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SYTYKE 10

K.-J. LEHTINEN & J. TANA

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PART I

EFFECTS ON SURVIVAL, GROWTH, PARASITES AND PHYSIOLOGICAL STATUS IN FISH EXPOSED IN MESOCOSMS TO EFFLUENTS FROM BLEACHED HARDWOOD KRAFT PULP PRODUCTION

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Abstract
Brood of the three-spined stickleback (*Gasterosteus aculeatus*) and juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed at mesocosm experiments to untreated and treated total effluents from two mills producing bleached hardwood kraft pulp with the sequence (C20+D80)(EOP)DED and O(D27,C68+D5)(EOP)D(EP)D respectively. The sticklebacks were exposed for 5.5 months and the rainbow trout for 8 weeks in pools connected to the outgoing water from the mesocosms. In addition to exposure to effluents one group of each fish species was exposed to a sterols, mainly sitosterol, a steroid compound from the wood raw material. Effects on growth, liver tissue structure and parasite frequency on the sticklebacs were obtained. In the rainbow trout effects on carbohydrate metabolism (increased liver glycogen) and liver tissue structure were observed. Responses on liver glycogen corresponded with vacuolization of the liver. The responses of both fish species were similar in the groups exposed to the mill effluents as in that exposed to sitosterol only.
The bile concentrations of resin acids and chlorophenols were low, especially in the groups exposed to treated effluents and gave no correlation with the effects noted.
Increased levels of sitosterol and other steroids were observed in the exposed trout. Also the level of bound cholesterol increased in both effluent and steroid treated fish, indicating that the cholesterol metabolism was affected. The mechanisms and the biochemical significance are so far not known.
Treatment had no obvious effect on the responses observed in the fish, indicating that responsible substances were not removed by external treatment. Total concentrations of AOX did not correspond with the effects noted.

Keywords
Bleaching, pulp, hardwood, environmental effects, mesocosms, fish, growth, histology, physiology, mortality

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Tiivistelmä
Kolmipiikin (*Gasterosteus aculeatus* L.) poikasia sekä kirjolohia (*Oncorhynchus mykiss*) altistettiin malliekosysteemikokeissa käsittelemättömiin ja ulkoisesti käsiteltyihin massateollisuusjätevesiin. Jätevedet olivat peräisin valkaistua sulfaattikoivumassaa tuottavista tehtaista. Tehtaat valkaisevat massaa seuraavilla sekvenseillä : A) (D80+C20)(EOP)DED ja B) O(D27,C68+D5)(EOP)D(EP)D.
Kolmipiikin poikaset altistettiin 5,5 kuukauden ja kirjolohet 8 viikon ajan altaissa, jotka oli kytketty malliekosysteemeistä poistuvaan veteen. Yhtä ryhmää altistettiin puuraaka-aineessa esiintyville steroideille (pääasiassa sitosterolille). Kolmipiikeissä havaittiin kokeen aikana kasvun kiihtymistä, maksamuutoksia, sekä lisääntynyt loismäärä. Kirjolohissa havaittiin muutoksia hiilihydraattiaineenvaihdunnassa (korkea maksan glykogeenipitoisuus) ja maksakudoksessa. Maksan glykogeenipitoisuudet korreloivat maksan vakuolisaation kanssa. Molempien kalalajien jätevesialtistuksesta saadut vaikutukset olivat samankaltaiset kuin pelkästään steroleille altistetuissa kaloissa havaitut vaikutukset.
Sappinesteen hartsihappo- ja kloorifenolipitoisuudet olivat alhaiset, varsinkin ulkoisesti puhdistetuille jätevesille altistetuissa kaloissa.
Altistetuista kirjolohista tavattiin kohonneita sitosteroli ja muita steroidipitoisuuksia. Myös konjugoituneen kolesterolin pitoisuus oli kohonnut sekä steroidille että jätevesille altistettujen kalojen sappinesteessä, mikä viittaa siihen, että kolesterolin aineenvaihdunnassa oli tapahtunut muutoksia. Takana olevat mekanismit, sekä biokemiallinen merkitys ovat toistaiseksi tuntemattomat.
Jätevesien ulkoisella puhdistuksella ei ollut ilmeisiä vaikutuksia kaloissa havaittuihin muutoksiin. Tämä viittaa siihen, että ulkoinen puhdistus ei poistanut vaikuttavia aineita jätevesistä. AOX:n kokonaispitoisuudet eivät korreloineet havaittujen vaikutusten kanssa.

Asiasanat (avainsanat)
Valkaisu, sellu, lehtipuut, malliekosysteemit, ympäristövaikutukset, kalat, kasvu, histologia, fysiologia, kuolleisuus

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Referat
Två blekprocesser var föremål för undersökningen. I det första alternativet blektes björkmassan enligt sekvensen (D80+C20)(EOP)DED (alt. A). I det andra alternativet blektes massan enligt sekvensen O(D27,C68+D5)(EOP)D(EP)D (alt. B).
Vid försöket exponerades yngel av storspigg (*Gasterosteus aculeatus*) samt regnbåge (*Oncorhynchus mykiss*) under 5,5 månader respektive 8 veckor i bassänger tillkopplade till utgående vatten från modellekosystem. Därtill exponerades en grupp av respektive fiskart för ett pulver innehållande steroider förekommande i träråvaran. Effekter på tillväxt, leverstruktur och parasitfrekvens erhölls hos yngel av storspigg. Hos regnbåge noterades effekter på kolhydratmetabolism (förhöjt leverglykogen) och leverstuktur. De leverstrukturella förändringarna korrelerade med de förhöjda leverglykogenvärdena. Responsen hos båda fiskarter var likartad i de grupper som exponerades för avloppsvatten som i den grupp som exponerades för steroider.
Koncentrationerna i gallvätska av konjugerade klorfenoliska substanser och hartssyror var låga, speciellt i de grupper som exponerades mot behandlade avloppsvatten och visade ingen korrelation med erhållna effekter. Förhöjda koncentrationer av sitosterol samt andra steroider observerades i exponerad regnbåge. Likaså ökade koncentrationen av konjugerat kolesterol i fiskgalla i såväl streroid- som avloppsvattenexponerade grupper, vilket indikerar att kolesterolomsättningen påverkats. Bakomliggande mekanismer och biokemiskt betydelse är okända för närvarande.
Externbehandling av avloppsvattnen hade ingen uppenbar effekt på noterade effekter, vilket indikerar att effektorsakande substanser inte avlägsnats vid behandlingen. Erhållna effekter korrelerade ej heller med doserade AOX-mängder.

Sakord (nyckelord)
Blekning, cellulosa, lövträd, modellekosystem, miljöeffekter, fisk, tillväxt, histologi, fysiologi, dödlighet

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Einflüsse auf Überleben, Wachstum, Parasiten und physiologischen Zustand in Fischen, die in einem Modellökosystem unbehandelten und behandelten Gesamtabwässern aus der Papierfaserstoffherstellung aus Laubholtz ausgesetzt wurden

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Zusammenfassung
Eine Brut von dreidornigen Stichlingen, *Gasterosteus aculeatus*, und junge Regenbogenforellen, *Oncorhynchus mykiss*, wurden in Versuchen im Modellökosystem unbehandelten und behandelten Gesamtabwässern aus zwei Fabriken ausgesetzt, in denen gebleichter Kraftzellstoff aus Laubholtz mit der Folge (C20+D80)(EOP)DED und O(D27,C68+D5)(EOP)D(EP)D hergestellt wurde. Die Stichlinge wurden für 4,5 Monate und die Regenbogenforellen für 8 Wochen in Becken ausgesetzt, die mit dem abgehenden Wasser aus dem Modellökosystem verbunden waren. Ausser der Aussetzung den Abwässern wurde aine Gruppe von jeder Fischart den Sterolen, hauptsächlich den Sitosterolen, einem Steroid aus dem Holtzrohmaterial, ausgesetzt. Es wurden Einflüsse auf Wachstum, Lebergewebestruktur und Parasitenhäufigkeit in den Stichlingen beobactet. In den Regenbogenforellen wurden Einflüsse auf den Kohlenhydratstoffwechsel (zugenommenes Leberglykolen) und auf die Lebergewebestruktur beobactet. Reaktionen beider Fischarten waren in den Gruppen, die den Fabrikabwässern ausgesetzt waren, ähnlich wie in denen, die nur dem Sitosterol ausgesetzt waren.
Die Harzsäure- und Chlorphenolkonzentrationen der Galle Waren niedrig, besonders in den Gruppen, die den behandelten Abwässern ausgesetzt waren, und gaben keine Korrelation mit den beobachteten Einflüssen.
Zugenommene Niveaus von Sitosterol und anderen Steroiden wurden in den ausgesetzten Regenbogenforellen beobachtet. Auch das Niveau von gebundenem Cholesterin nahm sowohl in abwasser- als in steroidbehandelten Fischen zu, was darauf hinwies, dass der Cholesterinmetabolismus beeinflusst war. Der Mechanismus und die biochemische Bedeutung sind bis jetzt nicht bekannt.
Die Behandlung hatte keinen deutlichen Einfluss auf die Reaktionen, die in den Fischen beobachtet wurden, was darauf hinwies, dass wirksame Stoffe nicht durch die äussere Behandlung entfernet waren. Die Gesamtkonzentrationen von AOX entsprachen nicht mit den beobachteten Einflüssen.

Stichwörter
Bleichen, Zellstoffe, Laubholz, Einflüsse, Fische, Wachstum, Histologie, Physiologie, Mortalität

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This is a report from a Finnish–Swedish joint–project on effects of pulp mill effluents in mesocosms. The work is part of a systematic research for evaluation of the environmental impact of effluents from pulp mills with different bleaching procedures and effluent treatment solutions.

The work was done at FERG's[1)] Baltic Sea Research Laboratory in co–operation with Åbo Akademi, Kuopio University and Helsinki University. The work was financed by the Finnish forest industry companies Kymmene Oy, Enso–Gutzeit Oy, Metsä–Botnia Oy, Veitsiluoto Oy and the SYTYKE–project. The Swedish financing part was the Swedish Industries Foundation for Water and Air Pollution (SSVL).

1) FERG = Finnish Environmental Research Group

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2 Chemical-analytical methods

1 INTRODUCTION

Since the 1970s, the processes for production of bleached kraft pulp have been changed towards lower use of elementary chlorine in the bleachery. Most bleacheries in the Nordic countries are today equipped with oxygen pre-delignification. Reinforced alcaline extraction using oxygen and/or peroxide is also becoming more frequent. All these measures are aimed at reducing or eliminating the environmental impact of bleached pulp production.

The fish populations constitute an important part of the aquatic ecosystem and at the same time they are an important food resource for man. Thus, the well-being of the fish populations in the long-term perspective is of great importance when the environmental impact of the pulp mill industry is discussed.

The effects on growth, mortality, incidence of parasites and infection, and the general physiological status of fish exposed to total effluents from the production of bleached softwood pulp, were studied in mesocosm in the 1980s (Lehtinen, 1989; Lehtinen et al. 1990). In total, six different mill effluents with different softwood pulp bleaching procedures were studied. The traditional bleaching sequence (C95+D5)EHDED was used as a reference. The results on fish from the studies mentioned above showed that the highest impact was obtained at exposure to effluent from the traditional bleaching sequence using a high proportion of elementary chlorine. The lowest impact was obtained in mesocosms exposed to externally treated effluent from a mill with the bleaching sequence O(C85+D15)EDED or untreated effluent from bleaching according to the sequence O(C52+D48)EDED.

Regarding possible effects of specific substances (such as chlorinated phenolics) the results did not show any clear correlation between concentrations and effects. The experiments also showed that effluents from processes producing chlorinated organic substances (measured as Total Organic Chlorine) around 2 kg/Adt of pulp induced small or no effects (excluding effects from chlorate) in the mesocosms (Rosemarin et al. 1990).

In 1986, an experiment using effluents from three different mills producing fully bleached hardwood pulp was performed (Hemming and Lehtinen 1988). The effluents were not externally treated. Despite the lack of physiological studies on fish, the results clearly indicated that effects on fish were prevalent. The environmental impact of these effluents, from processes with TOCl-discharges between 0.5-1.0 kg/Adt, was found to be of similar magnitude as that recorded with effluents from softwood kraft pulping having TOCl emissions of about 2 kg/Adt and higher (Lehtinen et al. 1991).

Thus, the general picture from experiments performed up to 1986in mesocosms showed a lack of correlation between biological effects and emissions of chlorinated organic compounds. The tested effluents from production of bleached hardwood pulp were untreated and information about the consequences of external treatment of such effluents on the general effect picture in mesocosms was lacking. Therefore, two effluents from production of bleached hardwood pulp with different bleaching processes and external treatment technique were tested in 1990. The effluents were tested before and after external treatment.

The present report is dealing with the effects on survival, growth and disease in the brood of three-spined sticklebacks, *Gasterosteus aculeatus* L., and the physiological status of rainbow trout, *Oncorhynchus mykiss*, exposed to these effluents.

2 MATERIAL AND METHODS

2.1 Processes and emissions

The two kraft pulp mills tested in the present work are called mill A and mill B. Untreated mill effluent is called "Au" and "Bu", and treated effluent "At" and "Bt" respectively.

Mill A is producing fully bleached hardwood pulp according to the sequence (C20+D80)(EOP)DED. The effluent is treated in an activated sludge plant with a residence time of about 24 hrs. Mill B is producing fully bleached market pulp using the sequence O(D27,C68+D5)(EOP)D(EP)D. The effluent is treated in an aerated lagoon with a residence time of about 8–9 days.

Production of pulp and process data are presented in table 1.

The details of sampling procedures are reported elsewhere (ÅF-IPK). A short description is given below.

The effluent samples where taken as grab samples at the respective mill under normal running conditions. The samples used in the mesocosm experiments were collected in 1 m^3 polyethylene containers. Enough time was allowed to pass between sampling of ingoing and outgoing water in the biological treatment plants in order to account for the residence time. Smaller samples for chemical characterization were taken with fixed intervals at strategic sampling points in the mills in order to control the process conditions.

Technical disorders at mill B made sampling of a representative treated effluent impossible. An untreated sample was consequently collected and transported to the laboratory, where it was treated in a pilot plant aerated lagoon activated with the bacterial sludge from mill B:s aerated lagoon. The efficiency of the treatment in terms of the reductions of BOD and COD, was very much alike that normally reported from the mill (Table 2).

The concentrations of chlorinated phenolics, resin acids, chlorinated resin acids and steroids are presented in Table 3.

Total gas-chromatographically eluted extractives are presented in table 4.

After sampling the effluents were transported to the laboratory. In order to eliminate the chlorate present in the effluents, the containers were sealed air tight to reach as low oxygen levels as possible during the first 24 hrs after sampling (cf. Rosemarin 1987).

At the laboratory the effluents were transferred to smaller, 30 l polyethylene containers and thereafter frozen in -30 °C. The effluents were placed in freezer within roughly 30 hours after sampling. Required amounts where thereafter thawed and used in the experiments.

The effluents were dosed using membrane pumps (Prominent Electronic) at 400

and 2000 times dilutions based on a normalized effluent volume of 50 m^3 t^{-1} pulp. Exposure of the mesocosms commenced on June 26, 1990 for mill A. Due to the technical difficulties at mill B effluent was obtained later and the exposure could not start before July 12. An extra control mesocosm pool was used in order to obtain data regarding the ecological events which took place during this two-week period.

Table 1. Production of pulp/day, effluent flow (m^3/t pulp) and some running data of the two different pulp mills tested.

	Mill A	Mill B
Production t_{90} d^{-1}	1 450	945
Effluent flow m^3 t^{-1}	40	54
Bleaching sequence	(D80+C20)(EOP)DED	O(D27,C68+D5)(EOP)D(EP)D
ClO_2 in D+C kg/t90	34.7	5.3
Cl_2 in D+C kg/t90	9.8	11.1
ClO_2 amount (%)	80	32
Kappa number to bleachery	15	13
Chlorine multiple	0.06	0.08
Active chlorine multiple	0.28	0.12
Total ClO_2 kg/t90	67	35
NaOH E1 kg/t90	19	11.1
NaOH E2 kg/t90	4	4.6
Oxygen E1 kg/t90	4.5	4.6
H_2O_2 E1 kg/t90	1.3	1.0
H_2O_2 E2 kg/t90	–	1.0
Viscosity dm^3 kg^{-1}	1 075	1 025

Table 2. COD, BOD_7, AOX and chlorate levels before and after treatment of Mill A:s and B:s effluents.

Mill	COD mg l^{-1}	BOD mg l^{-1}	AOX mg l^{-1}	Chlorate kg t^{-1} pulp
Mill A				
Untreated, Au	2100	800	39	5.8
Treated, At	910	100	23	–
Mill B				
Untreated, Bu	540	145	17*)	2.0
Treated (pilot), Bt	320	20	7.3	–

– = not detected

*) analyzed at STFI, Sweden. Other AOX-values analyzed at Enso-Gutzeit's Research Centre.

Table 3. Concentrations ($\mu g\ l^{-1}$) of chlorinated phenolics (Cl–P), chlorinated guaiachols (Cl–G), resin acids (RA), chlorinated resin acids (Cl–RA) and steroids in the untreated and treated effluents from mill A and B. Au = untreated mill A; At= treated mill A; Bu = untreated mill B; Bt = treated mill B.

	Cl–P	Cl–G	RA	Cl–RA	Steroids
Au	15	28	1 700	110	870
At	0.5	14	100	49	190
Bu	19	8	176	29	17
Bt	8	2	10	30	18

Table 4. Total amount of extractives eluted from the gas chromatograph from the untreated and treated effluents from mill A and B. Au = untreated mill A; At= treated mill A; Bu = untreated mill B; Bt = treated mill B.

Effluent	Total extractives ug l^{-1}
Au	21 900
At	6 900 – 12900
Bu	2 000 – 2 700
Bt	760 – 1 100

2.2 The mesocosm set–up

The mesocosms are made up of out–door circular pools with an inner polyethylene coating and a volume of 8 m^3. The pools are supplied with a continuous supply of brackish water (2.8 l min^{-1}), pumped from 10 meters depth from an unpolluted brackish water bay close to the laboratory in Nagu, SW Finland.

The bottom of each mesocosm pool was covered with a layer of pure sand without organic material. The total sand volume added to each pool was 0.25 m^3 giving a sediment thickness of about 3 cm. Bladder–wrack, *Fucus vesiculosus*, specimens were collected in the field using polyethylene 40x60 cm bags, which were lipped over the alga including the stone to which it was attached. The bag containing the alga and associated animals (see Lehtinen et al. 1988) was lifted to the surface, excessive water cautiuosly removed, and taken to the laboratory. The volume of every specimen was thereupon determined using the water displacement method. Bladder–wrack specimens were added evenly to each pool in the same region of the pools in order to obtain identical insolation for the plants. The number and kinds of different animal taxa were estimated and in case of some taxa lacking from a pool, these were added in similar numbers in all pools. Some free–swimming predator species (fish) were added in known numbers; one hundred stickleback, *Gasterosteus aculeatus*, brood and 7 bleaks, *Alburnus alburnus*, were placed into each pool (Fig. 1).

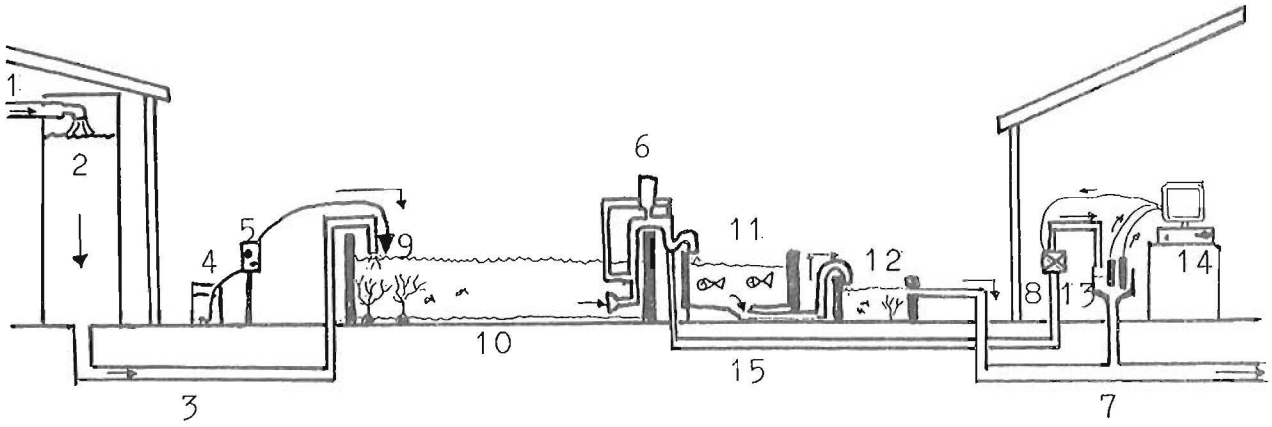

Fig 1. The mesocosm set-up

1. Incoming water
2. Seawater tank
3. PEH-pipe to the pools
4. Effluent container
5. Membrane pump
6. Siphon for out-going water
7. PEH-pipe for out-going water
8. Electric valve
9. Dosage of effluent water
10. Mesocosm pool
11. Rainbow trout tank
12. Stickleback tank
13. Registration electrodes
14. Computer

In order to enable periodic and specific controlled studies on growth, tissue structure, parasites and diseases in sticklebacks as well as the physiological status in rainbow trout, two additional smaller pools were connected to the out-going water from each of the mesocosms. One of the pools (500 l volume) was used for exposure of rainbow trouts, *O. mykiss*, (section 2.4 below) for physiological measurements (see Lehtinen et al. 1990 for details).

The other one, with a volume of 180 l was used for measurements of monthly growth, mortality, tissue structure and disease (section 2.3 below) of three-spined stickleback, *Gasterosteus aculeatus*, brood (see Lehtinen 1989 for details). The principal experimental set-up is presented in figure 1.

2.3 The stickleback experiment

A large number of maturing sticklebacks were collected by dip-nets from the brackish water bay outside the laboratory during the first week of June, 1990. The sticklebacks were stored in a 500 l pool with continuous renewal of brackish water. After the experimental set-up was finished (Fig. 1) and the systems stabilized (Rosemarin et al. 1990), three females and two males were distributed to each 180 l pool. The pools were supplied with a 3 cm thick sandsubstrate and a specimen of bladder-wrack attached to a stone in order to give shelter and material for nest-building. Soon after introduction to the pools the sticklebacks started to show territoriality and nestbuilding activities. Between June 18 and 20 stickleback larvae were detected in the pools. The larvae were counted and 50 individuals were returned into their respective pool and

100 to the mother mesocosm pool to serve as predators in the system. Due to the later start with Mill B:s effluents it is to be remembered that the fish populations exposed to these effluents were exposed for about two weeks less than the fish exposed to Mill A:s effluents.

The first measurement on growth and survival in all groups was made at commencement of the exposure with Mill A:s effluents i.e. on June 26th. The water level of the pools was lowered to a few centimeters and the fry caught with a dipnet. After capture the fish were individually weighed *in vivo* using a Mettler 300 electronic balance and thereupon returned into the pool. The fish where weighed every fourth week. The length was also measured after 8 weeks of exposure. Based on length and weight data the condition factor of the fish was measured. At the October measurements, 10 fish were randomly removed and fixed in Bouins solution for later histological and parasitological analysis.The fish were dehydrated, embedded in paraffin and sliced into 5 μm sections with a microtome and stained with hematoxylin–eosin (Romeis 1965). The entire experiment was maintained for 4.5 months. The fish fed on natural food produced in the system or introduced via in–coming seawater.

At the end of the experiment the remaining fish were frozen for later chemical analysis.

The length and weight of the fish in the big pools were measured at the end of the experiment and the condition factors established.

2.4 The rainbow trout experiment

In total 250 rainbow trout, *Oncorhynchus mykiss*, (mean weight 224±69 g) were evenly distributed into ten 500 l polyethylene pools connected to the outgoing water from the mesocosms. The water flow through the systems was 2.8 l min^{-1} , giving a retention time of about 3 hours. The experiment started on September 3 and ended on October 29, 1991. Sampling for physiological parameters was made after 2 and 8 weeks. At the two week sampling, only a restricted physiological program was carried out. Histology and livermetabolic parameters (glycogen, lipid and water content) were excluded. At the first sampling, 10 fish were analyzed and the remaining 15 after 8 weeks. In total 8 fish (3.2%) died during the exposure, 1 in Au and Bt, low dose, two in At high dose, and two in Bu high and low dose respectively. No fish died in the control group. The fish were daily fed *ad libitum* with commercial fish food

2.4.1 Sampling procedure

At sampling the fish was caught with a dipnet and stunned with a blow on the head. Blood was collected from caudal vessels with a disposable syringe. About 0.5 ml blood was taken using chrystalline ammonium heparin as anticoagulant. Blood hematocrit was determined immediately on this sample with a Compur Microspin hematocrit centrifuge. Moreover, blood smears were made for white blood cell

differential counts. The remaining blood sample was stored in a plastic tube at about +4 °C and transported to the laboratory for determination of hemoglobin and red blood cell numbers. Additionally about 1–2 ml blood was sampled without anticoagulant and transferred to a plastic tube where the blood was allowed to coagulate under 2 hrs. After this the sample was centrifuged and about 200 µl serum was collected into a plastic tube. After this the sample was centrifuged and serum was collected into plastic tubes and frozen on dry ice. The frozen samples were kept on dry ice until the analyses were carried out.

After blood sampling the fish was weighed and the length was measured. External and internal lesions were checked, whereupon the fish was opened, the sex determined and the gallbladder emptied using a 1 ml disposable syringe. The bile was collected in a 5 ml glass septum bottle in samples of 3/bottle.

The samples were frozen for later analysis of conjugated chlorophenolics and resin acids. The bile was also used for analysis of free and bound steroids and fatty acids in an attempt to find explanations for previously noted aberrations in growth and certain physiological responses in fish. After the bile sampling the liver was weighed, cut into two pieces, and stored in Eppendorf tubes frozen in liquid nitrogen for later analysis of enzyme activities and different metabolic parameters.

2.4.2 Analytical methods

Hematology

The hemoglobin concentration was determined spectrophotometrically according to the cyanmethemoglobin method. The number of red blood cells was counted according to a standard clinical–chemical method. The blood was diluted with an isotonic salt solution, whereupon the count was performed using a Clinicon cell counter. The erythrocyte constants MCHC (Mean Cell Hemoglobin Concentration), MCH (Mean Cell Hemoglobin) and MCV (Mean Cell volume) were calculated according to standard formulae.

Differential count of different blood cells (RBC, immature RBC, lymphocytes, granulocytes and thrombocytes) was made by counting under light microscope after staining (Pappenheim) (Sandoz, 1973). At least 2000 cells/ind. were counted. From the per centual proportion of different blood cells the number of leucocytes and immature red blood cells per liter blood was calculated.

The classification of immature erythrocytes was performed according to Härdig (1978).

Histology

Liver samples from the five first fishes sampled in each group were taken in order to compare the reactions between older fish like rainbow trout and fish larvae (stickle-backs) on the tissue level. The liver samples were treated identically with the stickleback samples.

Enzyme analysis

Analysis of aspartate aminortransferase (ASAT) and alanine aminotransferase (ALAT) was made at the department of clinical-chemistry at the Univ. of Agricultural Sciences, Uppsala, Sweden. A Corbas Mira autoanalyzer was used at the analysis.

