPase, SDH and chloride cells in juvenile atlantic salmon *Salmo salar* L. Comp. Biochem. Physiol. 77A, 9–12.

Lappivaara, J., Oikari, A., 1999. Altered challenge response in whitefish subchronically exposed in areas polluted by bleached kraft mill effluents. Ecotoxicol. Environ. Saf. 42, 212–222.

Laurent, P., Perry, S.F., 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tissue Res. 259, 429–442.

Lim, A.L., Ip, Y.K., 1989. Effect of fasting on glycogen metabolism and activities of glycolytic and gluconeogenic enzymes in the mudskipper *Boleophthalamus boddaerti*. J. Fish. Biol. 36, 349–367.

Lindström-Seppä, P., 1985. Seasonal variation of the xenobiotic metabolizing enzyme activities in the liver of male and female vendace (*Coregonus albula* L.). Aquat. Toxicol. 6, 323–331.

Lindström-Seppä, P., Oikari, A., 1989. Biotransformation and other physiological responses in whitefish caged in a lake receiving pulp and paper mill effluents. Ecotoxicol. Environ. Saf. 18, 191–203.

Martel, P., Kovacs, T., 1997. A comparison of the potential of primary- and secondary-treated pulp and paper mill effluents to induce mixed function oxygenase (MFO) activity in fish. Water Res. 6, 1482–1488.

Maule, A.G., Schreck, C.B., 1987. Changes in the immune system of Coho salmon (*Oncorhyncus kisutch*) during the parr-to-smolt transformation and after implantation of cortisol. Can. J. Fish. Aquat. Sci. 44, 161–166.

Maule, A.G., Schreck, C.B., Sharpe, C., 1993. Seasonal changes in cortisol sensitivity and glucocorticoid receptor affinity and number in leucocytes of coho salmon. Fish Physiol. Biochem. 10, 497–506.

Munkittrick, K.R., Sandström, O., 1997. Ecological assessment of pulp mill impacts: issues, concerns, myths and research needs. In: Proceedings of the Third International Conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents, Millenium, Rotarua, New Zealand, November 9–13, 1997, pp. 379–390.

Munkittrick, K.R., van der Kraak, G.J., McMaster, M.E., Portt, C.B., van den Heuvel, M.R., Servos, M.R., 1994.

Survey of receiving water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity and plasma sex sterols levels in white sucker. Environ. Toxicol. Chem. 13, 1089–1101.

Nelson, R.D., Quie, P.G., Simmons, R.L., 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115, 1650–1656.

Noll, H.U., 1974. L-(+)-lactate: determination with LDH, GPT and NAD. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, London, p. 1475.

Örså, F., Holmbom, B., 1994. A conventional method for the determination of wood extractives in papermaking process waters and effluents. J. Pulp Paper Sci. 20, J361–J365.

Owens, J.W., 1991. The hazard assessment of pulp and paper effluents in the aquatic environment: a review. Environ. Toxicol. Chem. 10, 1511–1540.

Pickering, A.D., Pottering, T.G., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem. 7, 253–258.

Salo, H.M., Aaltonen, T.M., Markkula, S.E., Jokinen, E.I., 1998. Ultraviolet B irradiation modulates the immune system of fish (*Rutilus rutilus*, Cyprinidae) I. Phagocytes. Photochem. Photobiol. 67, 433–437.

Savino, W., Dardenne, M., 1995. Immune-neuroendocrine interactions. Immunol. Today 16, 318–322.

Schrock, R.M., Beeman, J.W., Rondolf, D.W., Haner, P.V., 1994. A microassay for gill sodium, potassium-activated ATPase in juvenile Pacific salmonids. Trans. Am. Fish. Soc. 123, 223–229.

Scott, A.L., Klesius, P.H., 1981. Chemiluminescence: a novel analysis of phagocytosis in fish. Dev. Biol. Stand. 49, 243–254.

Slater, C.H., Fitzpatrick, M.S., Schreck, C.B., 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen. Comp. Endocrinol. 100, 218–225.

Södergren, A., 1993. Bleached pulp mill effluents. Composition, Fate and Effects in the Baltic Sea. Final Report from the Environment/Cellulose Project II, Swedish National Environmental Protection Board, Solna, 150 pp.

Soimasuo, R., Aaltonen, T., Nikinmaa, M., Pellinen, J., Ristola, T., Oikari, A., 1995a. Physiological toxicity of low-chlorine bleached pulp and paper mill effluent on whitefish (*Coregonus lavaretus* L. s.l.): a laboratory exposure simulating lake pollution. Ecotoxicol. Environ. Saf. 31, 228–237.

Soimasuo, R., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T., Oikari, A., 1995b. Biomarker responses along a pollution gradient: effects of pulp and paper mill effluents on caged whitefish. Aquat. Toxicol. 31, 329–345.

Soimasuo, M.R., Karels, A.E., Leppänen, H., Santti, R., Oikari, A.O.J., 1998. Biomarker responses in whitefish (*Coregonus lavaretus* L. s.l.) experimentally exposed in a large lake receiving effluents from pulp and paper industry. Arch. Environ. Contam. Toxicol. 34, 69–80.

Soimasuo M.R., Karels A.E., Oikari, A.O.J., 2000. Utility of fish biomarkers to assess effects of process alterations and secondary treatment of BKME by field and laboratory experiment. In: Stutridge, T.R., Gifford, J.S., Marvin, N.A., Slade, A.H. (Eds.), Environmental Fate and Effects of Pulp and Paper Mill Effluents. SETAC Press, Pensacola, FL, USA, in press.

Stegeman, J.J., Brouwer, M., di Giuilio, R.T., Förlin, L., Fowler, B.A., Sanders, B.M., van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein system as indicators of contaminant exposure and effect. In: Hugget, R.J., Kimerle, P.M., Mehrle Jr, P.M., Bergman, H.L. (Eds.), Biomarkers, Biochemical, Physiochemical, and Histological Markers of Anthropogenic Stress. Lewis Publishers, Chelsea, pp. 235–335.

Talal, N., 1992. Sex hormones and immunity. In: Roitt, I.M., Delves, P.J. (Eds.), Encyclopedia of Immunology. Academic Press, San Diego, pp. 1369–1371.

Tuurala, H., Soivio, A., 1982. Structural and circulatory changes in the secondary lamellae of *Salmo gairdneri* gills after sublethal exposures to dehydroabietic acid and zinc. Aquat. Toxicol. 2, 21–29.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.