Xenobiotic biotransformation was studied by measuring the activity of the enzyme 7-ethoxyresorufin-O-deethylase (EROD), as part of the mixed-function oxidase (MFO) system, and the conjugation enzyme UDP glucoronosyltransferase (UDPGT). The methodology, applied at the department of physiology at the University of Kuopio, Finland, was used (Lindström-Seppä 1990).

Other biological analyses

Liver-somatic index (LSI) and condition factors were calculated according to standard formulae. Liver lipid, glycogen, and water content were analysed at the department of Zoophysiology at Helsinki University, Finland, according to standardized methods (Soivio and Virtanen 1981).

2.5 Additional experiments

In parallel with exposure of three-spined stickleback brood and rainbow trout to total mill effluents from Mill A and B, an experiment using a steroid powder extracted from the black-liquor at a mill , was carried out with both fish species. The powder mainly contains beta-sitosterol (and to a lesser extent alfa-sitosterol and some other sterols), which is occurring in the wood raw material. The reason for performing the experiment with these particular substances are several: the sitosterol is to a large extent similar to cholesterol, which is the basic molecule for syntesis of several steroid hormones and also necessary for normal function of the cell membrane. If interference between cholesterol and sitosterol, or similar substances, would occur in metabolic processes, such mechanisms might serve as explanations for different responses seen in fish exposed to pulp mill effluents.

Sitosterol is by nature lipophilic, making it necessary to dilute the substance in a suitable extraction medium, in this case acetone. 1.5 g (theoretically, since other sterols were not accounted for) sitosterol was diluted in 1 l acetone. 12 ml of this solution was added per liter of distilled water, which initially was pumped into a small 180 l stickleback pool in a 5 $\mu g\ l^{-1}$ *nominal* concentration. The exposure started during the same period as the mill A exposure. Since no information about effective doses of sitosterol exists, the concentration was increased to 10 $\mu g\ l^{-1}$ at the August growth measurements of the sticklebacks in order to see whether any response of this would occur on the growth of the fish.

Since the sitosterol is lipophilic and uptake was suspected to be oral rather than through the gills of fish, it was decided that the rainbow trout was to be exposed

through the diet also. It was assumed that the sticklebacks accumulated sitosterol by ingestion of the zooplankton and other small organisms present in their testpool.

The rainbow trout food was prepared by spraying 15 ml of the original acetone solution containing sitosterol on a stainless steel tray covered with a layer of fish food pellets. Every batch of food received a theoretical dosis of 22.5 mg sitosterol. Food was prepared daily. Every food batch was left to dry over night in order to allow the acetone to evaporate. In total 1130 g prepared food was fed to the fish during the 8 week experiment. The mean daily consumption was 34 g of contaminated food for the whole group, which meant roughly 660 µg sitosterol per day. The theoretical individual dosis during the first 2 weeks was thus about 26 µg and after the first sampling about 45 ug for the remaining 15 fish due to lower appetite.

2.6 Chemical analyses

Chemical-analytical methods covering the whole project are given in appendix 2.

3 STATISTICS

Significant differences between mean values of physiological parameters in rainbow trout were established using Student's t-test and Cochrans correction of the variance of the means. Differences in mean weight and condition factors of three-spined sticklebacks between control and exposed groups were tested with logarithm transformed values using one-way Anova and Student's t-test (Harris 1975).

4 RESULTS

4.1 Growth of stickleback brood

Weight gain was measured four times during the experiment. Monthly mean weights of the diffrent groups are presented in Figures 2 and 3. Control values and data for sitosterol-exposed fish are presented in both figures for comparison. In July it can be seen from Figure 2 that both groups exposed to Au and At were stimulated significantly as compared to the control. The group exposed to sitosterol did not deviate significantly in this occasion. Fish exposed to Bu did not show any differences as compared to the control or the sitosterol-group. However, both groups exposed to Bt were significantly stimulated in July (Fig.3).

At the second observation (August) there were no significant deviations between control, sitosterol exposed fish and fish exposed to effluents except for the fish from the high dose of Bt and low dose of At.

The concentration of sitosterol was increased from 5 to 10 µg l^{-1} after the August measurements. From the data in Figures 2-5 it can be seen that the fish exposed to

this substance responded with a strong increase of the mean weight in September. It may be noted that all expoures resulted in a more or less strong growth stimulation. This stimulation persisted until the end of the experiment.

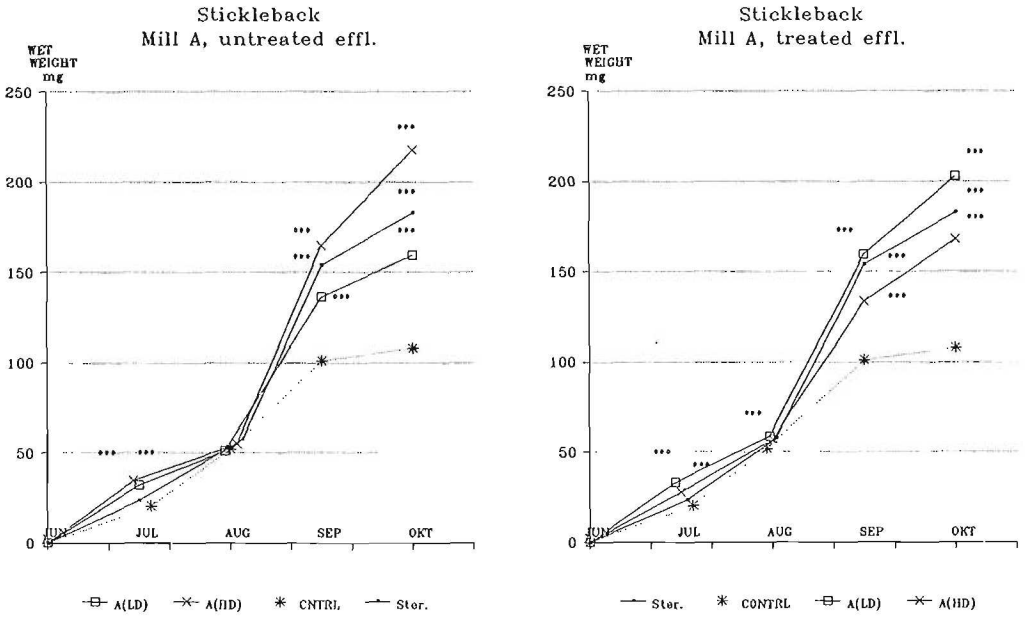

Fig. 2. Mean weight of sticklebacks exposed to effluents Au and At. Significances are calculated on differences from control and upon log transformed values: *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.

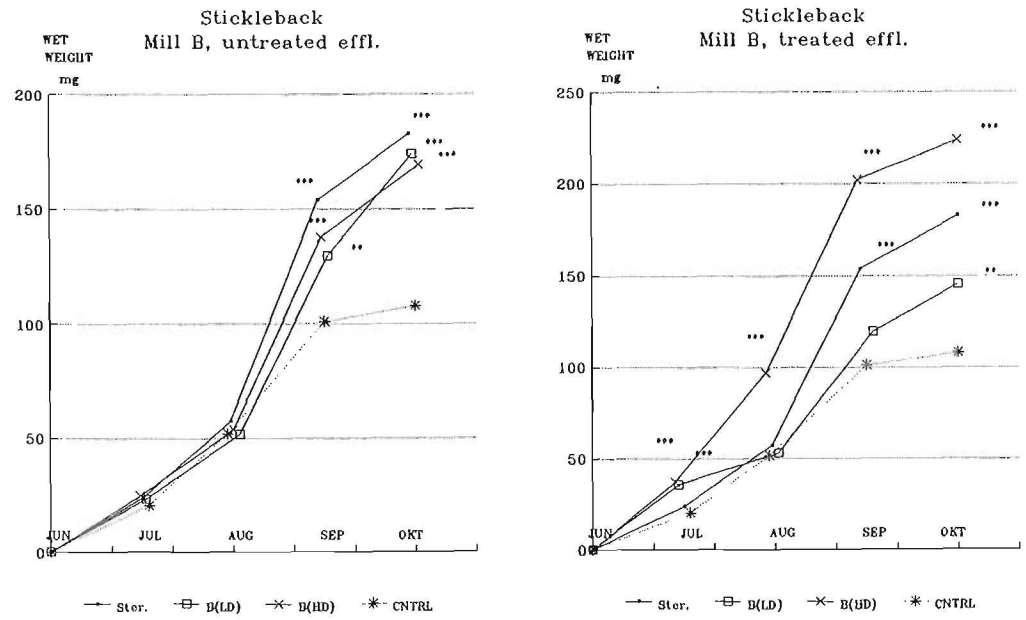

Fig. 3. Mean weight of sticklebacks exposed to effluents Bu and Bt. Significances are calculated on differences from control and upon log transformed values: *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.

The condition factors of the exposed fish (Figures 4 a. 5) exhibited stimulatory responses in the high dose of Au, At (both doses), Bu (both doses) and Bt (high dose). The fish in the low dose of Bt showed increased condition factor in September, whereafter a decrease was seen at the last observation in October. It is noteworthy that the fish exposed to sitosterol showed an identical response as the effluent exposed groups. It is also noteworthy that the fish from the mother mesocosms differed from their control in the same manner.

In this instance it should be noted that the stickleback were exposed to sitosterol in the small pools only. The particular mesocosm from which outgoing water was connected to the sitosterol group (shown in the figures most to the right, group SITO) (Fig. 4 and 5) was a clean system not exposed to sitosterol or any other contaminant. As shown from the figures, there was no difference between the control and the fish from this pool, further strengthening the assumption that the exposure to sitosterol was as effective as exposure to effluents in what regards stimulation of growth and condition factors.

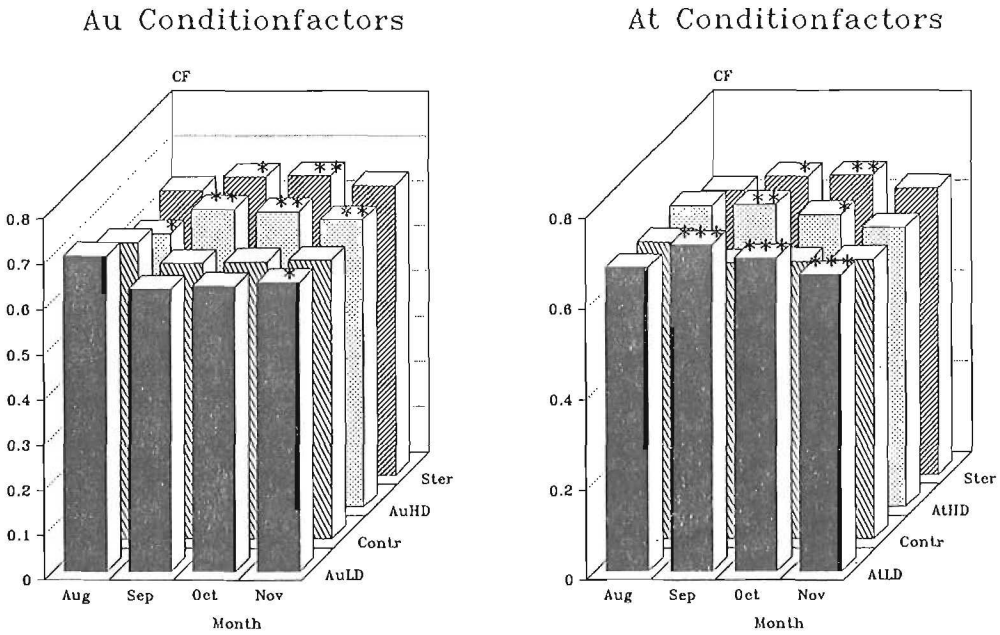

Fig. 4. Condition factors of sticklebacks exposed to effluent Au and At. November CF:s are calculated from the mother mesocosm pool.

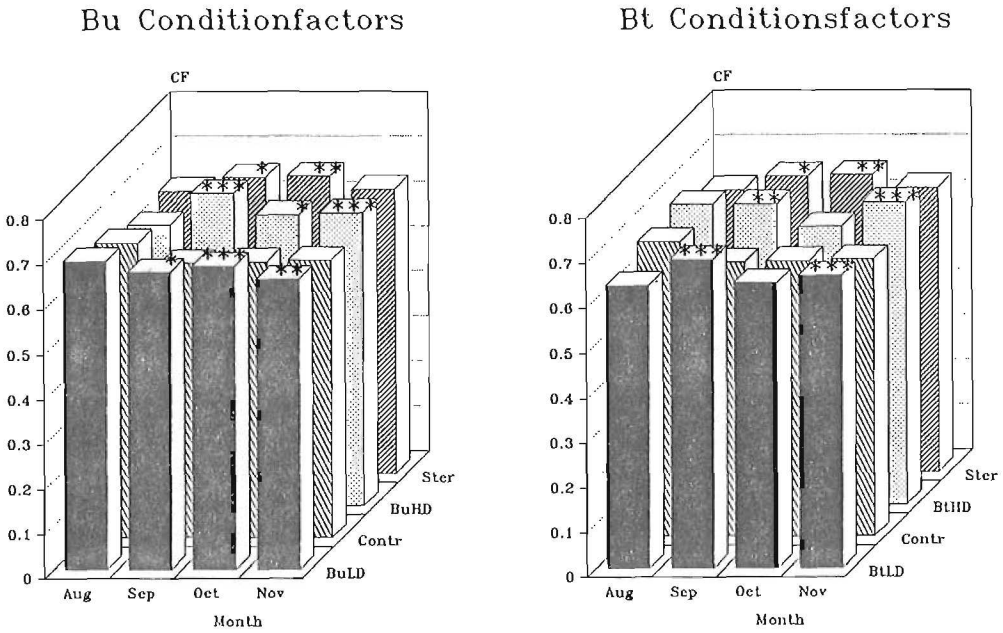

Fig. 5. Condition factors of sticklebacks exposed to effluent Bu and Bt. November CF:s are calculated from the mother mesocosm pool.

4.2 Liver histology and ectoparasites of stickleback brood

Analysis of liver tissue structure gave the following general results:

Mill Au, low dose:

The liver sections contained numerous pycnotic hepatocytes. In several cases necrotic areas occurred focally leading to enlarged sinusoids and mild hyperemia.

As with the rainbow trout (see below) vacuoles apparently containing glycogen occurred in these sticklebacks.

Mill Au, high dose:

The fish exposed in the high dose exhibited focal to massive necroses of the tissue. Some areas were fibrotic. Areas with liver cells with features similar to "cloudy swelling" , previously also observed in other experiments with effluent containig high levels of extractives, were observed. In two cases distinct areas with strongly stained basophilic cells similar to hepatomas were noticed. Additionally vacuoles containing fat occurred.

Mill At, low dose:

Some preparations revealed necrotic areas with pycnotic nuclei. The general liver structure was somewhat changed containing hepatocytes with abnormal size. Some fish livers had cells apparently containing glycogen. Additionally, some livers showed fibrotic areas in close association with enlarged sinusoids.

Mill At, high dose:

Areas with necrosis and pycnotic nuclei occurred rather commonly. Generally the fish in this group showed a disorganized structure of the liver with frequent glycogen vacuolization, enlarged sinusoids associated with hyperemic areas.

Mill Bu, low dose:

Also in this group livers with necrotic areas and pycnotic cells occurred. Hyperemia was frequent. The liver contained also frequently cells in a mitotic state indicating a highly active development of the organ or increased regenerative capacity. Vacuolization of the hepatocytes was not as frequent as within the groups above.

Mill Bu, high dose:

The livers in fish from this group were either focally or massively necrotic with consequent hyperemic and fibrotic areas.

Also areas with "cloudy swelling" occurred. Two fish contained deformed livers with strongly basophilc areas. Fat infiltrations occurred in some specimens. One non-vacuolized liver occurred with a strongly basophilic stained area.

Mill Bt, low dose:

Most of the livers had a normal structure with distinct nuclei. Only sporadic vacuolization was seen. Two livers were somewhat deformed and with areas containing pycnotic nuclei.

Mill Bt, high dose:

Necrotic areas with pycnotic nuclei were occurring sporadically. Otherwise the livers exhibited a rather normal structure. The level of vacuolization was varying with vacuoles possibly containing glycogen. One liver showed possibly signs of fat infiltration.

The sitosterol group:

Several livers with vacuoles occurred. Some fish exhibited limited necrotic areas containing enlarged nuclei and numerous mitoses and abnormal cell sizes.

In general it may be noted that liver structural changes occurred in all exposed groups including the group receiving sitosterol only. Fat infiltration was clearly observed in the high doses of mill Au and Bu. The livers of the exposed groups appeared with the histological technique to be in varying states of pathologic conditions and/or tissue repair since numerous actively dividing cells were seen in the testgroups. It may also be noted here that the present results are a reproduction of previously obtained results using stickleback brood in mesocosms exposed to untreated effluents from hardwood pulp production (Lehtinen et al. 1991). Also in that particular case fat infiltration was observed. In the present case fat infiltration was clearly established in fish exposed to the untreated effluents, whereas fish exposed to the treated effluents showed somewhat milder responses. Some examples of the histological alterations noted are shown in appendix 1.

Regarding the prevalence of ectoparasites it is noted that fish from all exposed groups including the sitosterol group were more infested with the ciliates *Apiosoma* and *Trichodina spp.* than the control (Table 5), which is consistent with previous findings (Lehtinen et al. 1984, Lehtinen 1989).

Table 5. Ectoparasites on three spined sticklebacks after 5 1/2 months exposure to sitosterol, untreated and treated effluents from mill A and B. +=1–15; ++=16–30; +++=>30 per 80x field.

	Apiosoma	Trichodina	Gyrodactylus
AuLD	++	+	–
AuHD	+++	+	–
AtLD	++	++	–
AtHD	+++	++	–
BuLD	++	++	–
BuHD	+	+++	–
BtLD	+++	+++	–
BtHD	++	–	+
Sito	++	++	+
CNTL	+	+	+

4.3 Mortality

The total mortality of the sticklebacks in the control mesocosm, were between 14 and 23 % (mean 19.7 %) (Fig. 6). In the sitosterol group the mortality was 38 % i.e. the highest noted in this experiment. Other groups showing higher figures than the control interval were Au LD; At LD; At HD and Bt LD.

4.4 Physiological responses in rainbow trout, *O. mykiss*

4.4.1 The exposure situation as described by analysis of conjugated compounds in fish bile

The levels of resin acids were significantly higher in the bile from fish exposed to untreated effluent from mill A, high dose, both after 2 and 8 weeks exposure (Fig. 9). It may also be noted that the level in the low dose was not in a steady state after 2 weeks, since the levels were still higher after 8 weeks exposure. The levels in the other groups were low and typically not significantly higher than natural background levels. The levels in fish exposed to treated effluents showed much lower bile concentrations of resin acids than fish exposed to untreated effluents. For the fish exposed to treated effluents the fish from At, high dose still showed the highest values both after 2 and 8 weeks exposure (Fig. 10).

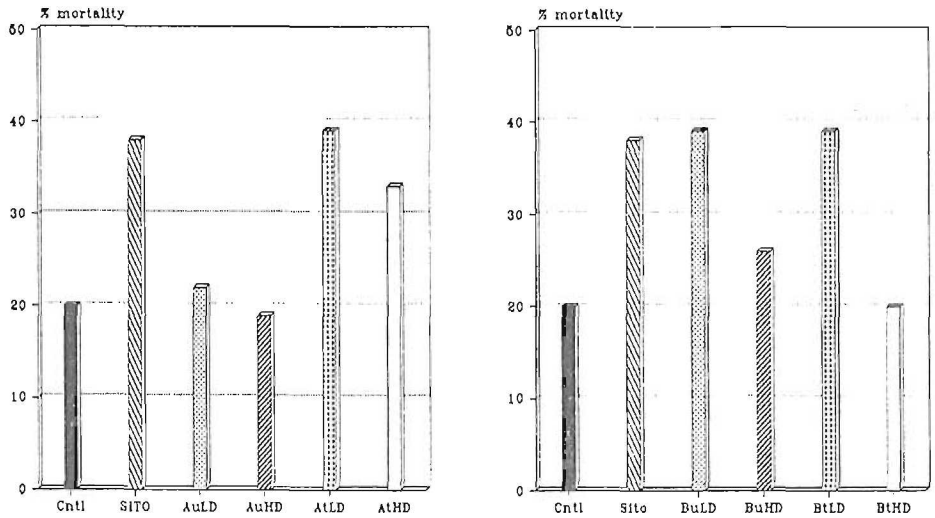

Fig. 6. Mortality in per cent of the sticklebacks exposed in the mesocosm pools. The sitosterol-group mortality value is counted from the small stickleback pool (see Fig.1).

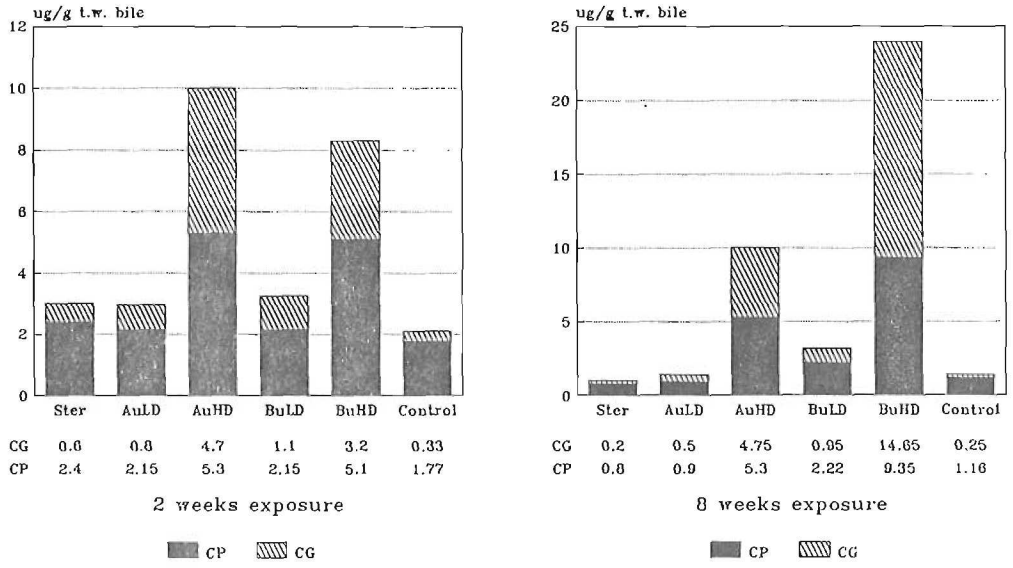

Fig. 7. Accumulation of chlorinated phenols (CP) and chlorinated guaiachols (CG) in rainbow trout biles exposed for 2 and 8 weeks by untreated effluents from Mill:s A and B.

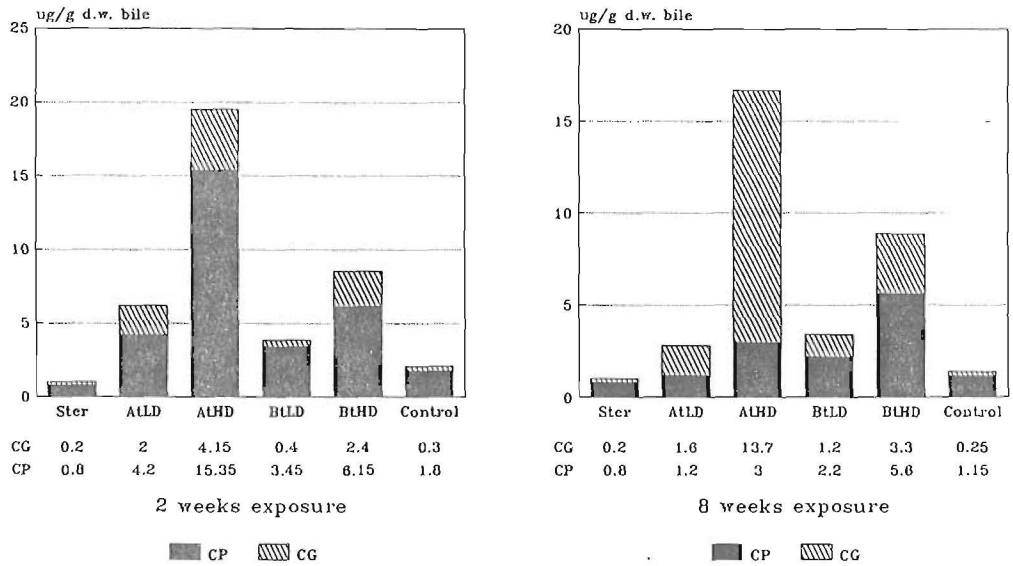

Fig. 8. Accumulation of chlorinated phenols (CP) and chlorinated guaiachols (CG) in rainbow trout biles exposed for 2 and 8 weeks by treated effluents from Mill:s A and B.

The low levels of chlorinated phenolics in the effluents (Table 3) were also reflected by the low bile concentrations (Figure 7 and 8). Moreover, very small differences were seen in bile concentrations between fish exposed to untreated and treated effluents. This is also a reflection of the small differences seen in the effluent concentrations (Table 3).

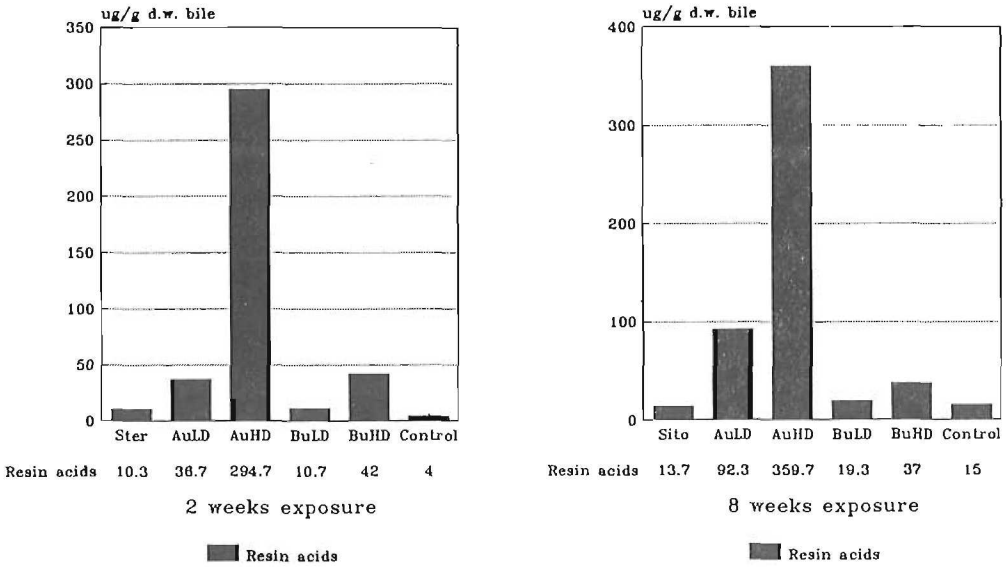

Fig. 9. Total resin acid accumulation in rainbow trout biles exposed for 2 and 8 weeks by untreated effluents from Mill:s A and B

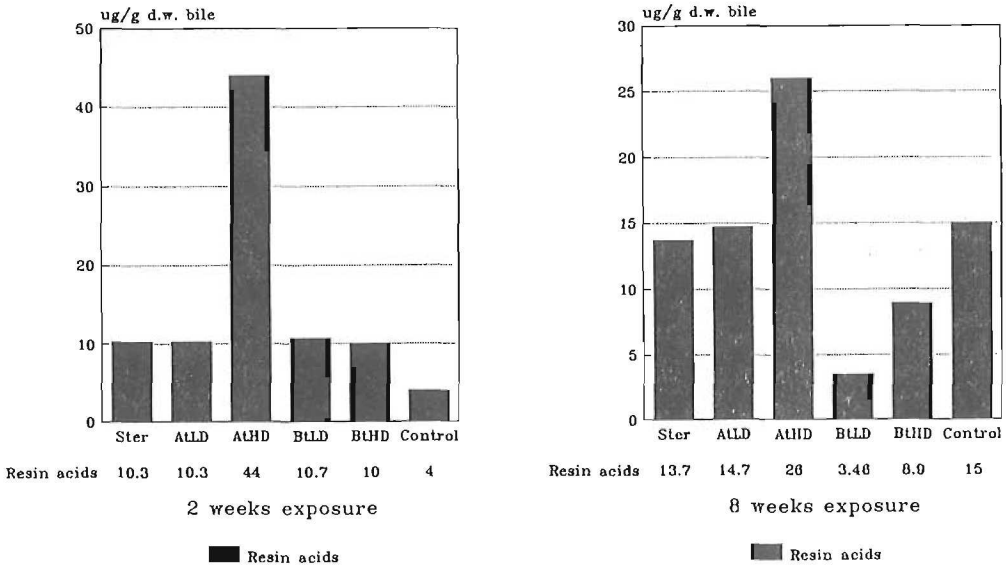

Fig. 10. Total resin acid accumulation in rainbow troat biles exposed for 2 and 8 weeks by treated effluents from Mill:s A and B.

4.4.2 Hematological effects.

The results from the hematological analysis is presented in Tables 6 to 13 .

Table 6. Hematological variables analyzed in rainbow trout exposed for 8 weeks to untreated effluent from mill A and to sitosterol. Values given as mean±s.d.

	AuLD n=15	AuHD n=14	Sitosterol n=15	Control n=15
Hct (%)	31.3±5.4	30.7±4.2	32.2±5.8	32.9±3.3
Hb (mM)	1.17±0.18	1.30±0.2	1.23±0.15	1.33±0.16
RBC 10^{12}/L	1.15±0.21	1.26±0.16	1.16±0.16	1.29±0.21
ImmRBC	2.4±1.4	1.8±0.6	2.1±0.82	1.6±0.7
MCHC (mM)	3.77±0.31	4.25±0.49	3.90±0.58	4.04±0.41
MCHx10^{-14}M	1.03±0.13	1.04±0.17	1.07±0.17	1.05±0.19
MCV 10^{-15}L	27.3±3.2	24.5±2.7	27.9±4.9	26.0±4.0

Table 7. White blood cell picture of rainbow trout exposed for 8 weeks for untreated effluent from mill A and sitosterol. Values are means±s.d.

	AuLD	AuHD	Sitosterol	Control
WBC 10^{10}/L	3.0±1.0	3.6±1.5	2.7±0.8	2.4±1.0
Lymphocytes	1.5±0.8	1.7±0.9	1.5±0.5	1.3±0.7
Granulocytes	0.6±0.2	0.9±0.6	0.7±0.6	0.6±0.4
Thrombocytes	0.9±0.5	1.0±0.8	0.5±0.2	0.6±0.4

Table 8. Hematological variables in rainbow trout exposed for 8 weeks to treated effluent from mill A. Values are given as means±s.d. Control values the same as in Table 6.

	AtLD n=15	AtHD n=13	Control n=15
Hct	31.0±4.0	33.0±4.3	32.9±3.3
Hb	1.17±0.16	1.14±0.12	1.33±0.16
RBC	1.24±0.14	1.30±0.17	1.29±0.21
immRBC	1.8±0.71	1.9±1.28	1.64±0.77
MCHC	3.78±0.31	3.49±0.21	4.04±0.41
MCH	0.94±0.08	0.88±0.08	1.05±0.19
MCV	25.0±2.0	25.4±2.2	26.0±4.0

Same units as in table 6.

Table 9. White blood cell picture of rainbow trout exposed for 8 weeks to treated effluent from mill A. Values given as means±s.d. Control values the same as in table 7.

	AtLD	AtHD	Control
WBC	3.1±1.2	2.9±0.7	2.5±1.0
Lymphocytes	1.5±0.6	1.7±0.4	1.3±0.7
Granulocytes	0.8±0.4	0.7±0.5	0.6±0.4
Thrombocytes	0.9±0.7	0.5±0.3	0.6±0.4

Same units as intable 7.

Table 10. Hematological variables in rainbow trout exposed for 8 weeks to untreated effluent from mill B. Values are given as means±s.d.

	BuLD n=13	BuHD n=13	Control
Hct	31.4±4.0	31.2±4.3	32.9±3.3
Hb	1.32±0.19	1.21±0.17	1.33±0.16
RBC	1.42±0.23	1.41±0.29	1.29±0.21
immRBC	2.4±1.7	2.0±1.1	1.6±0.7
MCHC	4.22±.55	3.91±0.64	4.04±0.41
MCH	0.92±0.12	0.89±0.20	1.05±0.19
MCV	22.3±2.7	22.7±4.6	26.0±4.0

Same units as in table 6

Table 11. White blood cell picture of rainbow trout exposed for 8 weeks to untreated effluent from mill B. Values given as means±s.d.

	BuLD n=15	BuHD n=13	Control
WBC	2.9±1.4	3.5±2.2	2.5±1.0
Lymphocytes	1.4±1.0	2.0±1.4	1.3±0.7
Granulocytes	0.6±0.6	0.8±0.7	0.6±0.4
Thrombocytes	0.9±0.6	0.7±0.7	0.6±0.4

Same units as in table 7.

Table 12. Hematological variables of rainbow trout exposed for 8 weeks to treated effluent from mill B. Values are given as means±s.d.

	BtLD	BtHD	Control
Hct	32.4±3.8	33.3±5.3	32.9±3.3
Hb	1.19±0.14	1.21±0.16	1.33±0.16
RBC	1.31±0.16	1.24±0.19	1.29±0.21
ImmRBC	1.69±1.34	1.68±0.75	1.64±0.77
MCHC	3.66±0.21	3.66±0.25	4.04±0.41
MCH	0.91±0.08	0.99±0.19	1.05±0.19
MCV	24.9±2.8	26.9±5.9	26.0±4.0

Same units as in table 6.

Table 13. White blood cell picture of rainbow trout exposed for 8 weeks for treated effluent from mill B. Values given as means±s.d.

	BtLD	BtHD	Control
WBC	3.2±1.8	2.3±0.9	2.5±1.0
Lymphocytes	1.9±1.2	1.2±0.5	1.3±0.7
Granulocytes	0.5±0.4	0.3±0.2	0.6±0.4
Thrombocytes	0.8±0.6	0.7±0.6	0.6±0.4

Same units as in table 7.

As can be sen from the tables, no statistical differences between the groups were seen regarding the red and white blood cell picture after 60 days exposure. Neither were differences recorded in fish exposed for 2 weeks (data not shown) indicating that the fish did not have to adjust for respiratory activity.

4.4.3 Liver metabolism and histology

Liver water, liver lipid, and liver glycogen content of the exposed fish are presented in Tables 14–17. For liver water and lipid content, no statistically significant differences were obtained. The liver glycogen content, however, showed a statistically significant increase as compared to the control in all groups except the high dose of treated mill B (BtHD), AuLD and the low dose of untreated mill B effluent (Tables 15 and 17). It may be noted that also the fish exposed to sitosterol had an increased liver glycogen content.

Moreover, high glycogen content (> 4%) corresponded well with vacuolization of the liver tissue in those trout investigated histologically (first five specimen /group). Also in this regard, the sitosterol exposed group was no exception, indicating similar biological responses to one single group of compounds and the total effluents tested.

The activity of the MFO-system measured as EROD-enzyme activity is also presented in tables 14 to 17. Generally the activity was lower in both control and exposed groups after 2 weeks than after 8 weeks possibly due to seasonal reasons. The only difference noted in the activity of this enzyme was a slight increase in the group exposed for two weeks to the high dose of effluent from mill A. After 8 weeks there was no significant differences between control and exposed fish. No differences were seen between sexes in their enzyme activity.

The activity of the conjugation enzyme UDP-GT did not differ between the groups at the two sampling occasions

The LSI-values did not show any differences. Neither did the condition factor show any statistically significant diffrences.

The serum ALAT-levels indicated that no acute liver damage occured in the rainbow trout studies (Fig. 11 and 12).

Table 14. Liver metabolic parameters in rainbow trout exposed for 2 and 8 weeks to untreated effluent from mill A and to sitosterol. Values given as mean±s.d. Statistical differences indicated with asteriscs. Values after 2 weeks exposure are given in parenthesis.

Liver	AuLD	AuHD	Sitosterol	Control
glycogen (%) [1]	4.4±0.5	5.7±0.7**	5.0±0.5**	3.2±0.3
lipid (%) [1]	2.9±0.1	2.9±0.3	2.7±0.1	3.0±0.2
water (%) [1]	74.5±0.2	74.5±0.4	74.5±0.3	74.5±0.3
LSI (%)	1.1±0.13	1.2±0.2	1.2±0.17	1.1±0.1
	(1.0±0.18)	(1.2±0.35)	(1.0±0.16)	(1.2±0.4)
EROD[2]	6.1±1.9	7.0±4.0	6.1±2.0	5.8±2.7
	(2.5±2.1)	(2.4±2.2)	(1.5±0.8)	(2.0±1.2)
UDPGT[2]	499 ± 84	538 ±100	541 ± 92	450 ±114
	(532 ± 92)	(419 ±109)	(475 ±102)	(489 ±108)

[1] 2 weeks samples not analysed
[2] pmol min^{-1} mg prot.

Table 15. Liver metabolic parameters in rainbow trout exposed for 2 and 8 weeks to untreated effluent from mill B. Values given as mean±s.d. Statistical differences indicated with asteriscs. Values after 2 weeks exposure are given in parenthesis.

Liver	BuLD	BuHD	Control
glycogen (%) [1]	3.6±0.7	6.0±0.9*	3.2±0.3
lipid (%) [1]	2.7±0.1	2.6±0.2	3.0±0.2
water (%) [1]	74.6±0.2	74.4±0.4	74.5±0.3
LSI (%)	1.1±0.2	1.2±0.3	1.1±0.1
	(1.3±0.8)	(1.1±0.2)	(1.2±0.4)
EROD[2]	4.0±1.6	6.4±2.5	5.8±2.7
	(2.7±1.3)	(2.9±4.1)	(2.0±1.2)
UDPGT[2]	472 ± 78	425 ±118	450 ±114
	(569 ±106)	(487 ±134)	(489 ±108)

[1] 2 weeks samples not analysed
[2] pmol min^{-1} mg prot.

Table 16. Liver metabolic parameters in rainbow trout exposed for 2 and 8 weeks to treated effluent from mill A. Values given as mean±s.d. Statistical differences indicated with asteriscs. Values after 2 weeks exposure are given in parenthesis.

Liver	AtLD	AtHD	Control
glycogen (%) [1]	6.5±0.9**	5.4±0.9*	3.2±0.3
lipid (%) [1]	2.5±0.1	3.0±0.6	3.0±0.2
water (%) [1]	74.7±0.3	73.9±0.5	74.5±0.3
LSI (%)	1.2±0.15	1.2±0.1	1.1±0.1
	(1.0±0.2)	(1.0±0.2)	(1.2±0.4)
EROD[2]	7.0±3.2	9.9±6.4	5.8±2.7
	(3.5±2.2)	(4.6±4.4)	(2.0±1.2)
UDPGT[2]	571 ±138	445 ± 85	450 ±114
	(419 ±109)	(474 ±104)	(489 ±108)

[1] 2 weeks samples not analysed
[2] pmol min^{-1} mg prot.

Table 17. Liver metabolic parameters in rainbow trout exposed for 2 and 8 weeks to treated effluent from mill B. Values given as mean±s.d. Statistical differences indicated with asteriscs. Values after 2 weeks exposure are given in parenthesis.

Liver	BtLD	BtHD	Control
glycogen (%) [1]	4.7±0.5*	4.0±0.5	3.2±0.3
lipid (%) [1]	2.7±0.2	2.8±0.2	3.0±0.2
water (%) [1]	74.7±0.2	74.7±0.3	74.5±0.3
LSI (%)	1.1±0.2	1.1±0.2	1.1±0.1
	(1.1±0.2)	(1.1±0.2)	(1.2±0.4)
EROD[2]	4.9±2.6	6.4±2.6	5.8±2.7
	(1.7±1.1)	(2.7±2.6)	(2.0±1.2)
UDPGT[2]	404 ±116	424 ±119	450 ±114
	(471 ± 60)	(399 ± 95)	(489 ±108)

[1] 2 weeks samples not analysed
[2] pmol min^{-1} mg prot.

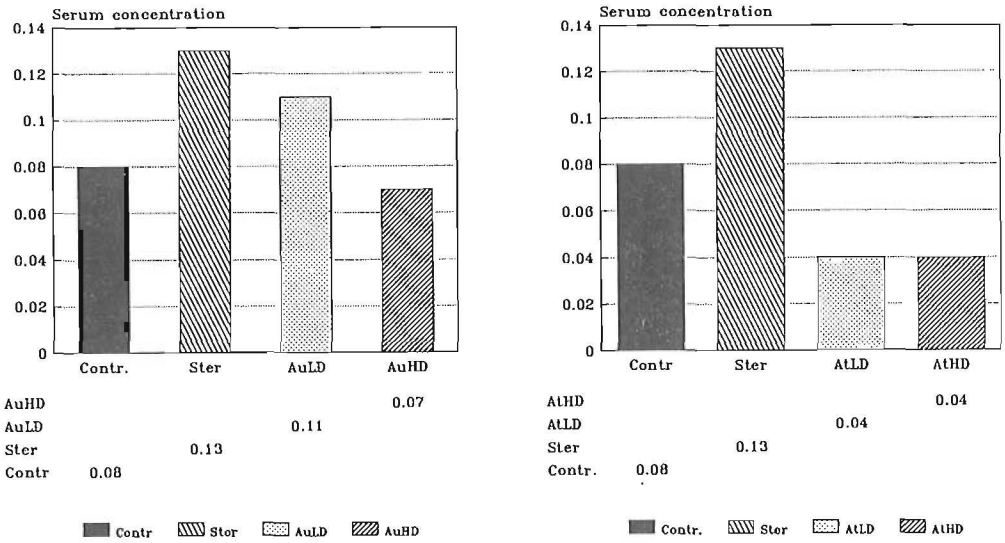

Fig. 11. Serum ALAT-levels in rainbow trouts after 8 weeks exposure to untreated and treated effluents from mill A.

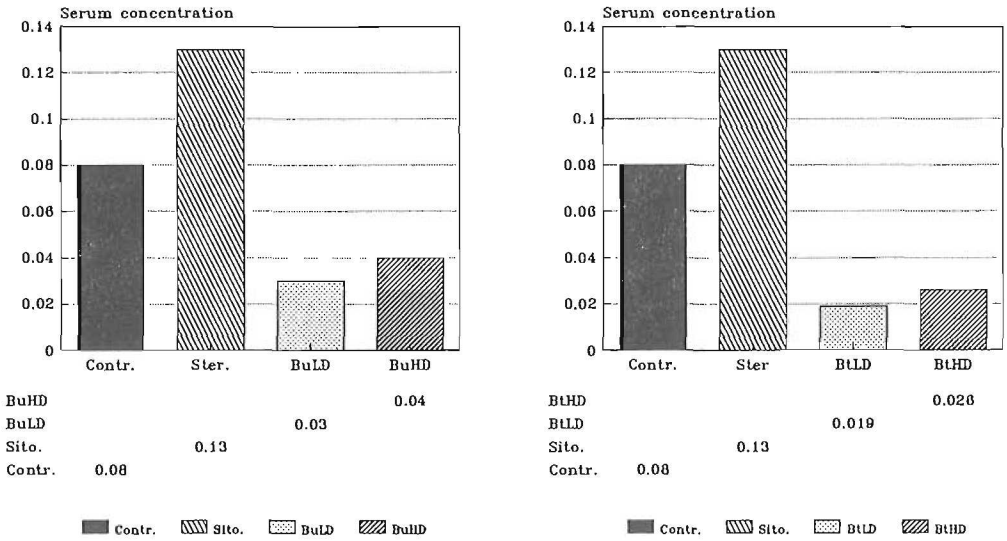

Figure 12. Serum ALAT-levels in rainbow trouts after 8 weeks exposure to untreated and treated effluents from mill B.

4.4.4 Levels of steroids in the bile and plasma

Analysis of steroids in the bile revealed that a number of different substances were present (Fig. 14). It was also confirmed that sitosterol was present in the bile of the fish exposed to sitosterol (doubled level compared to the control) as well as in some of the high doses of the effluents tested (low doses not analyzed) after 8 weeks exposure. In addition, it may be noted that at least two other steroids (camposterol and camposteron) were detected in all fish analyzed, both after 2 and 8 weeks exposure (Fig. 14). Moreover, it was noted that the level of bound cholesterol (esterified or conjugated) was higher than the control in the same fish (Fig. 13). The level of free cholesterol was unchanged.

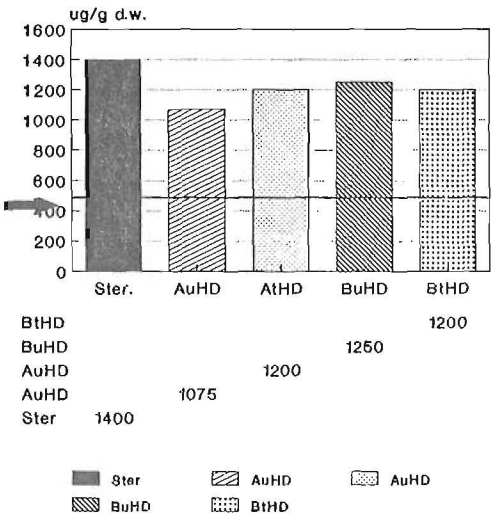

Fig. 13. Bile cholesterol levels in rainbow trouts after 8 weeks exposure to sitosterol and high dose of untreated and treated effluents from mill A and B. Control level indicated with arrow to the left in the figure.

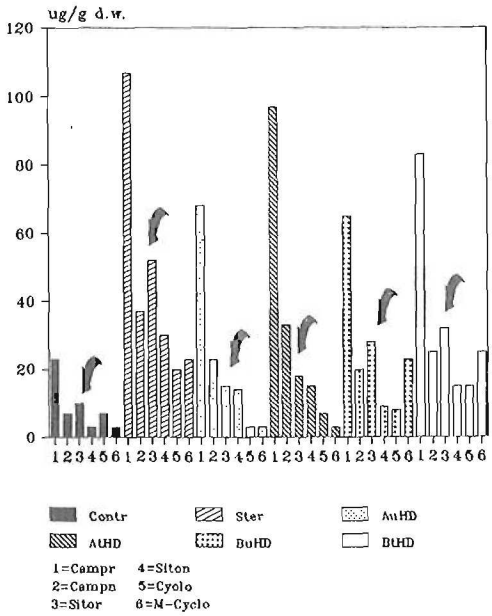

Fig. 14. Levels of different bile steroids found in rainbow trout after 8 weeks exposure to sitosterol (arrows) and high dose of untreated and treated effluents from mill A and B.

5 DISCUSSION

The alternating stimulation and inhibition of growth seen in the present experiment with the three-spined stickleback populations is a rather well-known phenomenon. The stimulatory effect of subinhibitory levels of toxicants was first observed by Schulz (1888), who demonstrated that toxic substances could stimulate respiration and growth in yeast. This type of response was established as the Arndt-Schulz law. Southam and Erlich (1943) found a similar stimulatory effect when working on the fungicidal properties of cedar heartwood extract, and they used the term "hormesis" to describe their observations.

Stebbing (1981) found that low concentrations of toxicants induced an initially strong stimulation and thereupon a lower inhibition of the growth rate in a hydroid colony. At moderate concentrations the growth started to oscillate around the control values before reaching steady state levels again. Alternating inhibitory and stimulatory reactions have also been obtained when the activity of a detoxification enzyme in fish, exposed to pulp mill effluent components, was studied (Tana 1988). Many other reports exist from experiments where both stimulation and inhibition of different responses in fish exposed to different toxicants have been obtained (McLeay and Brown 1974; Stoner and Livingston 1978; Bengtsson 1980; Oikari et al. 1985).

Bleached kraft mill effluents (BKME) are complex mixtures containing both chlorinated and non-chlorinated organic substances (Mäenpää et al. 1968; Holmbom and Lehtinen 1980; Niemelä 1990). The wood raw material itself used in the production contains considerable amounts of potentially bioactive substances aimed to protect the tree against insects and fungal attack. After digestion of the chips in the digester, part of the black liquor also containing such substances, is carried over to, and through the bleachery.

Since very similar effects on growth, condition factor and liver structure where obtained by exposure to the effluents as with the sterol compounds alone, the possible role of sitosterol and similar compounds present in the wood extractive as effect inducers must be considered.

The molecular structure of beta-sitosterol and other sterols is very similar to that of cholesterol (Fig. 15), which has an important role for the function of cell membranes (Chapman 1975, Jacob 1975). Cholesterol also serves as the precursor of the five major classes of steroid hormones: progestagens, glucocorticoids, mineralo-corticoids, androgens and estrogens. It is beyond the scope of this article to evaluate the possible interaction between sitosterol and cholesterol in detail. However, it may be speculated that sitosterol could act as a chemical double for cholesterol and interact in several metabolic pathways including steroid synthesis and catabolism.

Clear indications that some interference is occurring was gained by the steroid analysis of the bile in rainbow trout (Fig. 13 and 14). These analyses showed that sitosterol, apart from other steroids, was present in higher levels in exposed fish than in control fish. Furthermore, an increased cholesterol excretion in exposed fish was indicated by the increased level of bound cholesterol in the bile. Similar results have also been obtained in a dose-response mode from rainbow trout exposed to effluents

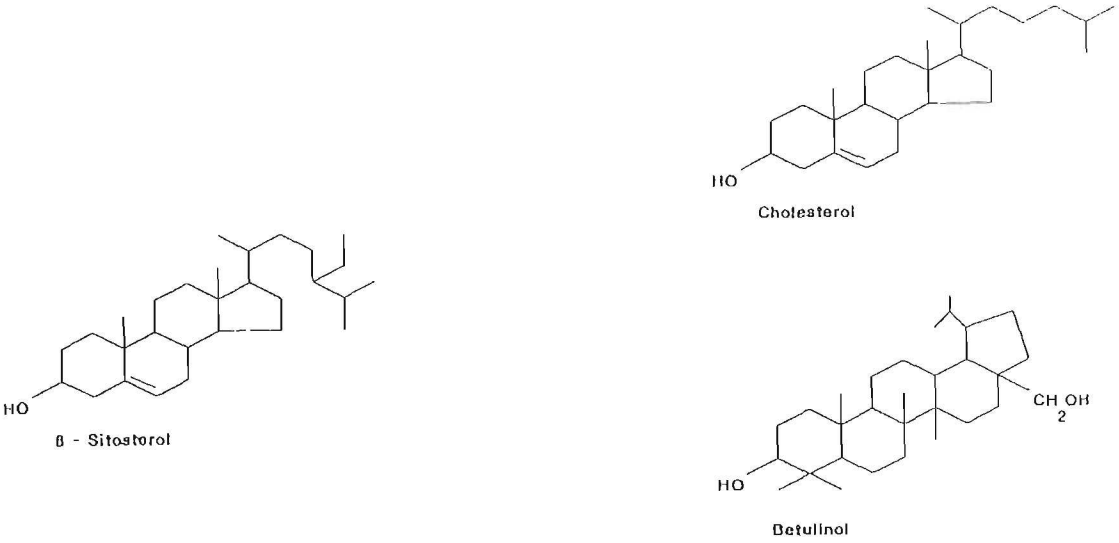

Fig. 15. A common triterpene and a steroid in wood and the cholesterol molecule.

from production of bleached softwood pulp (Lehtinen, unpublished results).

The significance of the findings discussed above are not clear. However, it was recently found by McMaster et al. (manuscript submitted for publ.) that fish caught downstreams a Canadian bleached pulpmill showed lower gonadosomatic indices (females) and reduced development of secondary sexual characteristics (males). Combined with this, the fish had significantly lower plasma steroid levels throughout the year in both sexes. The fish were also shorter, older and had decreased growth rate but increased condition factor.

It is noteworthy that similar results have also been reported from studies performed outside a Swedish pulp mill, i.e. low gonadosomatic indices and high condition factors. Contrary to the Canadian results, the growth rate was higher than in unpolluted areas, however (Södergren 1988). The increased condition factors and increased growth obtained in the present work are consistent with the findings in the Swedish field studies.

It is not known whether the sticklebacks during prolonged exposure would have shown similar signs of reproductory effects as in the Canadian study referred to above. More work is needed in this respect. The fact that fish in all groups, including the sitosterol (including other sterols) exposed one, showed some degree of liver histo-logical alterations, encompassing fat infiltration and necrosis, is an indication that the liver function could be disturbed and that sitosterol and similar substances may be responsible for this. Liver dysfunction caused by simulated unbleached kraft mill effluent resulting in jaundice after prolonged exposure, was noted by Oikari and Nakari (1982).

The fish in our experiment were too small to enable analysis of bilirubin levels in order to detect a possible jaundice. Although one should be cautious in making extrapolations between the two fish species tested in the present work, among other things due to the differences in age and time of exposure, no signs of disturbance in the red blood cell picture or in the plasma-ALAT level were noted in the rainbow

trout (see discussion below). A decreased number of red blood cells or reduced hemoglobin content might be a sign of an increased breakdown of red blood cells, which might result in increased bilirubin levels in plasma under liver dysfuntional conditions (Jacob 1975). Increased plasma-ALAT in man is a sign of membrane damage in the liver.

Regarding the incidence of parasite infection on the body surface of the stickle-backs, it may be noted that parasites/ectocommensals of previously described species (Lehtinen 1984) occurred in higher frequency in all exposed groups. The underlying mechanisms are not understood but may include both internal physiological/ biochemical as well as external environmental factors previously discussed in Lehtinen (1990) and Lehtinen et al. (1984).

The mortality figures do not correspond to exposure to treated or untreated effluents. The highest mortality was obtained in the groups exposed to sterols, Bu and Bt low dose. Mortality in the control group is somewhat higher than previously reported, but probably falls within the normal mortality range (Lehtinen 1989). The higher mortality figures for At were consistent with the results regarding inhibitive effects noted on total abundance and biomass of the invertebrate fauna in the same mesocosms (Lehtinen et al. 1991). The reasons for the higher mortality in the stickle-backs exposed to treated mill A effluent are not known presently.

As a conclusion regarding effects on the stickleback populations exposed to the effluents and sitosterol, it can be noted that deviations in growth pattern, liver histology and parasite infection were recorded. Owing to the fact that practically identical effects were obtained with sterols alone, these effects cannot be attributed to the presence of chlorinated organic matter. The nominal concentrations of total chlorinated phenolic compounds (chlorophenols and chloroguaiacols) in the high doses were in the order of 0.02–01 $\mu g\ l^{-1}$ test solution and the AOX concentrations (consideration taken to water use/t. pulp) in the order of 16–115 $\mu g\ l^{-1}$ (with varying degree of chlorination of the high molecular lignin). No relationship was observed between AOX-concentrations and intensity of effects, which is again strengthening the assumption that chlorinated substances present in the effluents tested had little or nothing to do with the effects noted. In this instance it should be stressed that the total amount of extractives was comparably high in the effluents. For untreated effluent from mill A they were at the same order of magnitude as AOX (Table 2 and 4).

The rainbow trout did not show any hematological responses on exposure in any group tested in the present experiment. This is consistent with results from previous investigations performed in connection with mesocosm studies. Effluents from processes using softwood and oxygen pre-bleaching in addition to either external treatment or high substitution of chlorine with chlorine dioxide were used in those experiments (Lehtinen et al. 1990). Usually hematological indices in fish exposed to highly diluted pulp mill effluents have responded within limits which may be considered adaptational. At lower dilutions of effluents or at high concentrations (sub-acute) of pure substances such as resin acids, effects on respiration and the number red blood cells have been obtained (Larsson et al. 1988, Oikari et al. 1984). Effects noted on fish hematology in studies of pulp mill effluents have, to our knowledge, always been accompanied by liver metabolic changes, and it is plausible that hematological

effects are not primary but secondary adaptations to for example liver metabolic ones (Lehtinen 1990).

The effects on carbohydrate metabolism i.e. significantly increased liver glycogen content in all groups except for the group Bt, high dose, are an indication of a possible disturbance of the hormonal regulation of the glycogen reserve (Larsson et al. 1988). Similar results were also obtained in rainbow trout exposed for 8 weeks to both total mill effluents as well as bleachery effluents from mills producing bleached soft-wood kraft pulp (Landner et al. 1991).

Contrary to these findings lowered liver glycogen levels were noted by Oikari and Nakari (1982) in fish exposed to simulated kraft mill effluent. The concentrations (75–150 μg l^{-1} total resin acids) were much higher than in our experiment (0.023–4.9 μg l^{-1} in the high doses) making it hard to compare the results. In cases where liver glycogen has been unresponsive or lowered, an increase of liver lipid content has usually been found (Oikari et al. 1985, Lehtinen 1991). The reasons behind these somewhat contradictory results are not clear but successive changes in liver metabolism, energy use and/or liver function may be considered plausible in the light of the results of Sandström et al. (1988) and Lehtinen (1989 and 1990). The role of sitosterol and similar substances would need to be given more emphasis in this respect.

The histological changes observed as vacuolization of the liver tissue corresponded with high glycogen values. If this is a consistent response, an increased vacuolization would be expected at glycogen values higher than 4%. Tentatively, this assumption would give the following expected numbers of fish with vacuolization of the liver tissue:

Sitosterol	7	(46%)
Au, Low dose	5	(33%)
Au, High dose	10	(66%)
At, Low dose	8	(53%)
At, High dose	6	(40%)
Bu, Low dose	5	(33%)
Bu, High dose	7	(46%)
Bt, Low dose	8	(53%)
Bt, High dose	5	(33%)
Control	3	(20%)

No effects on MFO-enzyme activity were observed in the fish from the present experiment except in one case (Au, high dose) after two weeks exposure, when a slight increase of the EROD enzyme activity was noted. The UDPGT-activity did not differ from the control for any of the groups tested.

Lack of induction of the EROD enzyme has been observed lately in field investigations outside Swedish pulp mills (Grahn et al. 1990), even at sites receiving effluents with rather high discharges of organic substance as well as AOX (Monfelt et al. 1990).

It has been suggested that the EROD enzyme generally may be responsive to bleached pulp mill effluents and chlorinated compounds (Lindström-Seppä 1990).

Increased activity of the enzyme has been obtained in several studies. However, recent results contradicting this general pattern have been gained (Landner et al. 1990). Also Andersson et al. (1987) did not obtain any induction in fish exposed to effluent from bleached hardwood pulp production. Effluents from bleached softwood pulp production and mixed hardwood/softwood effluent induced the EROD enzyme, on the other hand. However, the effluents tested by Landner et al. (1990) originated from softwood bleaching (4 different effluents). In one case only there was a slight increase in the EROD enzyme acticity. The lack of consistency regarding the induction of the EROD enzyme in fish may in many cases be assumed to depend, at least to some extent, on different strategies in the use of foam inhibitors and other auxiliary chemicals at different mills. This aspect has unfortunately been neglected so far when discussing effects on MFO-enzymes.

The lack of response in the activity of the UDPGT conjugation enzyme may be explained by the fact that the level of resin acids and chlorophenolics was so low that the livers of the exposed fish were able to excrete these substances at unchanged enzyme activities. It is known from several studies that the UDPGT activity is inhibited at exposure to resin acids and stimulated by chlorophenolics (Oikari et al. 1984; Tana 1988). Another possibility is that the enzyme activity oscillates in time as shown by Tana (1988), and did not deviate from the control at the moment of observation.

Our interpretation is that the effluents did not contain high enough levels of inducing substances to induce the enzymes assayed. Neither was the sitosterol inducing these enzymes. Since sitosterol (as well as several other sterols and sterons) was found in higher levels in the bile of both the sitosterol group as well as in the high doses of Bu and Bt it may be assumed that other cytochrome P-450 associated isozymes are processing these substances. In this instance it is noteworty that the fish exposed to mill B:s effluent had higher sitosterol levels although lower levels were in fact measured in the undiluted effluent as compared to the levels found in mill A:s effluent. Other sterols and sterons such as campesterol and camposteron were not detected in the effluents although they were found in the fish bile in higher levels than in the control. Niemelä (1990) qualitatively detected campesterol in birch kraft black liquor, however. Thus, it cannot be ruled out that both the sitosterol-powder as well as the effluents contained these compounds as seen from the amounts of total extractives from the effluents (Table 4). The lack of differences in bound cholesterol and other steroids in the bile between the sitosterol group and the other groups analyzed and between the groups exposed to treated and untreated mill effluents must be considered as remarkble. This indicates a common mode of action of substances present in the effluents, substances which are not eliminated during treatment. It may be held possible that such substances may be of steroid character and that sitosterol, at least in this case might be one major common effect elicitor. However, it is premature to make a final statement in this matter, not least since we do not know for certain if the effects noted in both the stickleback populations and in the rainbow trout are deleterious to the population. We recognize that the present results need further evaluation, also from the perspective of what future environmental priorities are to be made by the pulp and paper industry. In this respect it may be noted that the exposure

to chlorophenolics and resin acids, established by bile analysis, was approaching levels only slightly above, or at background levels found in watercourses in Nordic countries, i.e. roughly 10 and 50 μg g^{-1} d.w. (Lehtinen et al. 1988, Oikari and Holmbom,1986). The level of free+bound cholesterol in the bile of exposed fish in the present study were up to 1600 μg g^{-1} and about 500 μg g^{-1} in control fish, strongly indicating that the steroid turnover was affected. The sitosterol level in the bile of sitosterol exposed fish was about 50 μg g^{-1} d.w. as compared with about 15 μg g^{-1} for the control. The effects seen in both fish species exposed to sitosterol suggest that comparatively low levels of a substance with possible hormonal influence might be more effective than substances formed in the bleaching process.

6 CONCLUSIONS

Effects on growth, liver histology, and parasite frequency were obtained in the brood of the three-spined sticklebacks, *G. aculeatus*, exposed both to effluents from production of bleached kraft hardwood pulp and sitosterol. The significance of the growth effect is uncertain. Prolonged exposure encompassing the whole life cycle is required in order to evaluate if effects seen in field studies are reproducible.

No hematological responses nor effects on MFO-enzymes were noted in rainbow trout after 8 weeks exposure. Metabolic disturbance was indicated by increased liver glycogen content in the fish. This effect was accompanied by liver tissue vacuolization.

Metabolic interference of the effluents as well as by sitosterol was also indicated by increased bile levels of bound cholesterol and other steroids.

No acute liver damage was demonstrated, however, since serum ALAT did not deviate from control fish. It is not known wether the same picture was prevalent with the stickleback population. The growth abberration in these fish indicate a higher sensitivity of younger fish than of older ones.

Very small or negligible differences in the effect picture of the fish was noted between untreated and treated effluents and sitosterol. This is indicating common effect mechanism between the groups tested.

The fact that the effects were similar despite differing concentrations of sitosterol in the exposed groups and in the sitosterol group ewokes some questions regarding effective dose and sampling methodology of waste water samples due to the lipophility of the substance. Another reason behind the similar effects may be that other similar substances as sitosterol were acting together in the effluent groups or that the sitosterol powder contained impurities. However, as judged from the present results sitosterol might serve as a useful model compound to illustrate effect mechanism of hormone analogues in pulp mill effluents.

The exposure as evaluated by bile conjugate analysis of chlorophenols and resin acids was somewhat higher before than after treatment of the effluents. On the other hand, the concentrations were only slightly above natural background levels. A relationship between exposure to these substances and effects cannot therefore be established.

A relationship between AOX in the effluents and effects was not seen.

Due to the similarity of effects caused by sitosterol and the effluents regarding histology and steroid concentrations in bile as well as growth effects, we find it important that further research should be directed toward evaluation of the physiological role in fish of steroids and similar non-chlorinated natural substances present in the wood raw material.

GLOSSARY - ORDLISTA - SANASTO

Ad libitum	= efter aptit, vid matning av försöksdjur, ingen födo-restriktion = ravintoa annettu rajoittamatta kulutuksen mukaan
Anticoagulant	= substans som hindrar blod att stelna = hyytymisen estoaine
Abberration	= avvikelse = poikkeama
Brood	= avkomma = jälkeläiset
Bile	= gallvätska = sappineste
Bleak	= löja, *Alburnus alburnus* = salakka, *Alburnus alburnus*
Basophilic	= vävnad som färgas med basiskt färgämne = emäksisesti värjäytyvä kudos
Caudal vessel	= blodkärl i stjärtregionen hos bla fisk = kalan pyrstöosassa sijaitsevat verisuonet
Conjugate	= lipofil substans som gjorts hydrofil genom ett glucorunsyra via enzymatisk katalysator konjugerats till substansen = entsyymitoiminnan katalysoima glukuronihapon kautta rasvaliukoisesta vesiliukoiseksi konjugoitu yhdiste
Ciliate	= flimmerhårförsedd encellig organism = yksisoluinen siimaeliö
Catabolism	= nedbrytningsomsättning av substanser = yhdisteiden hajoamistapahtuma
Dysfunction	= nedsatt eller försämrad function hos ett organ ss. lever = elimen esim. maksan heikentynyt tai estynyt toiminta
Erythrocyte	= röd blodcell = veren punasolu
Ectoparasite	= på huden/ytan levande parasit = pintaloinen
Ectocommensal	= på huden/ytan levande organism som inte parasiterar direkt på värden = iho/pintaloinen, joka ei suoraan loisi isäntää
Focal	= regional, begränsad t.ex. vävnadsskada = paikallinen, rajoittunut esim kudosvaurio
Fibrotic	= ursprunglig vävnad som ersatts med fibrös vävnad = säie/sidekudoksella korvattu kudos
Gonado-somatic index	= könsorganens vikt som procent av fiskens totalvikt = sukuelinten prosentuaalinen paino
Histology	= läran om vävnaders byggnad = kudosoppi

Hematocrit	= den sammanpackade volymen av röda blodceller = punasolujen prosentuaalinen osuus
Hemoglobin	= gastransporterande protein i blodet = veren hapenkuljetusproteiini
Hepatocyte	= levercell = maksasolu
Hyperemia	= infiltration av blodkroppar i vävnaden = verentungos
Hepatom	= leversvulst = maksakasvain
Hormesis	= stimulatorisk respons vid låga koncentrationer av en toxisk substans = myrkyllisten yhdisteiden alhaisissa pitoisuuksissa aiheuttama stimuloiva vaste
Juvenile	= icke könsmogen = ei sukukypsä
Jaundice	= gulsot = keltatauti
Larva	= nykläckt yngel, ej metamorfoserat = vastakuoriutunut toukka, muodonmuutos läpikäymättä
Lesion	= vävnadsskada = kudosvaurio
Leucocyte	= vit blodkropp = valkosolu
Metabolism	= ämnesomsättning = aineenvaihdunta
Mortality	= dödlighet = kuolleisuus
Maturing	= mognande i bemärkelsen könsmognande = sukupuolisesti kypsyvä
Mitotic	= delning hos somatiska (icke-könsc.) celler = somaattisten solujen (ei sukupuolisolu) jakautuminen
MFO-enzyme	= enzym ingående i membranbundet cytocrom p-450 iso-enzym system = solun kalvorakenteissa sijaitsevaan P-450 isoentsyymi-järjestelmään kuuluva entsyymi
Necrotic	= död vävnad = kuollutta kudosta
Nuclei	= (plur.) cellkärnor = solutumat
Oral	= via munnen = suun kautta
Predator	= rovdjur = petoeläin

Pycnotic	= skrumpnad t ex. cellkärna i hepatocyt
	= kurttuinen (esim. maksasolun ydin)
Serum	= uppstående klar vätska efter att blodet stelnat
	= veren hyydyttyä muodostunut kirkas neste
Sinusoid	= blodkanal i lever
	= maksan verisuoni
Three-spined stickleback	= storspigg, *Gasterosteus aculeatus*
	= kolmipiikki, *Gasterosteus aculeatus*
Vacuole	= hålrum i cell
	= solurakkula

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APPENDIX 1/1

HISTOLOGY

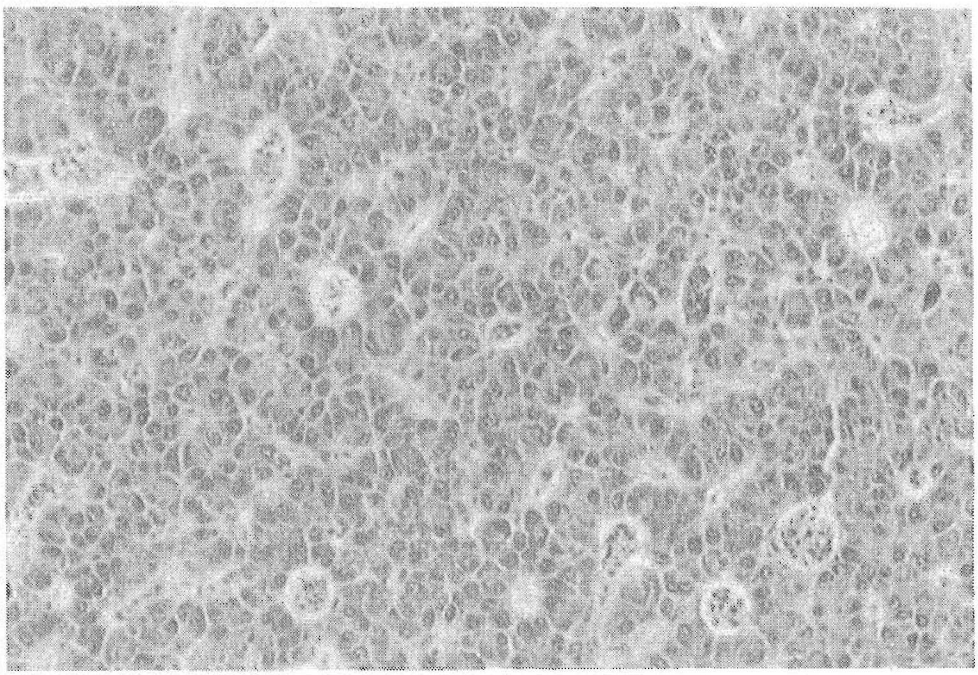

Figure 1. Liver section of an unexposed three-spined stickleback efter 5.5 months. The tissue is well stained with regular cell structure and well defined nuclei. Hematoxylin-Eosin. x20.

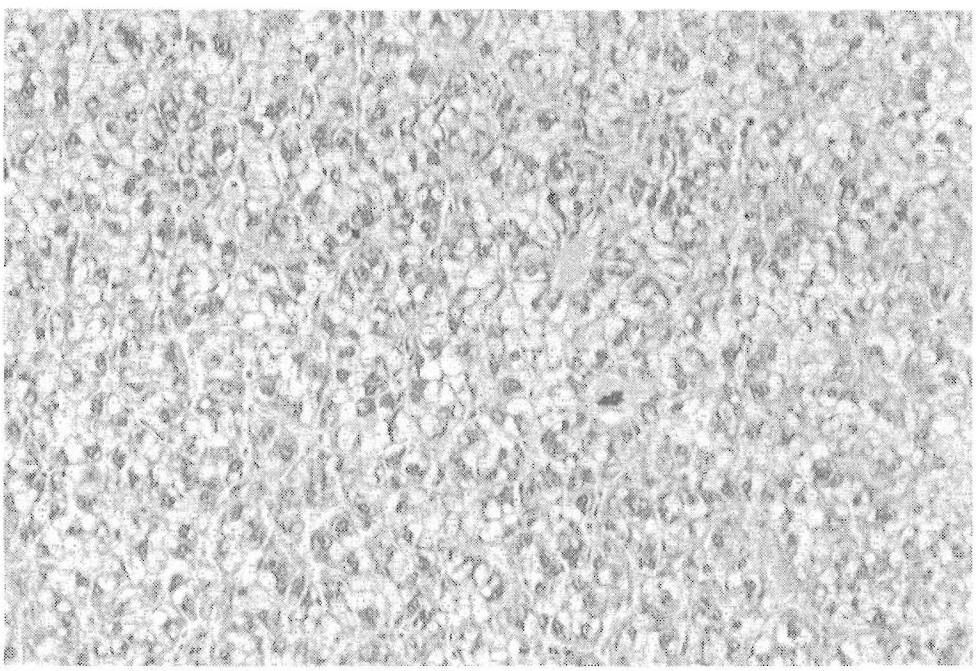

Figure 2. Liver section of a stickleback exposed to wood steroids. Extensive vacuolization probably due to high glycogen content is seen. Hematoxylin-Eosin x20.

APPENDIX 1/2

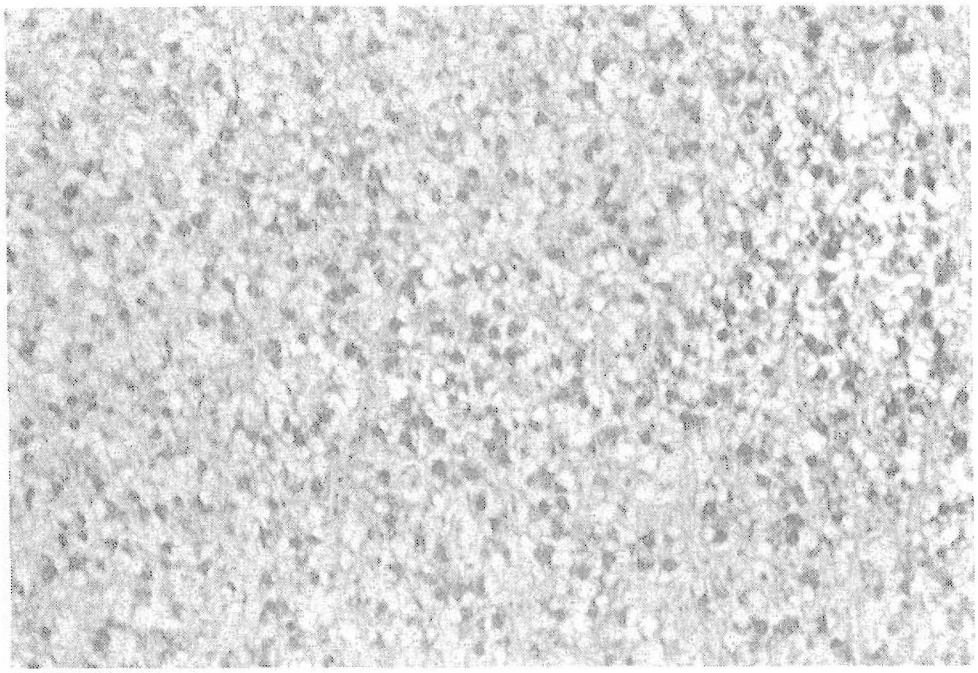

Figure 3. Liver section of a stickleback exposed effluent of mill At, HD. The tissue is extensively vacuolized with some degree of nuclear condensation. Hematoxylin–Eosin x20.

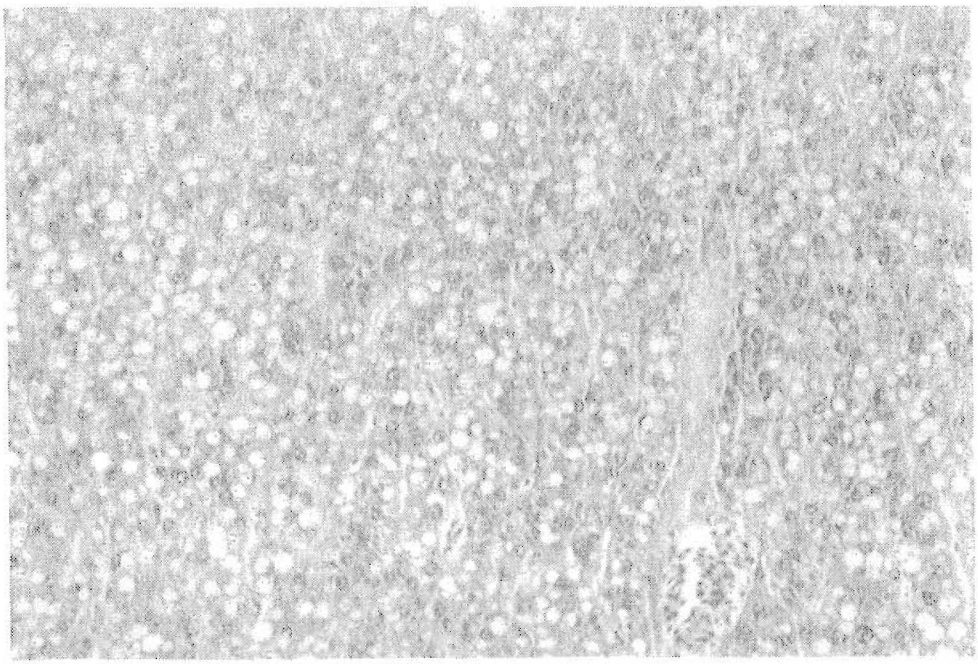

Figure 4. Liver section of a stickleback exposed to effluent from mill Bt, Hd. The cells contain vacuoles of round, smooth character, possibly containing fat. The nuclei appear round and distinct. Condensation or pycnosis is not seen. Hematoxyli–Eosin x20.

APPENDIX 2/1

Chemical–analytical methods.

Water analysis

Chlorinated phenolics analysis by in-situ acetylation and GC-ECD.

5 -10 mL industrial waste water samples (with added 2,6-dibromo phenol as internal standard) were buffered to pH 9 by adding a K_2CO_3/$NaHCO_3$ solution, acetylated with acetic anhydride and extracted with 1 mL hexane. The hexane extract was collected, concentrated under a gentle stream of nitrogen, and a 0.5 - 1.0 µL volume injected on-column for gas chromatographic analysis with electron capture detection (ECD). The column was a slightly polar 25 m capillary DB-5.

Resin and fatty acids (and sterols) analysis by solvent extraction and GC-FID.

50 -100 mL industrial waste water samples (with heptadecanoic acid as internal standard) were buffered to pH 9 by adding a K_2CO_3/$NaHCO_3$ solution and extracted twice with nearly equal volumes of MTBE (methyl *tert*-buthyl ether). The combined MTBE extract was concentrated to near dryness in a rotary vacuum evaporator, dissolved in a few mL diethyl ether/ methanol (9:1), methylated with diazo methane. The solvent was removed under a stream of nitrogen and the residue silylated by adding 50 - 100 µL BSTFA (bis-trimethylsilyl-trifluroacetamide) and heating at 70 °C for one hour. 0.7 µL was injected on GC using a splitless injection technique and the analytes detected with a flame ionization detector (FID). The column was an unpolar 25 m capillary DB-1.

Fish bile analysis

Total chlorinated phenolics, recoverable after alkaline hydrolysis.

600 µL bile samples were freeze dried in open test tubes, and wet and dry weight recorded. The dry residue was hydrolyzed by adding 2 mL of 0.5 M KOH in 90 % ethanol and heating for two hours at 70 °C. The solution was cooled, diluted with 3 mL distilled water, acidified (pH 2) by adding dilute sulfuric acid, and extracted three times with 3 mL diethyl ether. 2,6-dibromophenol and heptadecanoic acid was added as internal standards. The combined ether extracts were divided into two equal parts (one part for resin/fatty acid analysis).

The ether extract was concentrated under nitrogen to less than one mL (i.e. a water-rich residue) and 4 mL carbonate buffer added. Hexane was added and the mixture acetylated by addition of acetic anhydride. The hexane phase was analyzed by GC-ECD as above.

Total resin/fatty acids, recoverable after alkaline hydrolysis.

Hydrolysis and ether extraction as above. The ether extract was concentrated under nitrogen to less than one mL. Buffer solution (5 mL, pH 9) was added and the mixture extracted twice with 4 mL of MTBE. The MTBE was removed by a stream of nitrogen and the extract methylated and analysed by GC-FID as above.

APPENDIX 2/2

Sediment analysis

Total chlorinated phenolics and resin/fatty acids, recoverable after alkaline hydrolysis.

Sediments were freze dried and wet and dry weight recorded. 200 -300 mg dry sediment was weighted into a test tube, a small amount of ascorbic acid added, and hydrolysis performed by adding 2 mL of 2.5 M KOH in 50 % ethanol with simultaneous bubbling of nitrogen through the solution. Tubes were capped and heated at 70 °C for 16 -18 h. The solutions were cooled, diluted with distilled water, acidified by adding concentrated sulfuric acid (cooling), and extracted with diethyl ether and analyzed as for bile samples.

Extractable organic halogen, EOX.

A few grams of wet sediment was shaken (45 min) three times with 10 mL of a cyclohexane-isopropanol (1:1) mixture. The combined extract was washed by shaking three times with acidified $NaNO_3$ (0.25 M, pH 2), and then dried over $NaSO_4$. The volume of the final extract was recorded and a 5 mL aliquote taken for halogen determination with combustion/micro-coulometric halogen titration. The solvent was removed under a stream of nitrogen and a small volume of hexane/iso-oktane was added prior to injection into the furnace of a TOX apparatus.

Total organic material, determined as loss on ignition.

Dry sediment was heated for 3 h in 500 °C, and the loss of weight recorded.

Biological material analysis

Total chlorinated phenolics and resin/fatty acids, recoverable after alkaline hydrolysis.

The analytical procedure was the same as for sediments except that wet sample material was used, and a mechanical homogenization was performed after addition of the alkaline ethanol-water solution.

Extractable organic halogen, EOX.

The analytical procedure was the same as for sediments except that wet sample material was used, and a mechanical homogenization was performed after addition of the cyclohexane-isopropanol solution.

PART II

CHEMICAL CHARACTERIZATION AND EFFECTS IN MESOCOSMS OF EFFLUENTS FROM BLEACHED HARDWOOD KRAFT PULP PRODUCTION

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Abstract
Two different bleaching sequences [mill A: (C20+D80)(EOP)DED and mill B: O(D27,C68+D5)(EOP)D(EP)D] were tested. The effluent from alternative A was treated in an activated sludge plant and from alternative B in a pilot plant aerated lagoon.
The total volume of bladder–wrack (*Fucus vesiculosus*) was decreased in the exposed pools, however, probably due to animal grazing. Periphytic algae was shown to increse in exposed pools as consequence of increased introduction of nutrients. The bladder–wrack was nitrogen–deficient, showing that this alga was unable to compete succesfully for nutrients with fast growing periphytic annual algae. The annual *Spirogyra* green alga occured randomly in some pools, one control included. It was clearly indicated that occurence of *Spirogyra* moderated the responses of the invertebrate fauna in exposed pools. Crustaceans belonging to the *Gammaridae* family showed stimulatory growth responses in all doses, regardless of treatment or no treatment of the effluents.
Sediment EOX–concentrations increased significantly in all exposed mesocosms. The lowest concentrations were found in pools recieving treated mill A and B effluents. Fish exhibited increased EOX–levels only in mesocosms recieving treated and untreated mill A effluent. An EOX budget calculated on the total mass of organic sediment, *Gammarus spp, Cardium sp.,* and fish (stickleback) showed clearly that most of the EOX–pool was associated with sediment and *Cardium sp.*, whereas *Gammarus spp.* and sticklebacks contained much less. The total amount of AOX dosed and the total EOX in the mesocosms did not correlate with effects seen on the animal communities.

Keywords
Effluents, hardwood, kraft sulphate pulp, environmental effects, mesocosms

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KUVAILULEHTI

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Toukokuu 1992

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K.-J. Lehtinen, J. Tana, P. Karlsson, C. Engström, K. Mattsson, S. Hemming, J. Hemming ja A.-L. Fugleberg

Julkaisun nimi (myös ruotsinkielinen)
Lehtipuumassan tuotannosta aiheutuvien jätevesien vaikutuksista malliekosysteemissä

Julkaisun laji
Tutkimusraportti

Toimeksiantaja

Toimielimen asettamispvm

Julkaisun osat
Osa I: S. 3–54 Summary: S. 109–125
Osa II: S. 55–108 Yhteenveto: S. 127–144

Tiivistelmä
Tutkimuksessa testattiin lehtipuumassan kahta eri valkaisutapaa: tehdas A (C20+D80)(EOP)DED ja tehdas B (D27,C68+D5)(EOP)D(EP)D. Tehtaan A jätevesi oli käsitelty aktiivilietelaitoksessa ja tehtaan B jätevesi keinotekoisessa ilmastetussa lammikossa.
Rakkolevän (*Fucus vesiculosus*) kokonaismäärä altistetuissa malliekosysteemialtaissa pieneni, joka voi johtua siitä, että levissä elävät eläimet söivät rakkolevää. Perifyyttisen levän määrä lisääntyi altistetuissa altaissa, johtuen lisääntyneestä ravinteiden määrästä. Rakkolevä on typpirajoitteinen, joka osoittaa, että tämä levä ei pysty menestyksekkäästi kilpailemaan ravinteista nopeasti kasvavien levien kanssa. Viherlevä *Spirogyra* esiintyi satunnaisesti eräissä altaissa, toinen vertailuallas mukaanluettuna. *Spirogyra*n esiintymisen voitiin selvästi osoittaa lieventävän jätevesien selkärangattomille aiheuttavia vasteita. *Gammaridae*–suvun äyriäisissä voitiin todeta kasvun kiihtymistä kummassakin laimennuksessa, huolimatta siitä olivatko altistavat jätevedet käsiteltyjä tai käsittelemättömiä.
Sedimentin EOX–pitoisuudet kohosivat selvästi kaikissa jätevesille altistuneissa altaissa. Alhaisimmat pitoisuudet todettiin altaissa, jotka altistuivat käsitellylle ja käsittelemättömälle tehtaan A jätevedelle. Orgaanisen sedimentin, *Gammaruksien, Cardiumin* ja kalojen (kolmipiikki) kokonaisbiomassaan perustuva laskelma EOX–taseesta osoitti selvästi, että suurin osa EOX:stä oli sitoutuneena sedimenttiin ja *Cardiumeihin* kun taas *Gammaruksissa* ja kaloissa EOX:ää oli paljon vähemmän. Altaisiin annosteltu kokonaismäärä AOX:ää ja malliekosysteemeistä mitatut kokonais–EOX:t eivät korreloineet eläinyhteisöissä todettuihin vaikutuksiin.

Asiasanat (avainsanat)
Ympäristövaikutukset, jätevesi, lehtipuut, sellu, malliekosysteemit

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Kemisk karakterisering och effekter i modellekosystem av avloppsvatten från produktion av blekt björksulfatmassa

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Referat
Två blekprocesser var föremål för undersökningen. I det första alternativet blektes björkmassan enligt sekvensen (D80+C20)(EOP)DED (alt. A). I det andra alternativet blektes massan enligt sekvensen O(D27,C68+D5)(EOP)D(EP)D (alt. B). Avloppsvattnet i det förstnämnda fallet externrenades i en aktivslammanläggning, medan det i det andra renades i en pilot luftad damm.
Effekter på blåstång (*Fucus vesiculosus*) uppstod i form av minskad biomassa, sannolikt till följd av ökad betning av tånglevande djur. Påväxtalgernas biomassa ökade, vilket berodde på ökad närsalttillförsel. Blåstången var emellertid kvävebegränsad, vilket visade att denna alg inte kunde konkurrera med mera snabbväxande alger om tillgängliga närsalter. Den ett-åriga algen *Spirogyra sp.* förekom slumpmässigt i några bassänger, inklusive en kontroll. Klara indikationer på att *Spirogyra* modererade effekterna på ryggradlösa djur i exponerade bassänger. Stimulatoriska effekter på tillväxten hos märlkräftor (*Gammarus spp.*) erhölls i samtliga fall, oberoende av om externrening tillgripits eller inte.
Exponeringen orsakade förhöjda EOX-halter i sedimenten i samtliga doser. De lägsta halterna noterades i bassänger som mottog behandlade avloppsvatten från de två testade processerna.
Förhöjda EOX-halter i fisk noterades endast i de grupper som exponerats för avloppsvatten från A (behandlat och obehandlat). En beräknad EOX-budget, baserad på den totala sedimentmassan, *Gammarus spp., Cardium sp.* och fisk, visade klart att det mesta av EOX var bundet till sedimentet och *Cardium sp. Gammarus spp.* och fisken innehöll mycket mindre mängder EOX. Den totala doserade mängden AOX korrelerade inte med de noterade effekterna på djursamhällena.

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Miljöeffekter, avloppsvatten, lövträd, cellulosa, modellekosystem

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Chemische Charakterisierung und Einflüsse in einem die Ostseeküste darstellenden Modellökosystem von behandelten und unbehandelten Abwässern aus der Papierfaserstoffherstellung

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Zusammenfassung
Es wurden zwei unterschiedliche Bleichfolgen [Fabrik A: (C20+D80)(EOP)(DED) und Fabrik B: O(D27,C68+D5)(EOP)D(EP)D] getestet. Das Abwasser aus der Alternative A wurde in einer Belebtschlammanlage und aus der Alternative B in einem künstlichen belüfteten Teich behandelt.
Die Gesamtmenge vom Blasenstrang nahm jedoch in den ausgesetzten Becken, vermutlich deswegen ab, dass im Blasenstrang lebende Tiere den Blasenstrang frassen. Es wurde gezeigt, dass die periphytische Alge sich in den ausgesetzten Becken infolge zugenommener Einführung der Nährstoffe vermehrte. Der Blasenstrang war arm an Stickstoff, was zeigte, dass diese Alge nich imstande war, erfolgreich um die Nährstoffe mit den schnell wachsenden einjährigen Algen des Pflanzenplanktons zu bekämpfen. Die einjährige *Spirogyra*–Grünalge kam gelegentlich in einigen Becken vor, einschliesslich eines Kontrollbeckens. Es wurde deutlich gezeigt, dass das Vorkommen von *Spirogyra* die Reaktionen der Invertebrata in den ausgesetzten Becken milderte. Krebstiere der *Gammaridae*–Gattung zeigten stimulierende Wachstumsreaktionen in allen Dosen, ungeachtet der Behandlung oder Nicht–Behandlung der Abwässer.
Sedimentäre EOX–Konzentrationen nahmen bemerkenswert in allen ausgesetzten Becken zu. Die niedrigsten Konzentrationen wurden in Becken gefunden, die behandelte Abwässer von den Fabriken A und B aufnahmen. Fische wiesen zugenommene EOX–Niveaus nur in Becken auf, die behandeltes und unbehandeltes Abwasser aus der Fabrik A aufnahmen. Eine EOX–Bilanz, bezogen auf die Gesamtmasse vom organischen Sediment, von *Gammarus* spp., *Cardium* sp. und von Fischen (Stichling), zeigte deutlich, dass der grösste Teil von dem EOX–Becken mit Sediment und *Cardium* sp. verbunden war, während *Gammarus* spp. und Stichlinge viel weniger EOX aufwiesen. Die gesamte dosierte AOX–Menge und die gesamte EOX–Menge in den Modellökosystemen entsprachen nicht den Einflüssen, die in den Tiergemeinschaften beobachtet wurden.

Stichwörter
Einflüsse, Abwässer, Laubholz, Kraftzellstoff, Modellökosystem

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This is a report from a Finnish–Swedish joint project on effects of pulp mill effluents in mesocosms. The work is part of a systematic research for evaluation of the environmental impact of effluents from pulp mills with different bleaching procedures and effluent treatment solutions.

The work was done at the Baltic Sea Research Laboratory (FERG)*) in co–operation with Åbo Akademi, Kuopio University and Helsinki University. The work was financed by the Finnish forest industry companies Kymmene Oy, Enso–Gutzeit Oy, Metsä–Botnia Oy, Veitsiluoto Oy and the SYTYKE–project. The Swedish financing part was the Swedish Forest Industries Foundation for Water and Air Pollution (SSVL).

*)FERG= Finnish Environmental Research Group

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1 Chemical-analytical methods

1 INTRODUCTION

One central problem in current ecotoxicological research is to identify, within the natural ecosystem, such species or subsystems that may have a key function for the integrity of the whole system or may be particularly sensitive to pollutants. In the Baltic Sea, the bladder-wrack, (*Fucus vesiculosus* L.), is the dominant structural element in the shallow, hardbottom ecosystem and as such, it plays an important role for the function of this subsystem as well as of adjacent subsystems (Jansson et al. 1982). The bladder-wrack zone is a prominent spawning, nursery and/or feeding area for a great number (>70%) of the macroscopic animal species, including fishes, living in the Baltic Sea (Haage, 1975).

Traditionally, ecotoxicological tests are of single-species character and batteries of different organisms from different taxonomic levels are used in order to identify weak links of the ecosystem. However, information on interspecific effects and their relative importance for the ecosystem integrity are not gained from single species tests. In order to meet the demand for a better knowledge about interspecific/indirect effects of toxicants a model ecosystem simulating the littoral, bladder-wrack zone of the Baltic Sea was developed in the middle of the 1970s (Notini et al. 1977). Since then the model ecosystems have successfully been used to test ecological effects of oil and dispersants, arsenic and trichloro-guaiacol (Notini and Hagström, 1974; Linden et al., 1987; Rosemarin and Notini, 1989).

In the 1980s the impact of pulp mill effluents on this particular subsystem was studied using this technique. The complete results have been published by Rosemarin et al.(1986); Lehtinen et al.(1988); Lehtinen (1989); Lehtinen et al.(1990); Lehtinen (1990); Rosemarin et al. (1990) and Lehtinen et al. (1991).

The objective of the investigations referred to above was to evaluate the significance of bleach process modifications on the environmental impact of bleached kraft pulp mill effluents. The technology for production of bleached chemical pulp have changed significantly during the 1980s. Most bleacheries in the Northern countries are equipped with oxygen pre-delignification. Elementary chlorine is increasingly substituted with chlorine dioxide and the alcaline exctraction stage is fortified with oxygen or peroxide. External effluent treatement in aerated lagoons or activated sludge plants is also widely used.

In the present work two total effluents from production of bleached hardwood kraft pulp were tested. One of the mill is using oxygen pre-delignification and 30 % chlorine dioxide in the first bleaching step followed by an oxygen and peroxide reinforced alcaline extraction.

The present report is dealing with the effects of the effluents on invertebrate macrofauna and algae as well as the occurrence and distribution in the mesocosms, of componds formed and/or released in the process.

Effects on growth, tissue structure, parasite infection and physiological status of fish are reported elsewhere (Lehtinen et al. 1991b).

2 MATERIAL AND METHODS

2.1 Processes and emissions

The two kraft pulp mills tested in the present work are called mill A and mill B. Untreated mill effluent is called "Au" and "Bu", and treated effluent "At" and "Bt" respectively.

Mill A is producing fully bleached hardwood pulp according to the sequence (C20+D80)(EOP)DED. The effluent is treated in an activated sludge plant with a residence time of about 24 hrs. Mill B is producing fully bleached market pulp using the sequence O(D27,C68+D5)(EOP)D(EP)D. The effluent is treated in an aerated lagoon with a residence time of about 8-9 days. Production of pulp and process data

are presented in table 1.

The sampling procedures are described below.

The effluent samples where taken as grab samples at the respective mill under normal running conditions. The samples used in the mesocosm experiments were collected in 1 m^3 polyethylene containers. Enough time was allowed to pass between sampling of ingoing and outgoing water in the biological treatment plants in order to account for the residence time. Smaller samples for chemical characterization were taken with fixed intervals at strategic sampling points in the mills in order to control the process conditions.

Technical disorders at mill B made sampling of a representative treated effluent impossible. An untreated sample was consequently collected and transported to the laboratory, where it was treated in a pilot plant aerated lagoon, activated with the bacterial sludge from mill B:s aerated lagoon. The efficiency of the treatment in terms of the reductions of BOD and COD, was very much alike that normally reported from mill B (Table 2).

The concentrations of chlorinated phenolics, resin acids, chlorinated resin acids and steroids are presented in Table 3.

Total gas–chromatographically eluted extractives are presented in table 4.

The effluents used for the model ecosystem experiments were transported to the laboratory in 1 000 l polyethylene containers. In order to eliminate the chlorate present in the effluents, the containers were sealed air tight to reach as low oxygen levels as possible during the first 24 hrs after sampling (cf. Rosemarin 1987). At the laboratory the effluents were transferred to smaller, 30 l polyethylene containers and thereafter frozen in −30 °C. The effluents were placed in freezer within roughly 30 hours after sampling. Required amounts where thereafter thawed and used in the experiments.

Effluent dosage was performed using membrane pumps (Prominent Electronic) at 400 and 2000 times dilutions based on a normalized effluent volume of 50 $m^3\ t^{-1}$ pulp. Exposure of the mesocosms commenced on June 26, 1990 for mill A. Due to the technical difficulties at mill B, effluent was obtained later and the exposure could not start before July 12. An extra control mesocosm pool was used in order to obtain data regarding ecological development taking place during this two–week period.

Table 1. Production of pulp/day, effluent flow (m^3/t pulp) and some running data of the two different pulp mills tested.

	Mill A	Mill B
Production $t_{90}\ d^{-1}$	1 450	945
Effluent flow $m^3\ t^{-1}$	40	54
Bleaching sequence	(D80+C20)(EOP)DED	O(D27,C68+D5)(EOP)D(EP)D
ClO_2 in D+C kg/t90	34.7	5.3
Cl_2 in D+C kg/t90	9.8	11.1
ClO_2 amount (%)	80	32
Kappa number to bleachery	15	13
Chlorine multiple	0.06	0.08
Active chlorine multiple	0.28	0.12
Total ClO_2 kg/t90	67	35
NaOH E1 kg/t90	19	11.1
NaOH E2 kg/t90	4	4.6
Oxygen E1 kg/t90	4.5	4.6
H_2O_2 E1 kg/t90	1.3	1.0
H_2O_2 E2 kg/t90	–	1.0
Viscosity $dm^3\ kg^{-1}$	1 075	1 025

Table 2. COD, BOD_7, AOX and chlorate levels before and after treatment of Mill A:s and B:s effluents.

Mill	COD mg l^{-1}	BOD mg l^{-1}	AOX mg l^{-1}	Chlorate kg t^{-1} pulp
Mill A				
Untreated, Au	2100	800	39	5.8
Treated, At	910	100	23	–
Mill B				
Untreated, Bu	540	145	17*)	2.0
Treated (pilot), Bt	320	20	7.3	–

– = not detected
*) analyzed at STFI, Sweden. Other AOX-values analyzed at Enso-Gutzeit's Research Centre.

Table 3. Concentrations (μg l^{-1}) of chlorinated phenolics (Cl-P), chlorinated guaiachols (Cl-G), resin acids (RA), chlorinated resin acids (Cl-RA) and steroids in the untreated and treated effluents from mill A and B. Au = untreated mill A; At= treated mill A; Bu = untreated mill B; Bt = treated mill B.

	Cl-P	Cl-G	RA	Cl-RA	Steroids
Au	15	28	1 700	110	870
At	0.5	14	100	49	190
Bu	19	8	176	29	17
Bt	8	2	10	30	18

Table 4. Total amount of extractives eluted from the gas chromatograph from the untreated and treated effluents from mill A and B. Au = untreated mill A; At= treated mill A; Bu = untreated mill B; Bt = treated mill B.

Effluent	Total extractives ug l^{-1}
Au	21 900
At	6 900 – 12900
Bu	2 000 – 2 700
Bt	760 – 1 100

2.2 The mesocosm set-up

The mesocosms are established in circular pools with an inner polyethylene coating and a volume of 8 m^3. The pools, placed in the open air, are supplied with a continuous flow of brackish water (2.8 l min^{-1}), which is pumped from 10 meters depth from a brackish water bay close to the laboratory in Nagu, SW Finland.

The principal experimental set-up is presented in Figure 1.

The bottom of each mesocosm pool was covered with a layer of pure sand without organic material and macroscopic organisms. The total volume of sand added to each pool was 250 l giving a sediment thickness of about 3 cm. Bladder-wrack, (*F. vesiculosus*), specimens were collected in in the field using 40x60 cm polyethylene bags which were slipped over the alga including the stone to which it was attached. The bag containing the alga and associated organisms was cautiously lifted to the surface, excessive water removed, and taken to the laboratory. The volume of every specimen was thereupon determined by the water displacement method. Bladder-wrack specimens were introduced evenly (about 7 l/pool) in the same region of each pool in order to obtain identical insolation for the plants. The number and kinds of animal taxa were estimated, and in case of some taxa lacking or occurring in lower numbers in a pool, additional organisms were added to reach similar levels in all pools. Some free-swimming predator species (fish) were added in known numbers; one hundred stickleback (*Gasterosteus aculeatus*) brood and 7 bleaks, (*Alburnus alburnus*) were introduced into each pool. Twenty five pre-weighed specimens of blue mussels, (*Mytilus edulis*) were also placed in 10x25x75 cm plastic lid-equipped

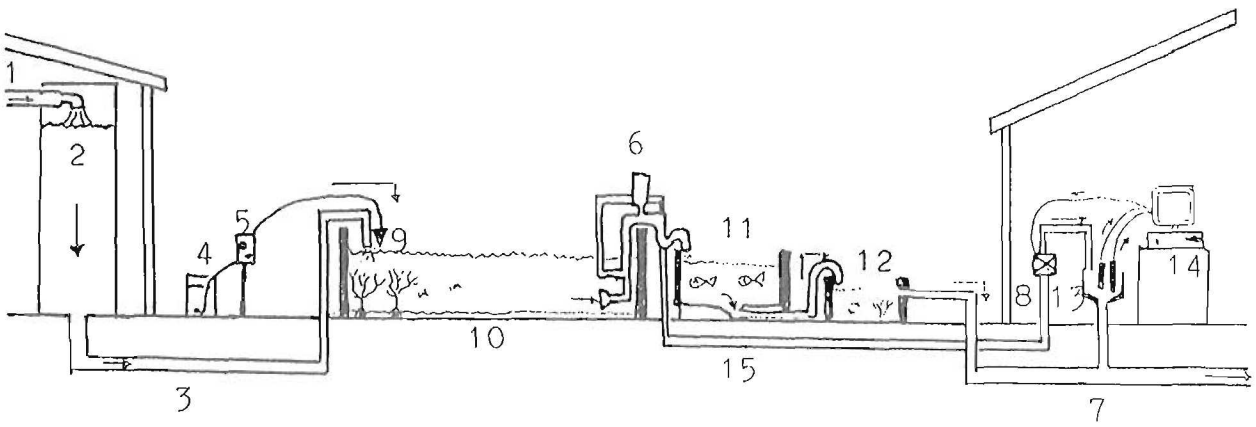

Fig. 1. The mesocosm set-up

1. Incoming water
2. Seawater tank
3. PEH-pipe to the pools
4. Effluent container
5. Membrane pump
6. Siphon for out-going water
7. PEH-pipe for out-going water
8. Electric valve
9. Dosage of effluent water
10. Mesocosm pool
11. Rainbow trout tank
12. Stickleback tank
13. Registration electrodes
14. Computer

boxes with six 5 cm holes covered with a (1 mm mesh size) plastic net for controlled growth measurements. Six 20x20 cm polyethylene squares equipped with a weight were placed into each pool on a cord attached in N–S direction just below the water surface for quantification of periphyton growth. The pools were left to stabilize for 2–4 (mill B) weeks before exposure to effluents started.

2.2.1 Sampling and analyses

The effluent exposure was maintained until November 12, when the experiment was terminated and final sampling for the different biological and chemical analyses was done.

Apical growth of the bladder–wrack as well as growth of the blue mussels were measured monthly.

At the end of the experiment, five specimens of bladder–wrack were sampled, using the bag technique, for analysis of the associated invertebrate fauna abundance and biomass. The rest of the bladder–wrack biomass was measured in the same way as at the start of the experiment and apical fronds were sampled for later tissue N/P analysis. The algae sampled for fauna measurements were later pulled together with the remaining bladder–wrack biomass in order obtain total biomass after exposure.

Sediment fauna was sampled by taking 8 separate 12 cm diameter cores from each pool. Results regarding abundance and biomass were compared on a pool unit basis by pulling together the bladder–wrack and sediment data.

The fish were collected individually after the water level was lowered to a few centimeters. All remaining fish were counted, their weight and length measured, whereupon they were frozen for later chemical analysis.

After samples for quantitative biological analyses were taken, samples for chemical analysis were collected from the crustacean, mollusc, fish, algal, and sediment component, in order to evaluate the distribution of various compounds in the system.

Chemical analysis was also made on undiluted effluents, in order to calculate total theoretical dosed amounts. In this way information on the persistence and bioaccumulation of substances in the systems was obtained. For information on analytical methods, see appendix 1.

Water samples for nutrient analysis were taken in the beginning, in the middle, and at the end of the experiment from the mesocosms as well as from the incoming water in order to check the nutrient level of the systems.

Physical–chemical parameters including light were monitored semi–continuously by a computerized system allowing for an estimate of the total primary production of the systems.

Samples for chemical analysis of chlorinated material (AOX), chlorophenolics, resin acids and steroids were taken monthly from undiluted effluents used in the experiment, i.e. effluent aging was accounted for.

2.3 Statistics

Mean values of all parameters measured in effluent exposed pools were compared with the corresponding mean values in control pools using Students' t-test. Levels of significance for differences between pools were set at ***= $p<0.001$; **= $p<=0.005$ and *= $p<0.05$.

3 RESULTS

3.1 Distribution of contaminants in the mesocosms

The total amounts of some important substance groups dosed into the different high dose pools (400 times dilution) during the experiment are presented in Table 5.

From the table it may be noted that DHA contributed with about 50% to the total resin acids dosed to the pools receiving untreated effluents from mill A and B. It may also be noted that mill A:s effluent contained much higher amounts of resin acids than mill B. Activated sludge treatement of mill A:s effluent resulted in an 83 % decrease of RA. The reduction in RA after treatment of mill B effluent in aerated lagoon was 94 %.

The total eluted extractives were also high for mill A before treatment, but treatment resulted in a 56 % reduction. The corresponding figures for mill B were generally low. The reduction was about 50% after treatment in the pilot plant aerated lagoon (Table 4).

The total amount of AOX dosed to the high doses of mill A were much higher than for mill B. For mill A there was a 40 % lower dosage of AOX after treatment (At). For mill B the reduction was 23 %.

Table 5. Total amounts of resin acids (RA) (DHA= dehydroabietic acid presented separately), steroids (Ste), eluted extractives (EL), chlorinated phenols (CP), chlorinated guaiacols (GP) and AOX introduced into the high dose pools respectively. Differences in water flow per ton pulp for the two mills have accounted for in the calculations.

	Au	At	Bu	Bt
RA (mg)	2100	350	240	13
DHA(mg)	1300	35	100	9
Ste(mg)	1100	460	25	25
EL (g)*	27	12	3	0.2
CP (mg)	18	0.7	26	11
CG (mg)	33	19	35	2.6
AOX (g)*	47	28	13	10

*=note that figures are given in grams.

3.1.1 Contaminants in sediment, invertebrates, fish and algae

The concentration of EOX (Extractable Organic Halogen) in different compartments of the mesocosms exposed to the high doses of the effluents tested are presented in Table 6.

There was a clear increase of EOX in all sediment samples as a result of effluent exposure. The gammarids showed an increase in EOX content in the pools exposed to untreated and treated effluent from mill A, whereas gammarids exposed to mill B effluents did not show any increase. The sediment dwelling filtrating mussel *Cardium sp.* showed increased values in all types of effluents, indicating the importance of contaminated suspended material as a contributor to the build–up of the body–burden in filtrating organisms.

The fish component showed increased values of EOX in the pools exposed to effluents from mill A only.

The concentration in individual organisms is interesting from an ecotoxicological point of view. For the whole ecosystem, it is interesting to know the distribution of the total amount of lipophilic chlorinated material in different compartments. In order to obtain a picture of this, the total of amounts EOX in the different mesocosm compartments given in Table 6 were calculated (Table 7).

The total amounts of AOX (Adsorbable organic halogen) dosed to the different pools are given in Table 5. The total amount of AOX dosed for example to AuHD was 47 g over the experimental period. This pool also contained most EOX in the compartments analyzed. It is noteworthy that the BuHD sediment contained more EOX relative to the total amount dosed (17 g) as compared with AuHD. Moreover, it can be noted that external treatment lowered the total amount of EOX accumulated in the systems (relates to both mills) and that the sediment is an important sink together with filtrating organisms such as *Cardium sp.* It might be expected that the fewer organisms present in the sediment compartment, the more material is built up in the sediment in an area with similar hydrological conditions as the mesocosms (> 2 d residence time). As compared with the total amount of AOX introduced into the pools, it may also be noted that only a very small fraction was recovered as EOX in the compartments analyzed, showing that the major part of AOX (>95%) is leaving the system or is broken down.

Table 6. Concentration of EOX in sediment, *Gammarus spp.*, *Cardium sp.*, and three–spined stickleback in mesocosms exposed to treated and untreated kraft pulp mill effluents. Sediment figures given as organic material. Biological tissue figures given as fat weight.

Component ($\mu g\ g^{-1}$)	Control	AuHD	AtHD	BuHD	BtHD
Sediment	0.86	20	11	17	9
Gammarus spp.	140	650	260	100	90
Cardium sp.	460	2200	1500	950	1100
Stickleback	68	350	120	74	68

Table 7. Total EOX in sediment, gammarids, *Cardium sp.*, and three-spined stickleback. Numbers given as mg per total organic material (sediment) and as mg/ whole population biomass d.w. (animals). The amount of organic material and biomasses/pool are given at the bottom of the table.

Compartment	Control	AuHD	AtHD	BuHD	BtHD
Sediment	5.3	146	104	146	56
Cardium sp.	0.65	9.9	4.6	8.2	9.7
Gammarus spp.	0.8	5.0	0.3	0.3	0.1
Stickleback	0.06	0.25	0.06	0.04	0.05
Organic sediment (kg d.w.)	6	7	10	9	6
Cardium (g d.w)	5	15	10	29	30
Gammarus (g d.w.)	88	73	26	27	15
Stickleback (g d.w)	6	5	4	4	6

Regarding specific substances in the same ecosystem compartments as above, no increased levels of chlorophenolics or resin acids were detected as compared with control values. The only exception was an increased amount of dehydroabietic acid in the sediment of the pools exposed to untreated effluents:

Control: 2.6 mg
AuHD: 5.1 mg
AtHD: 2.5
BuHD: 71 mg
BtHD: 3.3 mg

3.2 Effects on primary production of the mesocosms

The primary production of the mesocosms is illustrated by the difference in the levels of pH and dissolved oxygen between the incoming and outgoing water from the pools. The curves for incoming seawater, two control pools and the pools receiving treated and untreated effluents from mill A and B, during a representative period at the end of September are presented in Figures 3 – 5.

The light energy is shown in Figure 6.

In comparison with the incoming seawater it can be seen that there is a net increase of pH and oxygen in the two control pools. It can also be noted that the two controls are similar with regard to their primary production. Moreover, the temperature shows somewhat smaller fluctuations compared with the incoming reference water. The oxygen curve is illustrating the diurnal fluctuation of oxygen; the lowest parts of the curves are a function of a higher respiration during the dark period.

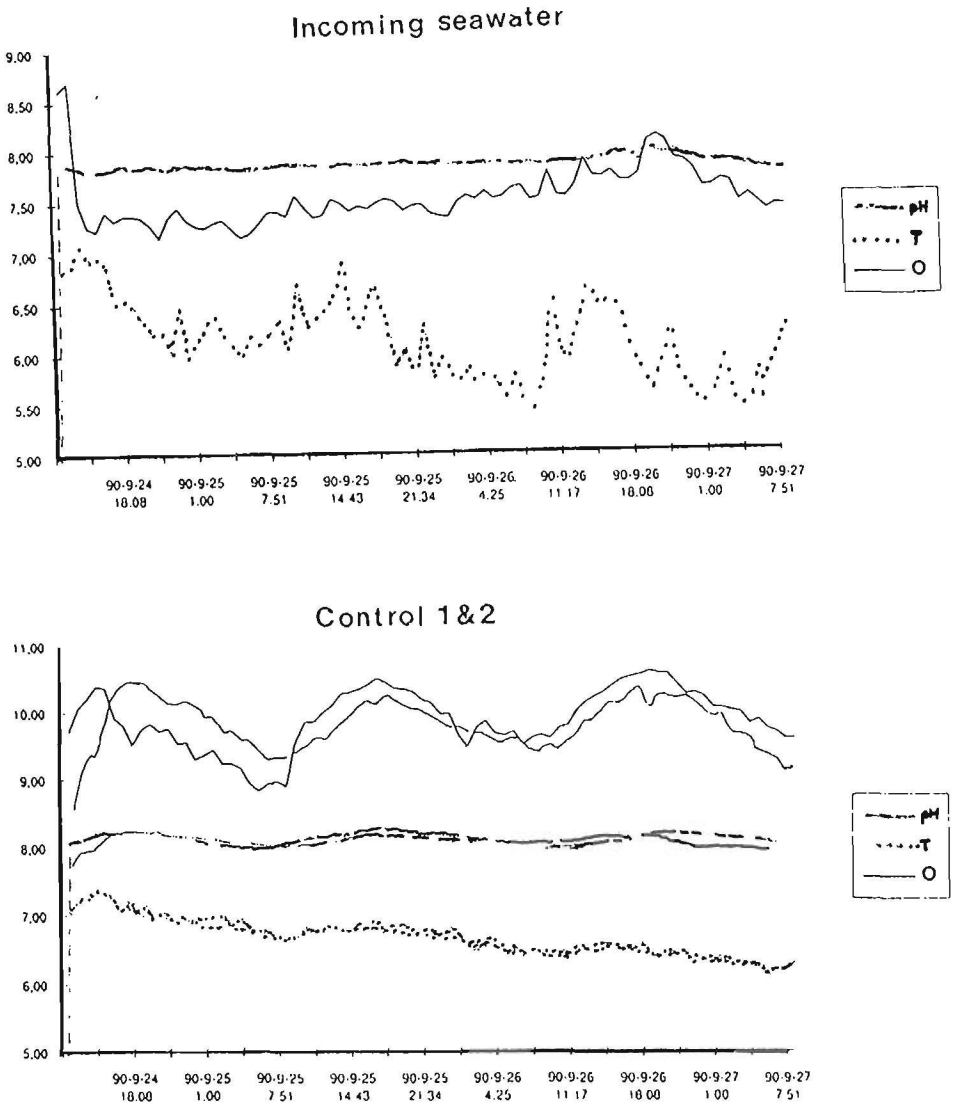

Fig. 3. pH, temperature (T), and dissolved oxygen (O) in incoming seawater and control mesocosm pools in late September, 1990.

The untreated effluent from mill A (Au) gave very similar results as the controls. This was also the case with the treated effluent of mill A (At). Neither effluent induced any community shift from autotrophy to heterotrophy, since there was a net oxygen production at a pH above 8. As to the untreated effluent of mill B, no differences compared with the controls were noted. The drastic drop of the dissolved oxygen in Figure 5 is due to a clogged sampling tube to the computer system. As can be seen, the values rapidly rose to normal in the morning when the disorder was eliminated.

Treated effluent (high and low dose) of mill B exhibited a clear stimulation of the primary production.

The primary production of algae is to a large extent regulated by the levels of available phosphorus and nitrogen besides necessary trace elements (Table 8).

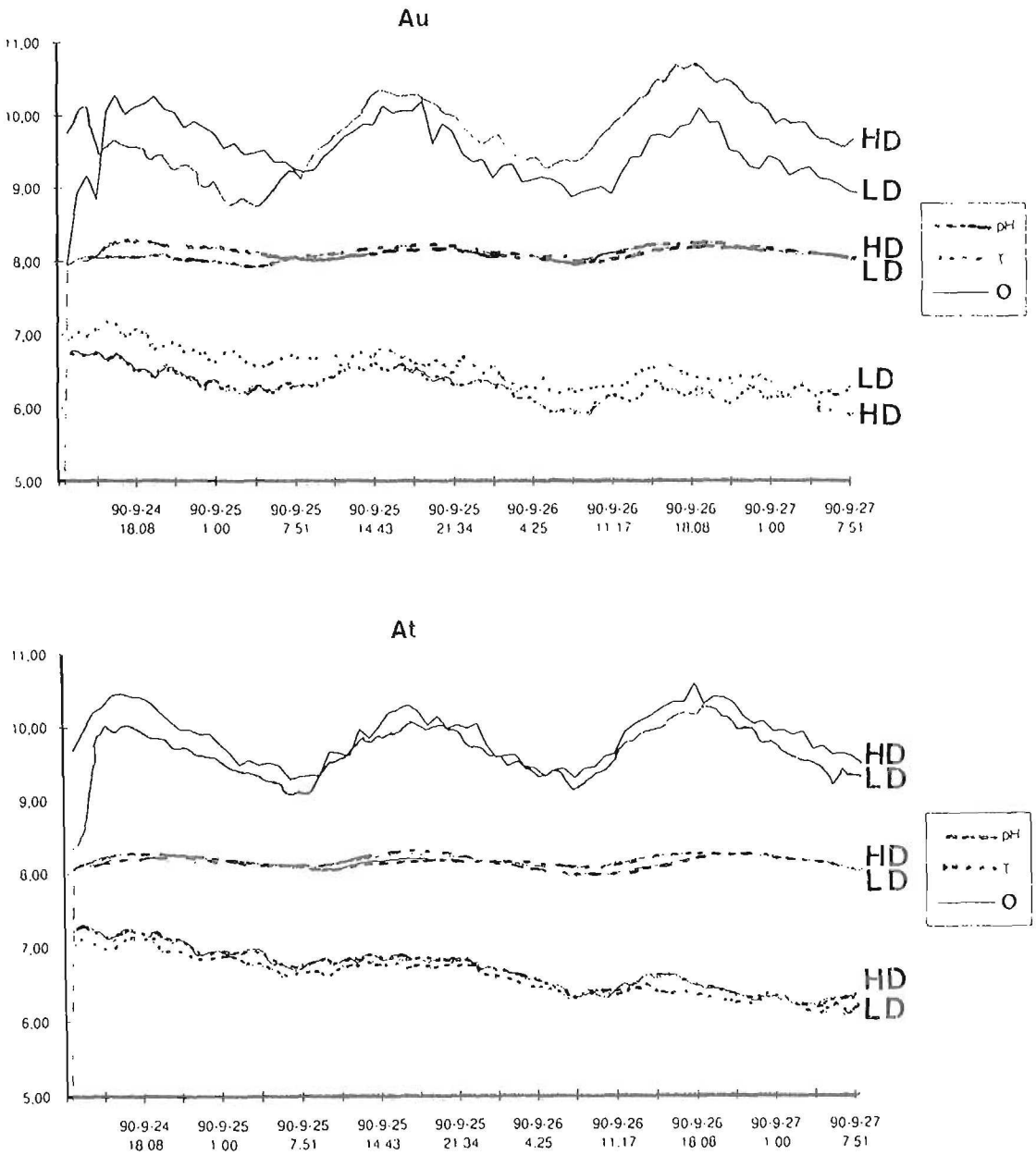

Fig. 4. pH, temperature (T), and dissolved oxygen (O) in mesocosms receiving untreated (Au) and treated (At) effluent from mill A in September 1990. HD=High Dose; LD=Low Dose. Note the similarity with controls.

No reliable values on phosphorus were obtained at analysis. The results have therefore been omitted from the presentation. Reliable values on total phosphorus content in incoming seawater and control water were previously obtained in 1989. Those figures are presented in Table 8 in order to give an indication of the levels that may occur in this water.

The nitrate levels are lowest in July–August, whereupon they start to increase in the autumn. This is due to a slower primary production with decreasing temperature. There is also an increased ammonium level due to increased breakdown of organic material. This picture was consistent in most effluents and doses. In Au,LD ;HD and

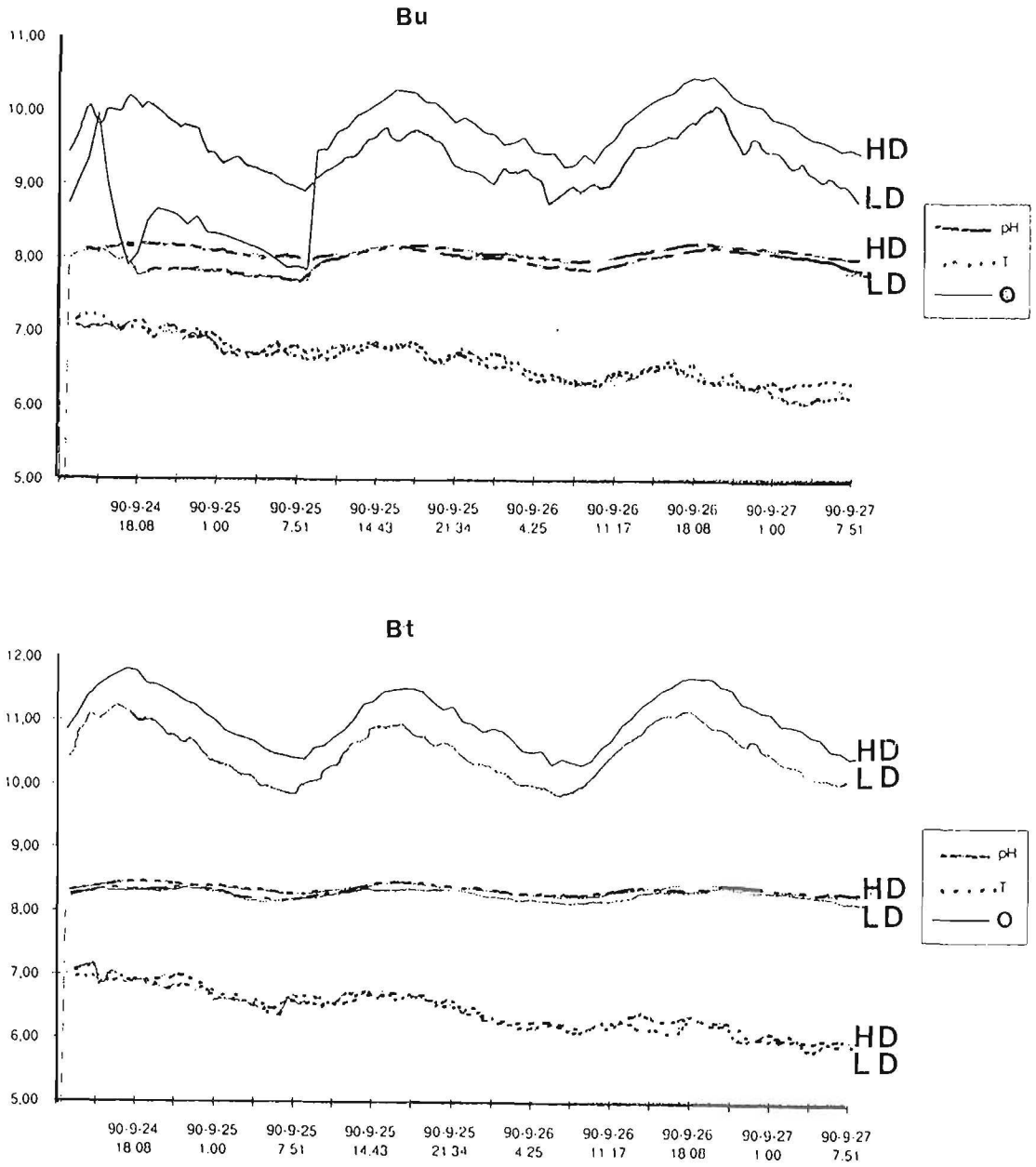

Fig. 5. pH, temperature (T), and dissolved oxygen (O) in mesocosms receiving untreated (Bu) and treated (Bt) effluent from mill B in September 1990. HD=High Dose; LD=Low Dose.

Bu,HD the levels were low in October and November, however, possibly indicating a slower bacterial activity. The treated effluents from both mills exhibited an increased ammonium and an increased nitrate level as well.

The concentrations of inorganic nutrients in the water illustrate the actual levels available for plants. However, different plants possess different strategies for nutrient uptake, in order to cope with competing species. The bladder-wrack is a perennial, slow-growing alga, which is a poor competitor for nutrients in the summer. In order to see whether some plant component is nutrient deficient, it is usually informative to analyze the N/P quotient in plants, in this case the bladder-wrack. In Table 9 the

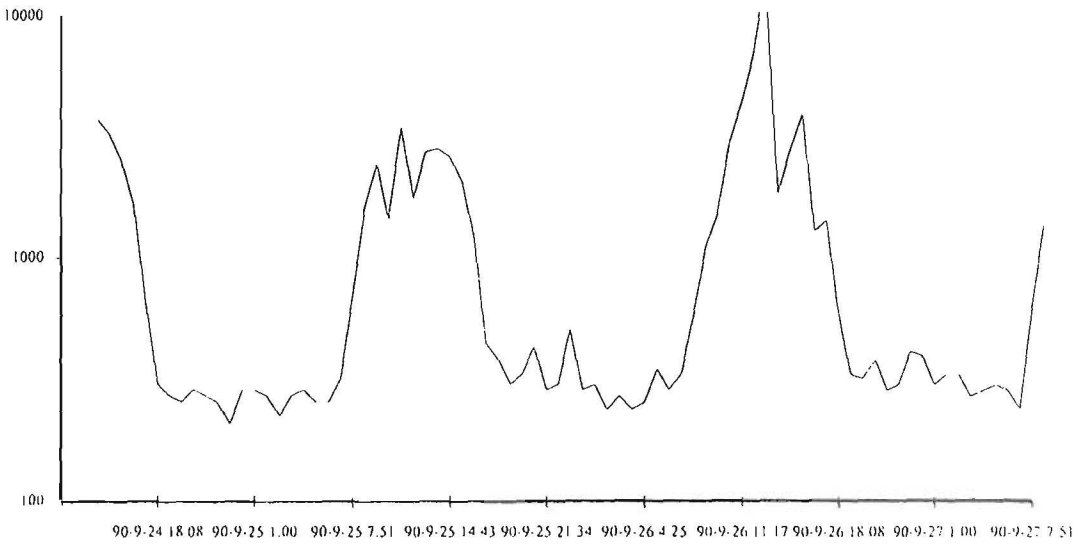

Fig. 6. Incoming light energy over the mesocosm pools in September 1990 (mV cm^{-2}).

N/P quotients in apical fronds of bladder-wrack from the mesocosms are shown, together with the value at the experimental start.

The chlorate concentrations in freshly sampled effluents at the mills were at the detection limit for the treated effluents of both mills. For the untreated effluents, the concentrations were 140 mg l^{-1} for Au and 37 mg l^{-1} for Bu respectively. However, since no significant effects were seen on apical growth or N/P quotients in the bladder-wrack for the groups receiving untreated effluents, it may be noted that the air-tight sealing of the tanks during transport to the laboratory was enough to eliminate harmful effects of chlorate on the bladder-wrack. Thus, effects seen on both bladder-wrack and invertebrate populations may be assumed not to be directly or indirectly caused by chlorate in this experiment.

As can be seen from the table, the quotients decreased in all groups during the experiment. The normal values in nature are usually around 20 (Atkinson and Smith, 1983). From the values obtained it is evident that the bladder-wrack in all mesocosms, including the controls, were nitrogen deficient. From this it is also evident that the dosage of the effluents did not cause neither inhibition of the nitrogen metabolism due to chlorate in the bladder-wrack nor stimulation due to water nitrogen levels. The only deviation from this is the higher N/P quotient in bladder-wrack from the pool receiving untreated effluent from mill A (Au,HD), which might be an indication of a somewhat slower nitrogen metabolism due to sub-lethal concentrations of chlorate. The possible inhibition by chlorate was not high enough to affect apical growth, however (see Figs 8 and 9).

The levels of phosphorus and nitrogen in undiluted effluents are given in table 10.

Table 8. Concentrations of phosphorus, nitrate, nitrite and ammonium in the incoming seawater, controls and exposed pools.

	P (µg l^{-1})	NO_2 (µg l^{-1})	NO_3 (µg l^{-1})	NH_4 (µg l^{-1})
Incoming seawater[1)]				
July	25	<1.0	–	–
August	25	1.0	18	26
October	16	1.0	30	11
Control[1)]				
July	22	<1.0	11	12
August	25	1.0	15	17
October	17	1.0	19	6
November	–	2.0	20	32
Au, LD				
July	–	<1.0	15	17
August	–	<1.0	18	14
October	–	1.0	20	6
November	–	1.5	13	9
Au, HD				
July	–	1.0	13	19
August	–	1.0	15	10
October	–	1.0	24	7
November	–	1.5	14	6
At, LD				
July	–	1.0	9	18
August	–	1.0	10	7
October	–	1.5	26	10
November	–	1.5	24	28
At, HD				
July	–	<1.0	11	9
August	–	1.0	15	12
October	–	1.5	27	8
November	–	2.0	33	32
Bu, LD				
July	–	–	–	–
August	–	1.0	26	10
October	–	1.0	26	10
November	–	1.5	14	12
Bu, HD				
July	–	–	–	–
August	–	1.0	9	13
October	–	1.0	9	16
November	–	2.0	16	7
Bt, LD				
July	–	–	–	–
August	–	<1.0	11	8
October	–	1.0	15	7
November	–	1.5	23	59
Bt, HD				
July	–	–	–	–
August	–	<1.0	15	8
October	–	1.0	16	8
November	–	2.0	24	26

[1)] Values refer to analyses performed in previous experiments 1989.
– = not analyzed

Table 9. N/P quotients in apical fronds of bladder–wrack at start and at the end of the exposure in November.

	N/P quotient (mmole/mmole)
Start in June	18.5
At end, November:	
Control (n=3)	6.1
Au,LD	8.3
Au,HD	11.8
At,LD	5.9
At,HD	3.8
Bu,LD	5.4
Bu,HD	5.8
Bt,LD	6.5
Bt,HD	4.1

Table 10. Concentrations of total– and inorganic phosphorus and total nitrogen, nitrate, nitrite and ammonium in undiluted effluents from mill A and B. Values are in mg l^{-1}.

	Au	At	Bu	Bt
Total–P	2.1	0.6	2.8	2.5
PO_4 – P	1.8	0.5	2.6	2.5
Total–N	17.0	3.0	1.0	1.1
NO_3 – N	0.7	<0.5	0.7	1.0
NO_2 – N	0.01	0.006	<0.003	0.004
NH_4 – N	12.0	0.3	0.01	0.02

The total dosage of P (roughly the same as PO_4) and nitrate to pools receiving 400 times diluted effluents over the experimental period was roughly calculated as follows:

	Total–P	Nitrate
Au:	2.5 g	0.9 g
At:	0.7 g	<0.6 g
Bu:	3.5 g	0.9 g
Bt:	3.4 g	1.3 g

Using a background seawater concentration of 20 μg l^{-1} of phosphorus and 15 μg l^{-1} of nitrate, the total amount dosed through the diluting water was roughly 11 g P and 9 g nitrate for mill A and 10 g P and 8 g nitrate for mill B. Two remarks regarding these findings can be made: (i) despite a rather remarkable contribution of phosphorus (22–35 %) to the systems receiving effluents from Au, Bu and Bt there were no significant stimulatory responses in the growth of bladder–wrack or in total primary production and (ii) the contribution of nitrogen was not enough to keep the N/P quotients at the normal levels found in field populations of bladder–wrack, i.e. these populations were strongly nitrogen deficient.

3.2.1 Effects on bladder–wrack and periphyton algae

The total volume (as per cent of control values) of bladder–wrack from the effluent exposed pools is presented in Fig. 7.

The total volume of the alga decreased significantly (> 20 %) in Au,LD; At,LD;At,HD and in Bu,HD and Bt,LD. There was no dose–response relationships except for At and Bu, and it may be assumed that secondary mechanisms such as grazing of invertebrates may have contributed to the effects observed. An assumption of prevailing secondary effects is strengthened by the fact that there was no significant differences in growth of the apical fronds between controls and exposed pools (Figs. 8 and 9). The apical frond is the location of growth in the bladder–wrack, whereas lower, older parts are subject to mechanical deterioration or animal grazing.

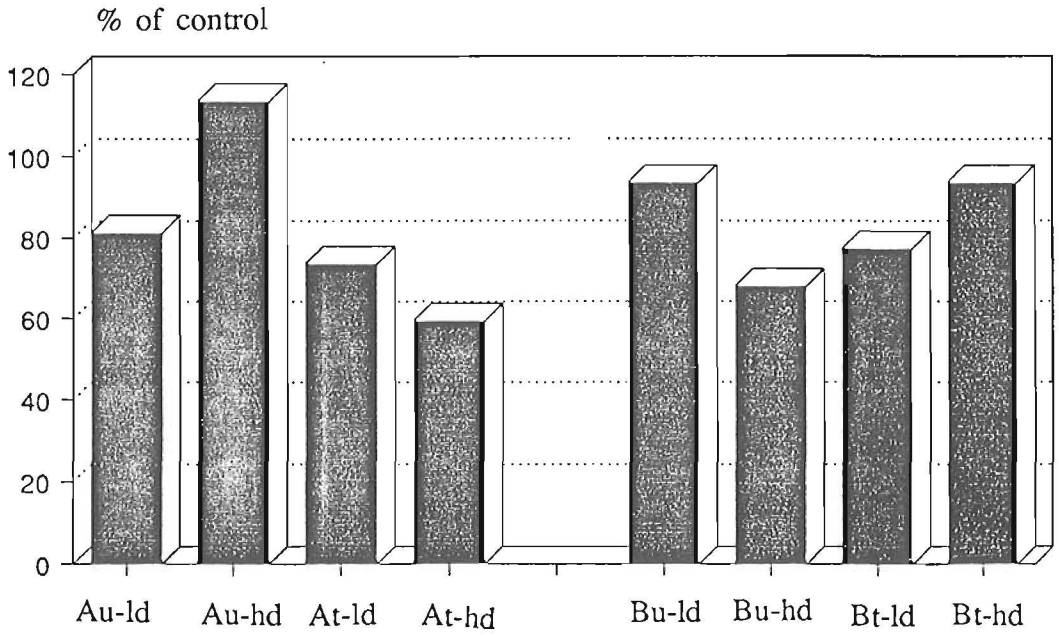

Fig. 7. Total volume of bladder–wrack (*F. vesiculosus*) exposed in mesocosms during 5.5 months to total effluents from production of bleached hardwood kraft pulp. Values given as per cent of control.

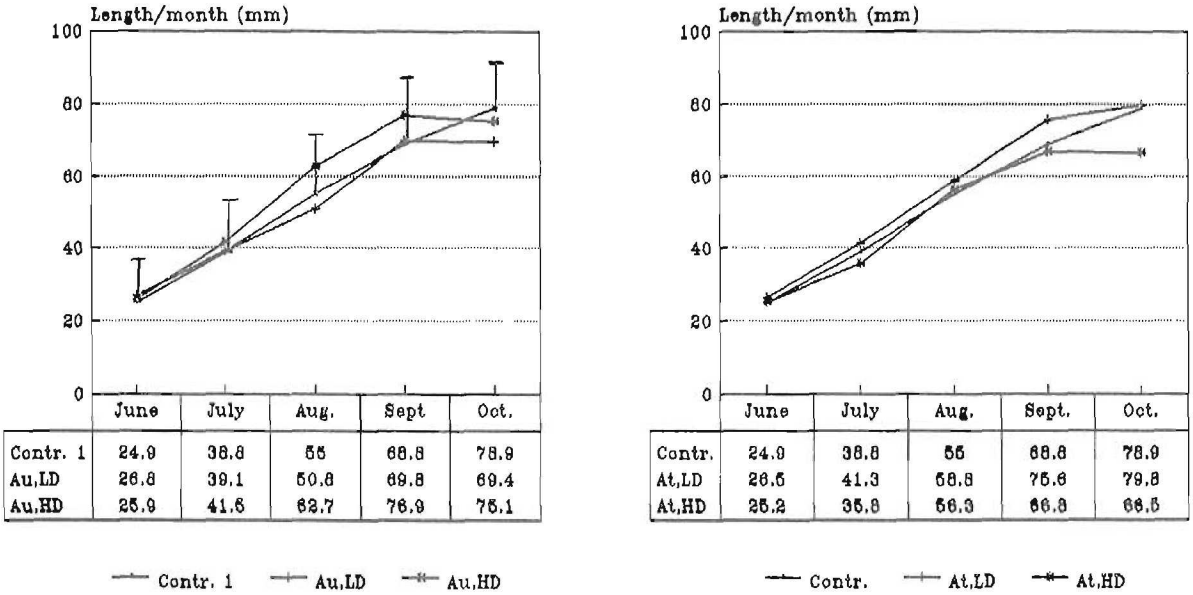

Fig. 8. Apical growth of bladder-wrack between June and October in mesocosm pools exposed to effluents from production of bleached hardwood kraft pulp (mill A). Values are given as mean length of 50 measurements per pool and month. Bars of control pool values indicate standard deviation of the mean.

Some of the mesocosms were invaded by the *Spirogyra sp*. green alga in the early autumn. This occurred in one control, in effluent pool Au,HD and Bt,LD;HD. The invasion is presumably not exposure derived but rather a result of randomness. The biomass was semiquantitatively measured and there were no correspondance seen between biomass and primary production. A possible correlation might have been prevalent between the ammonium values (control pool 32 $\mu g\ l^{-1}$ BtHD 26 $\mu g\ l^{-1}$ and Bt,LD 59 $\mu g\ l^{-1}$) and the biomass of *Spirogyra*. The ammonium values are signs of a higher degradative activity of organic material.

The *Spirogyra* biomasses at the end of the experiment were:

Au,HD : 80 g
Bt,LD: 106 g
Bt,HD: 29 g
Control: 84 g

Since the animal biomasses were influenced by the occurrence of *Spirogyra*, the effluent exposed *Spirogyra* pools were compared with the control pool containing *Spirogyra* (see below results on invertebrate abundance and biomass, Figs 21-22).

Two samplings for periphyton biomass were made during the experiment, the first in the middle of September and the second in November at the end of the experiment. The biomass from the different effluent receiving pools are presented in Figure 10 as per cent of control values/m^2.

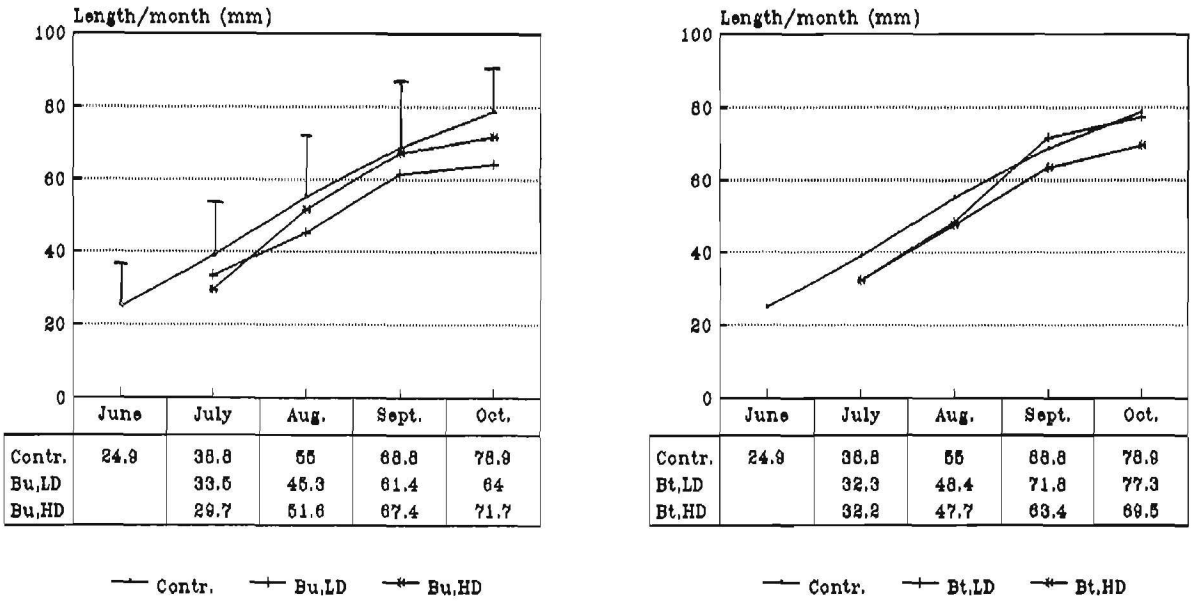

Fig. 9. Apical growth of bladder-wrack between July and October in mesocosm pools exposed to effluent from production of bleached hardwood kraft pulp (mill B). Note one missing measurement due to later exposure start of mill B. Values are given as mean length of 50 measurements per pool and month. Bars of control pool values indicate standard deviation of the mean.

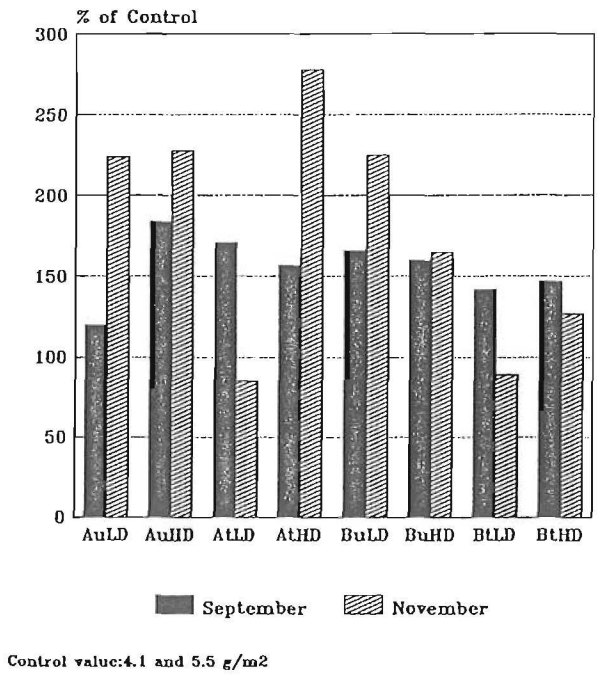

Fig. 10. Periphyton biomass as % of control biomass/m^2 (d.w) in September and November from mesocosms exposed to treated and untreated effluents from mill A and B.

The periphyton biomass increased in all treatements except for the low doses of treated effluents of mill A (At,LD) and B (Bt,LD). The results indicate that periphyton algae, contrary to the bladder-wrack, were favoured by exposure to effluents and it may be assumed that these algae were able to compete for the nutrients present in the water. The biomass in the low dose pools with mill A and B treated effluent showed small differences as compared with the control. This may be considered consistent with the fact that very little nitrogen was added to these pools (120 and 260 ug respectively) with the effluents over the experimental period. Moreover, it is noteworthy that the pool BtLD was colonized by *Spirogyra*, which certainly also competed for nutrients. This might also be an explanation for the relatively low periphyton biomass of the high dose of Bt.

All in all, regarding the plant communities, it may be concluded that there were some shifts in bladder-wrack biomass, which were compensated for by the periphyton community. In the case where *Spirogyra* occurred, the periphyton community responded to this with a relative decrease. The total stimulation in oxygen production in Bt (Fig. 5), although a relatively small deviation as compared with the control, might have been a result of an increased production by planktic algae.

3.3 Effects on the bladder-wrack-associated invertebrate comunity

3.3.1 Gammarus spp

Abundance

Five different species of the *Amphipod* crustacean family Gammaridae occur along the Finnish coast. Four of these spend part of their life cycle in the bladder-wrack zone (Fenchel and Kolding 1979). In the present experiment mainly two species were included i.e. *Gammarus zaddachi* (more abundant) and *G. oceanicus*.

As to the total abundance of gammarids in the bladder-wrack at the end of the experiment, all effluent reveiving pools except Bu,LD showed a clear stimulation compared with the controls, which contained 113 and 68 individuals respectively (Fig. 11). However, if the total abundance of gammarids at the end of the experiment is compared with the situation at the start of the experiment, a notable decrease can be seen.

In most cases <10 % of the total number (and < 50% of the biomass) of the animals initially introduced into the pools at the beginning of the experiment remained there. This is probably due to natural seasonal changes, since the reduction in the field was of the same magnitude.

In order to gain more detailed information regarding gammarid abundance, the number of animals were related to the dry weight of the bladder-wrack they lived on. This gives a measure of the "community crowding" and is presented as per cent of control (Fig. 12). By using the abundance material in this way, statistically useful data are gained since several bladder-wrack specimens per pool were analyzed separately. The community crowding in two controls and in the field is presented in table 11.

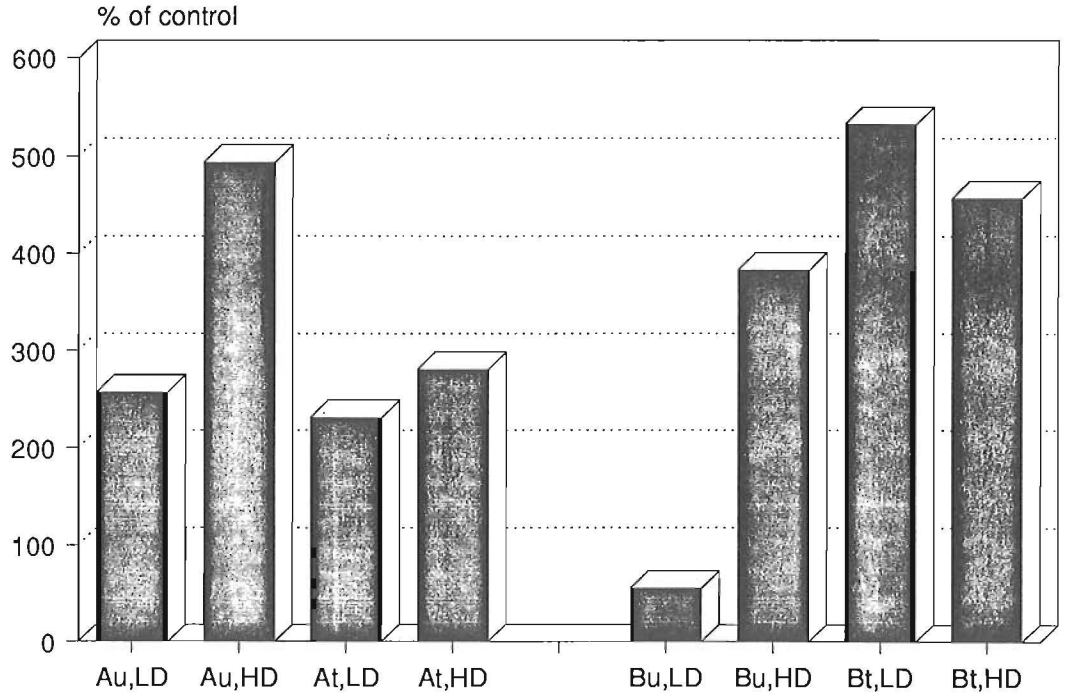

Fig. 11. Total number of *Gammarus spp.* in the bladder-wrack as per cent of controls in mesocosms exposed to effluents from production of bleached hardwood kraft pulp.

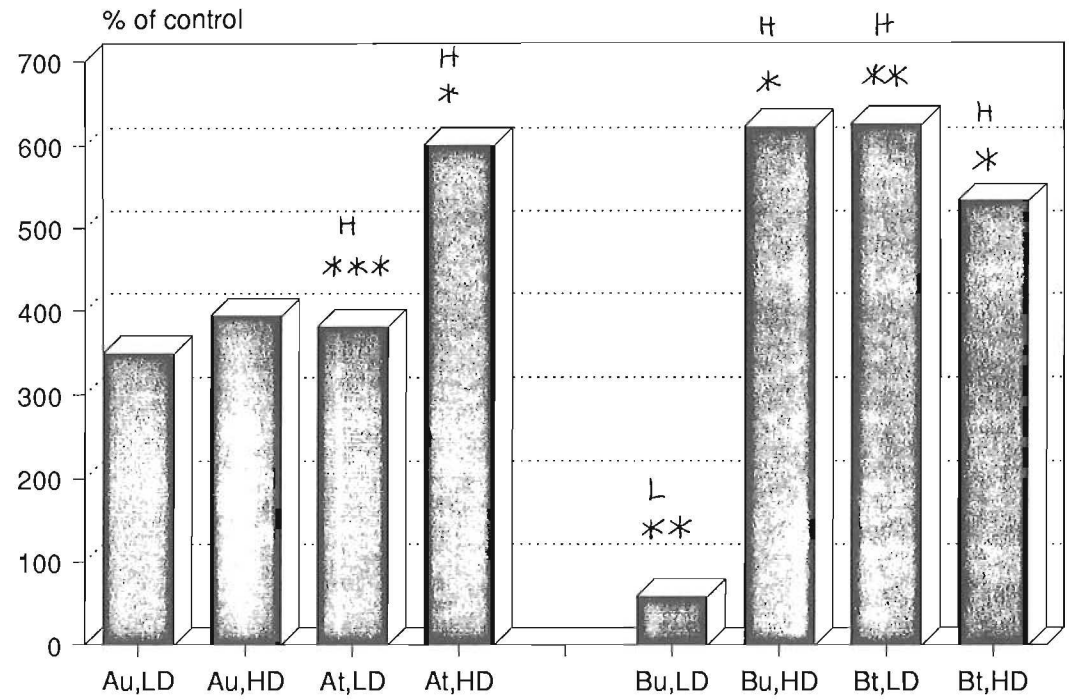

Fig. 12. *Gammarus spp.* crowding/unit bladder-wrack of mesocosms exposed to effluents from production of bleached hardwood kraft pulp (values given as per cent of control). Statistically significant deviations from controls indicated with asteriscs. ***=p< 0.001, **=p<0.005, *=p<0.05

Table 11. Community crowding, i.e. number of gammarids/100 g d.w. bladder-wrack in control pools and in the field on three different occasions.

	n/100 g bladder-wrack
Control 1, start 19.7	253
Control 1, end, 15.11	8
Control 2, end, 15.11 (Spirogyra invasion)	9
Field 19.7	176
Field 15.11	14
Field 1.12	26

Both doses of the treated effluents of mills A and B caused an increased crowding of gammarids on the bladder-wrack. In the high dose of Bu a significant crowding of gammarids was noticed, in the low dose of Bu the crowding was significantly inhibited. Untreated effluent from mill A did not show any statistically significant deviations from the control.

Growth

Since every individual of the gammarid populations from the analysed bladder-wrack material was weighed and the length measured, an evaluation of effects on growth was possible.

In most of the exposed groups statistically significant differences occurred as compared with relevant controls (controls with and without *Spirogyra*), showing a skewed size distribution (Figs 13 and 14).

In most cases where skewed size distributions were observed the response was of stimulatory character. The pool exposed to the low dose of untreated effluent from mill B (BtLD) was the only pool with no statistically significant deviation from the control. The strong inhibitory effect seen in the high dose of mill Bu is probably connected with the high proportion of juveniles. A similar explanation might be given for the small mean length of the gammarids from At,LD. For the rest of the effluent exposures a varying degree of growth stimulation is seen. This was particularly conspicuous in At,HD and in both doses of Bt.

The gammarids were further divided into 9 length classes and 11 weight classes respectively (Figs 15 and 16).From this material it can be seen that the size distribution for the gammarids exposed to At both doses, Au,LD and BuHD, largely are following the controls except for the occurrence of one lenght-class more (> 26 mm) in both doses of At. In Au,HD and especially in both doses of Bt a much more conspicuous shift towards bigger sizes seemed to occur, however. In these pools, the number of length classes increased by one and the weight classes by 2 (180-199 mg and > 200 mg, respectively).

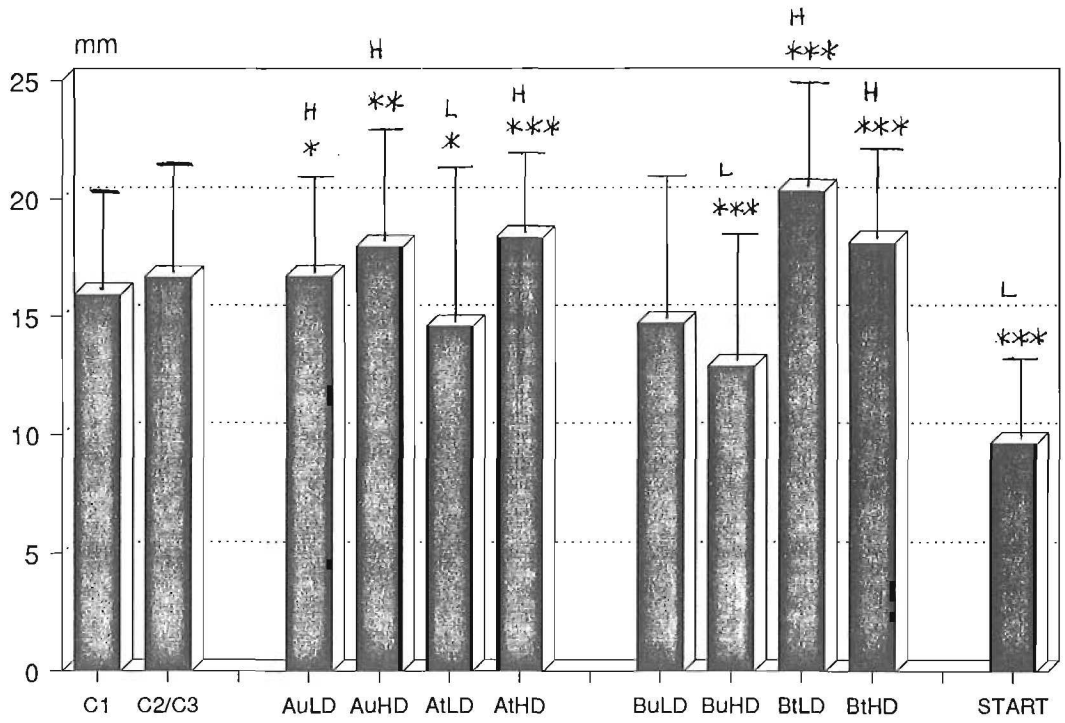

Fig. 13. Mean length±S.D of *Gammarus spp.* exposed for 5.5 months in mesocosms to effluents from production of bleached hardwood kraft pulp. Significance levels the same as in figure 12.

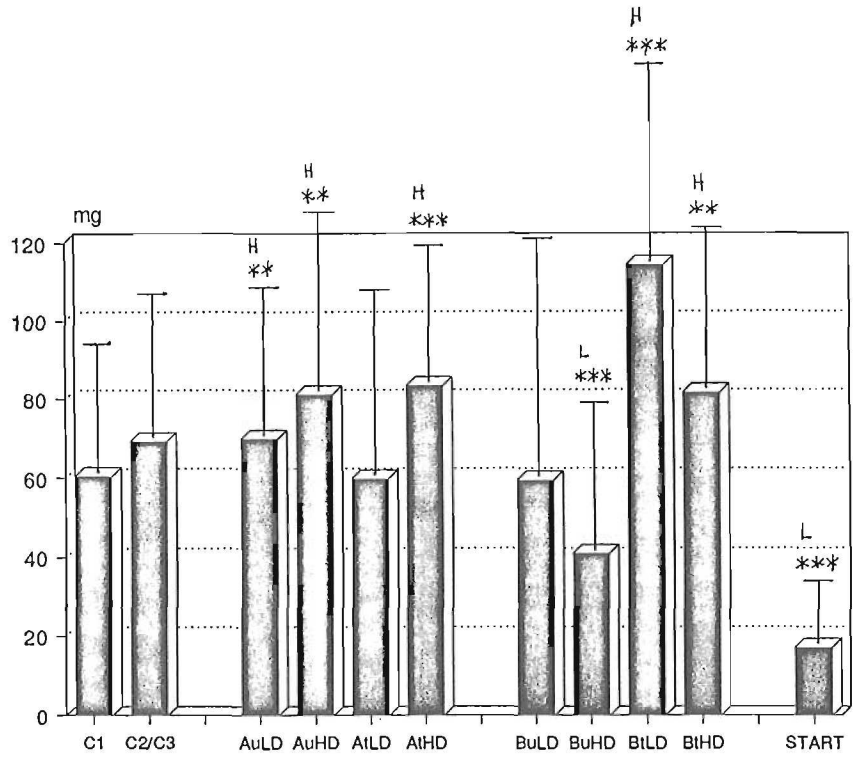

Fig. 14. Mean weight±S.D. of *Gammarus spp.* exposed for 5.5 months in mesocosms to effluents from production of bleached hardwood kraft pulp. Significance levels the same as in figure 12.

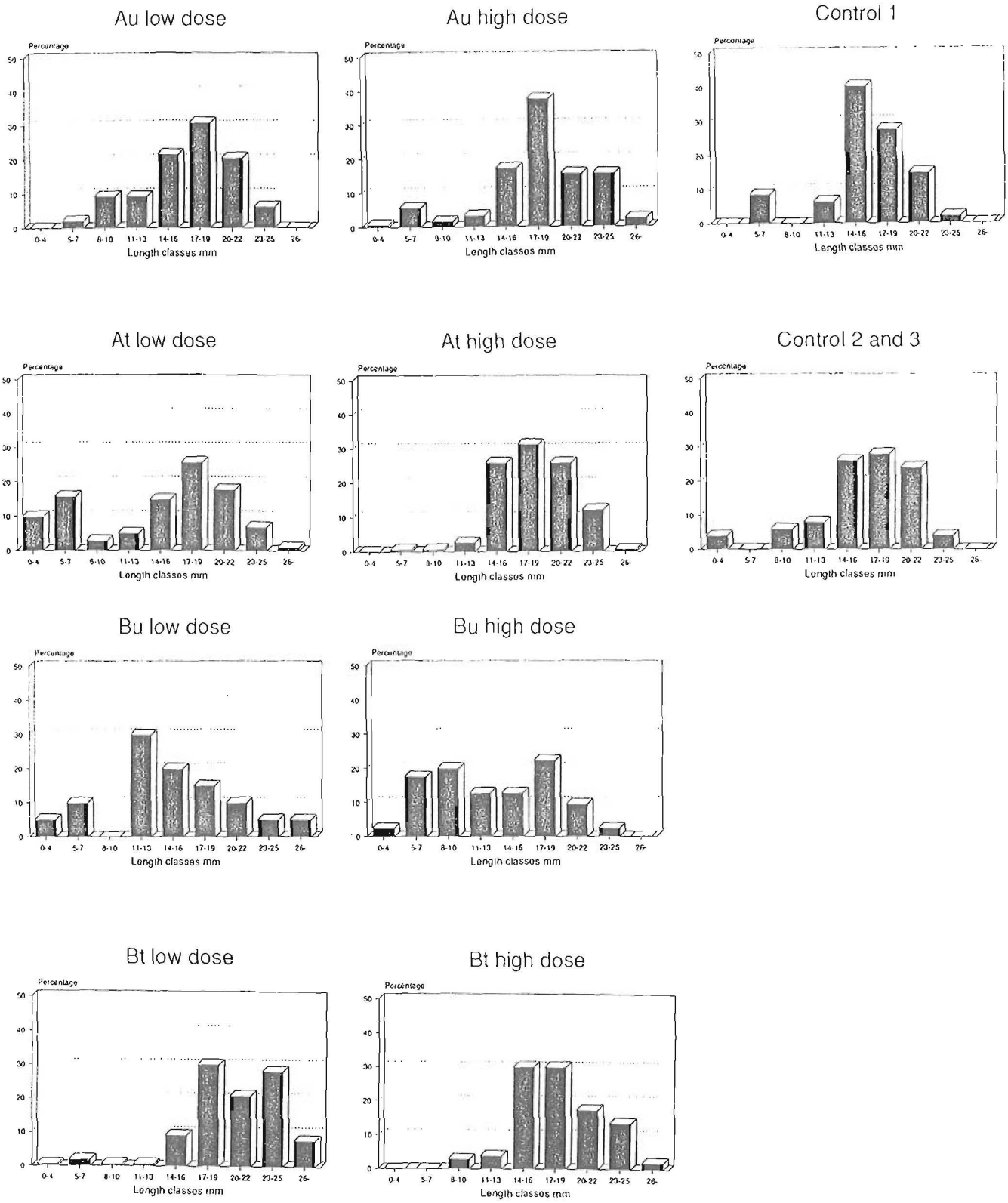

Fig. 15. Length distribution of *Gammarus spp.* exposed in mesocosms to effluents from production of bleached hardwood kraft pulp. Controls 2 and 3 with *Spirogyra*.

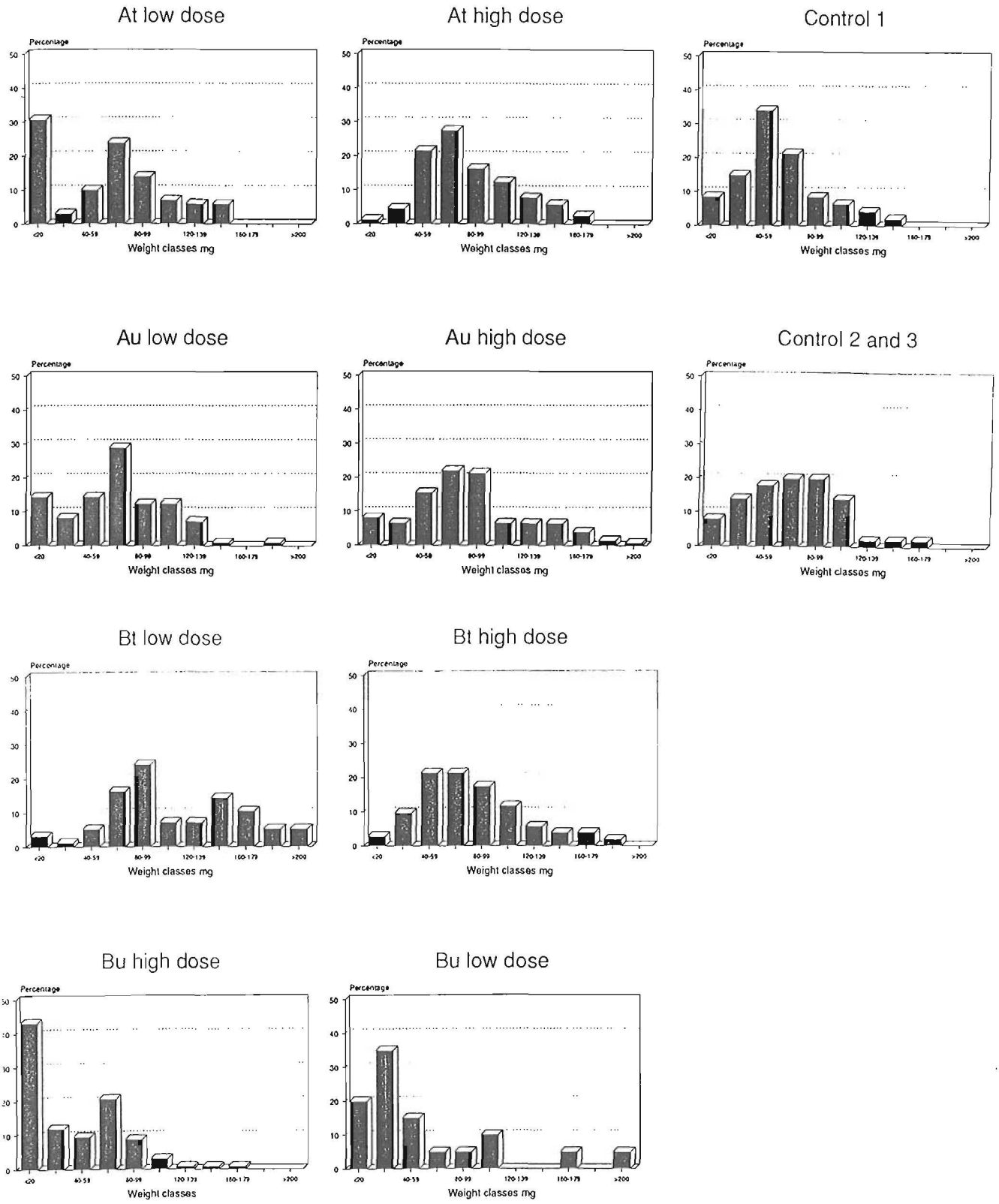

Fig. 16. Size distribution of *Gammarus spp.* exposed in mesocosms to effluents from production of bleached hardwood kraft pulp. Controls 2 and 3 with *Spirogyra*.

Reproduction

In order to obtain an indication of the reproduction success of gammarids, the percentage of juveniles, i.e. individuals <= 10 mm, was calculated (Table 12).

The number of juveniles varied strongly between the different pools and no consistent trends are obvious. The total number in the controls and the field were very similar in the autumn, but the number of juveniles were much higher in the latter case. When examining the percent figures, it can be seen that the groups exposed to mill At,HD and those exposed to both doses of Bt show a lower fraction of juveniles. The absolute numbers of juveniles do not deviate much from the control, however.

The *Spirogyra* invasion does not seem to influence the fraction of juvenile gammarids at the end of the experiment.

Table 12. Number of individuals in two size classes/100 g d.w. Fucus and percentage of juveniles in the mesocosms at the end of the experiment (if not otherwise mentioned).

	n	<=10 mm	>10mm	Juveniles %
Control 1	47	4	43	8.5
Control 2 *(Spiro)	50	5	45	10.0
Control 1 (Summer)	531	340	191	64.0
AuLD	97	11	86	11.3
AuHD (Spiro)	122	10	112	8.2
AtLD	101	29	72	28.7
AtHD	153	2	151	1.3
BuLD (Spiro)	20	3	17	15.0
BuHD	125	50	75	40.0
BtLD (Spiro)	153	5	148	3.3
BtHD (Spiro)	103	3	100	2.9
Field, summer	183	170	13	92.9
Field, autumn	58	44	14	75.9

*) = *Spirogyra* invasion of pool.

3.3.2 *Idotea sp.*

Two species of the isopod crustacean Idotea, *I. chelipes* and *I. baltica* (of totally three species along the Finnish coast) were included in the mesocoss experiment. In general, the total number was low, only around 100/pool at the start of the experiment. Since a strong decrease in number occurred in all mesocosm pools during the course of the experiment, the material does not permit any far-reaching conclusions.

3.3.3 Blue mussel, *Mytilus edulis*

The filter-feeder *M. edulis* did not exhibit any significant effects regarding abundance in exposed mesocosms as compared with unexposed control pools, indicating that most effluents tested did not induce effects on mortality or reproduction. However, in both doses of Bu, a moderate stimulation of the abundance seemed to occur.

On the other hand, the growth of the blue mussels was affected by the effluent exposure. This was noted both in the mussels repeatedly measured during the experiment (Figs 17 and 18) and in those measured at the end of the experiment. Mean length, mean weight and weight/length ratio were the parameters analyzed. The latter parameter may be considered a condition factor of the animals. As compared to the situation at the experimental start the mussels grew 1.2–2.1 mm by length and 25–40 mg by weight.

The mean length was significantly lower in AuLD, BuLD and BtLD, whereas mean growth was stimulated in AtLD and AtHD. Regarding mean weight and weight/length ratios AtLD shows a stimulation only, whereas AuLD, BuLD, BuHD and BtLD were inhibited. This indicates that less amount soft tissue in relation to shell mass of the blue mussels occurred in the latter four doses as compared with the control. This also indicates some metabolic interference due to the exposure to the effluents.

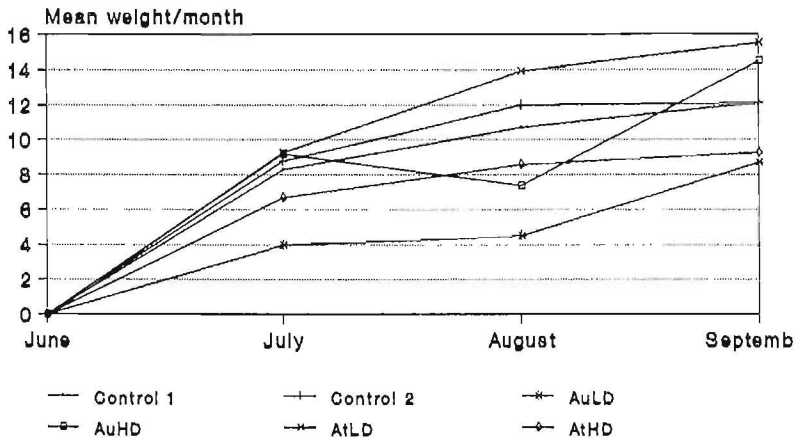

Fig. 17. Mean weight/month as per cent of start weight of blue mussels in mesocosms exposed to effluents from bleached hardwood kraft pulp production (mill A).

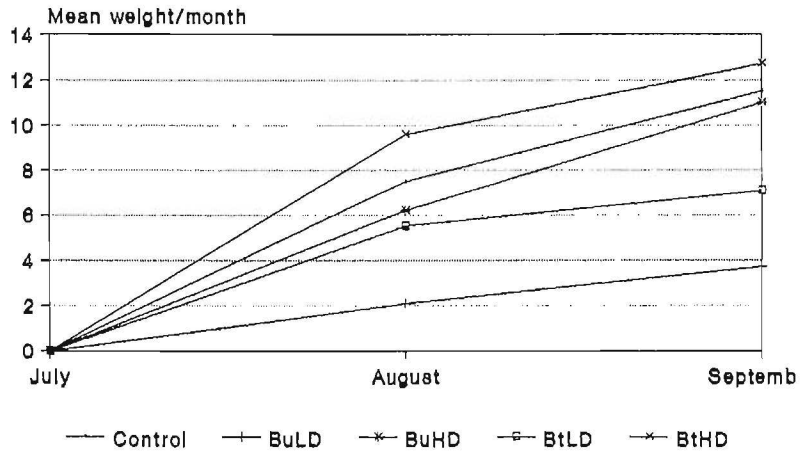

Fig. 18. Mean weight/month as per cent of start weight of blue mussels in mesocosms exposed to effluents from bleached hardwood kraft pulp production (mill B).

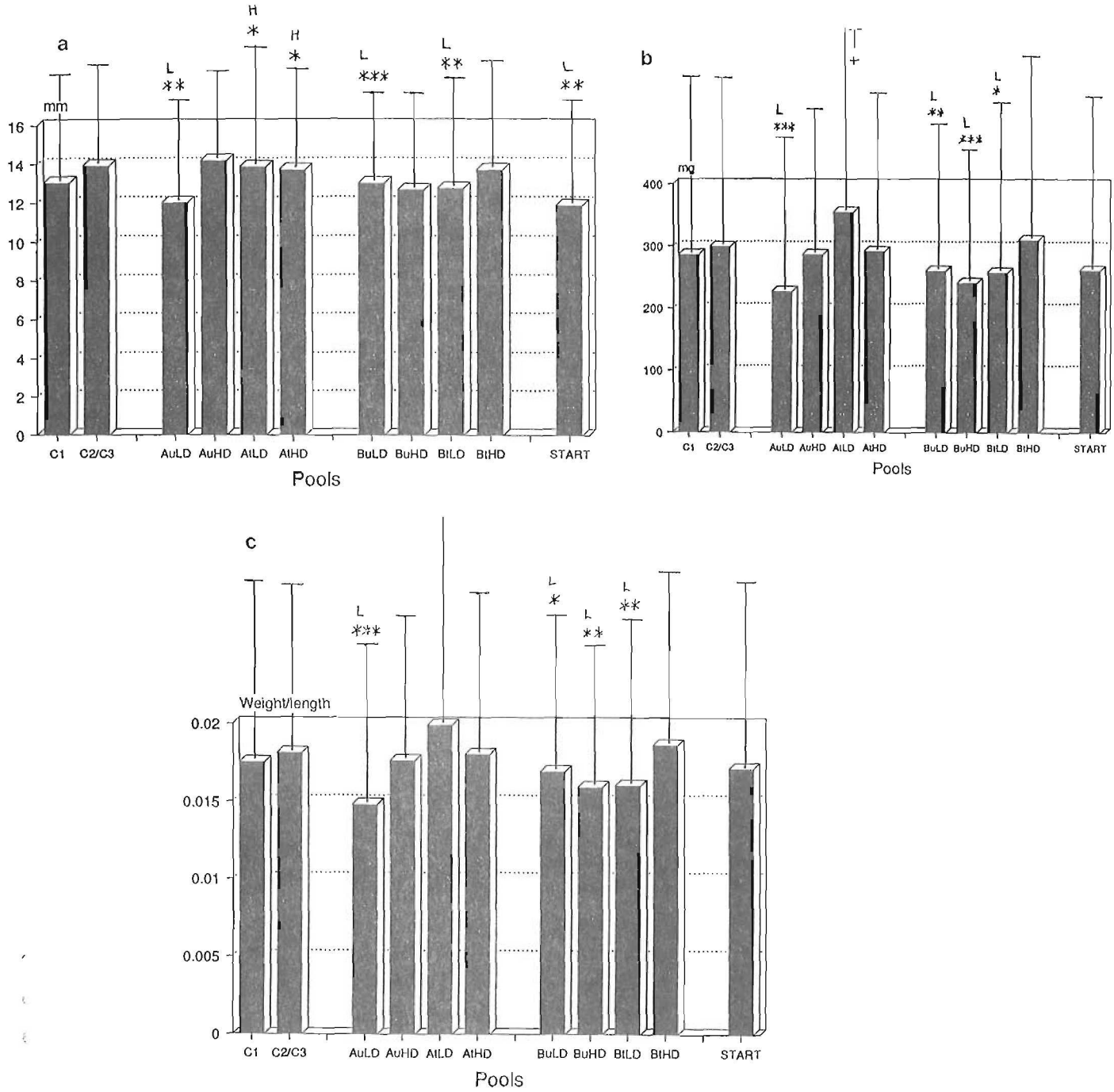

Fig. 19. Size (mean length (a), mean weight (b) and weight/length ratio (c) of blue mussel exposed in mesocosms to effluents from production of bleached hardwood kraft pulp. Significance levels same as in fig.12.

3.3.4 *Theodoxus fluviatilis* (Gastropoda)

The total abundance of the snail, *T. fluviatilis*, was stimulated in all exposed pools invaded by *Spirogyra* as compared with the corresponding *Spirogyra* control pool, whereas an inhibition of this species was noted in pools without *Spirogyra* (Fig. 20).

In AtHD the mean weight of both juvenile and adult *Theodoxus* was stimulated.

3.3.5 Total bladder-wrack associated invertebrate abundance and biomass

The total abundance and biomass of the bladder-wrack associated fauna are presented in Figures 21 and 22 . Values are given as per cent of control. Included in the figures are *Gammarus spp.*, *Idotea spp.*, *Mytilus edulis* and *Theodoxus fluviatilis*, the shrimp *Paleaemon adspersus*, the snail *Lymnaea spp.* and the mussel *Cardium*. The three last species occurred only sporadically in the bladder-wrack and their contribution is thus restricted to the biomass and not to abundance.

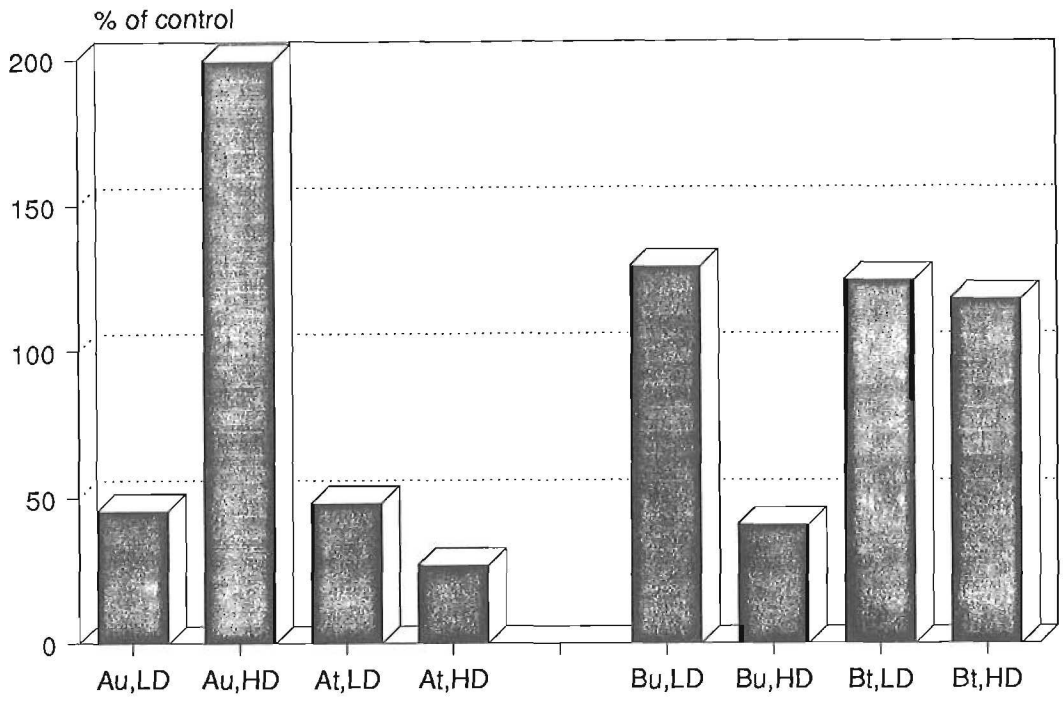

Fig. 20. Total abundance of *Theodoxus fluviatilis* as per cent of control in mesocosms exposed to effluents from production of bleached hardwood kraft pulp.

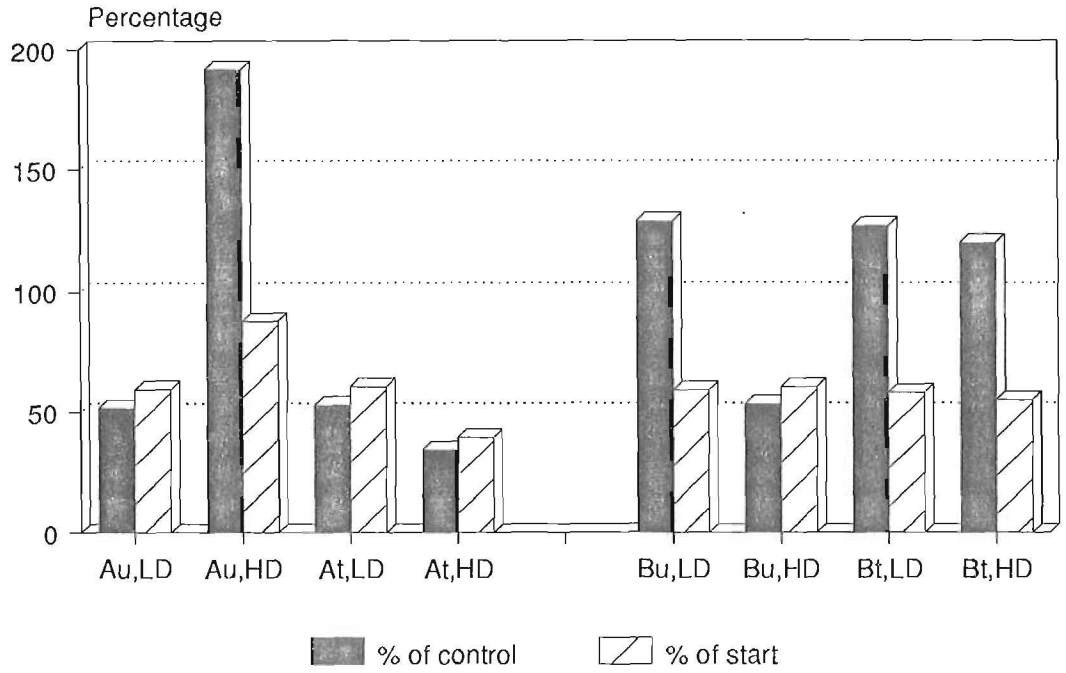

Fig. 21. Total bladder-wrack associated invertebrate macrofauna. Abundance as per cent of control and as per cent of start values in mesocosms exposed to effluents from production of bleached hardwood kraft pulp.

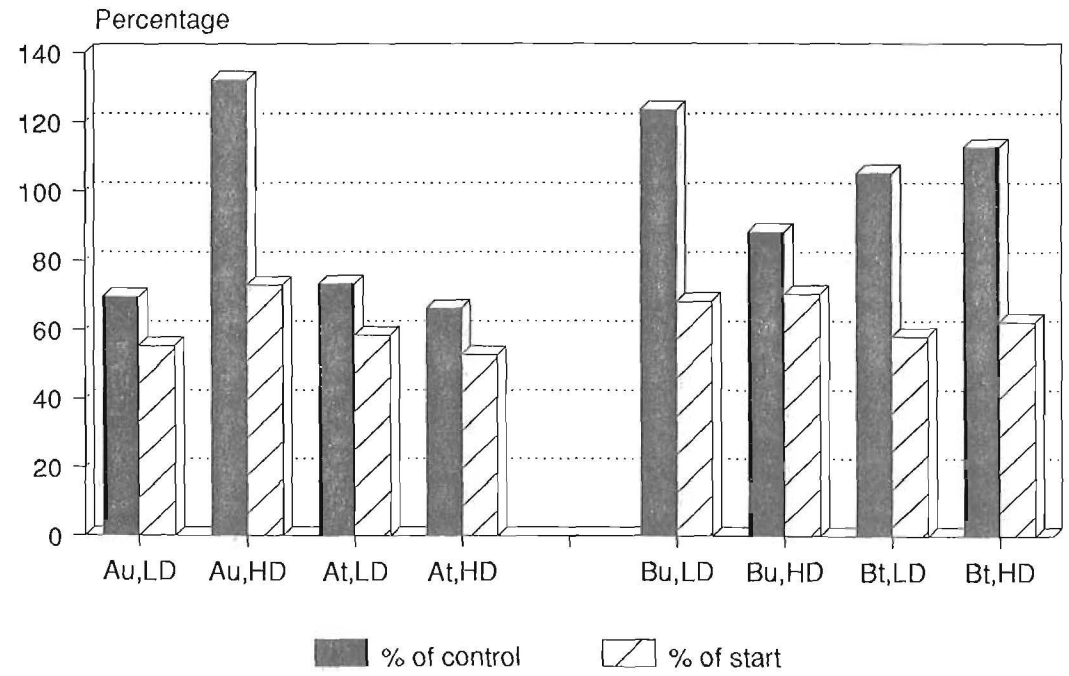

Fig. 22. Total bladder-wrack associated invertebrate fauna. Biomass in mesocosms exposed to effluents from production of bleached hardwood kraft pulp. Values given as per cent of control and as per cent of start values.

The figures for total abundance at the end of the experiment varied between 2600 (AtHD) and 7700 (Control 1) and the biomass varied between 200 (AtHD) and 300 g (wet weight) (Control 1). The controls containing *Spirogyra* had a considerably lower invertebrate fauna abundance and biomass than the control without *Spirogyra* (3000 ind. and 210 g respectively). However, invasion of *Spirogyra* in combination with effluent exposure seemed to have a stimulatory effect on the bladder–wrack fauna.

Generally there was a strong reduction of the fauna in all pools, except in the control 1–pool, in both abundance and biomass at the end of the exposure as compared with the situation at start.

If the situation at the end of the exposure is compared between the different pools, total abundance in AuHD was considerably, stimulated, while the abundance in BuLD and BtLD;HD was stimulated but less prominently. Thus, pools containing *Spirogyra* showed an increase of the number of animals in the bladder–wrack. Other pools, without *Spirogyra* were inhibited with AtHD as the extreme as compared with the control without *Spirogyra*. Also regarding biomass, pools with *Spirogyra* were stimulated and pools without *Spirogyra* inhibited. It may be noted in this instance that a high number of individuals depended upon high frequency of young *Theodoxus*, whereas high biomass correlated with high numbers of bigger size classes of blue mussels.

3.4 Effects on sediment fauna

The sediments of the mesocosms contained several animal species, which colonized the sediment under the course of the experiment. The animals were introduced as larvae via the incoming seawater and colonized the sediments if the conditions in the pools were favourable. In addition, some animals found in the sediment originated from the bladder–wrack.

The animal fraction contributing most to total sediment fauna abundance consisted of insect larvae as well as some *Oligocheatae* (to a minor extent). Since the number of individuals belonging to these groups very much depended upon random–ness and their contribution to the biomass was negligible, they have been omitted from the results. Total abundance and total biomass of mussels and snails in the sediments at the end of the experiment are presented in Fig. 23.

The total abundance was dominated by the Baltic mussel, *Macoma baltica*. The total biomass was dominated mainly by *Cardium sp.* and blue mussels. Other sediment dwelling species were the snails *Hydrobia spp.*, *Theodoxus fluviatilis* and *Lymnaea spp.*

In addition some gammarids occurred in this sub–system, especially in AuLD, AuHD, AtHD, BuHD and in Control 1 (without *Spirogyra*)

As was the case with the bladder–wrack invertebrate community, the same trends were noticeable regarding sediment fauna, i.e. a stimulation in exposed mesocosms containing *Spirogyra* and an inhibition in varying degree in mesocosms not containing Spirogyra. The controls with and with out *Spirogyra* differed most. The calculated

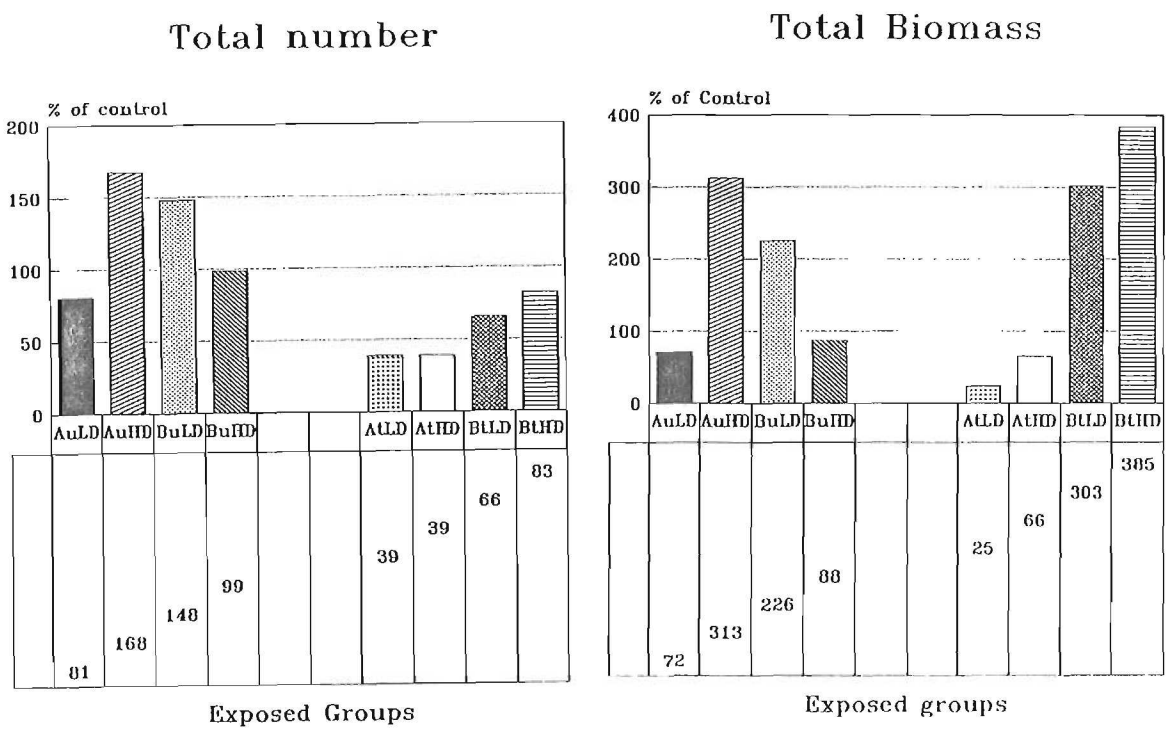

Fig. 23. Total number (abundance) and total biomass of sediment fauna as per cent of control in mesocosms exposed to effluents from production of bleached hardwood kraft pulp.

values for the control without *Spirogyra* were 125 000 individuals with a biomass of 660 g wet weight, which can be compared with 24 000 ind. and 190 g, respectively, in the control containing *Spirogyra*.

The strongest inhibition both with regard to abundance and biomass was seen in both doses of At. The strongest stimulation of abundance was observed in AuHD, whereas the most conspicious increase of biomass was noted in BtHD.

3.5 Total mesocosm invertebrate fauna

The total standing stock of invertebrates in the mesocosms at the end of the experiment embraces both the bladder–wrack and the sediment fauna. As expected, the same tendencies as for the different subsystems were observed regarding this parameter, i.e. stimulation in exposed *Spirogyra* pools and inhibition of pools without *Spirogyra* (Fig. 24).

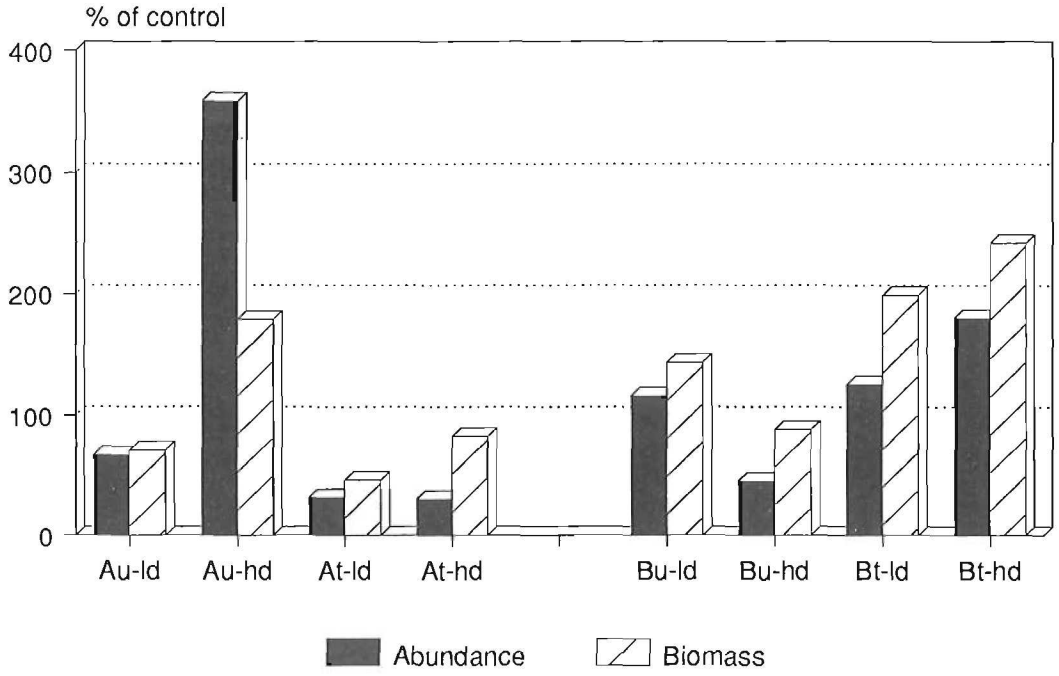

Fig. 24. Total fauna abundance and biomass as per cent of control in mesocosms exposed to effluents from production of bleached hardwood kraft pulp.

4 DISCUSSION

4.1 General

The complex nature of bleached kraft mill effluents (BKME) containing both stimulatory (such as nutrients) and inhibitory (toxic) substances makes environmental impact assessments of BKME based on field studies difficult to perform in a reliable way.

Moreover, the complexity of the aquatic ecosystems receiving BKME is also high, which makes extrapolations of results from single species toxicity tests difficult. Particularily, regarding single species testing, the question may be asked whether the exposure situation is realistic, since the system lacks "competing" compartments (animals, plants) for the toxicant or other substances present in the effluent. Thus, the organism being tested might be exposed at an unrealistically high level. This might not be prevalent in the real situation, where biomass represented by other species will compete for the substances occurring in solution in the water or adsorbed to particles. Regarding nutrients, it is well established that aquatic plants possess different strategies for nutrient uptake (Wallentinus 1983, Tamminen 1990). As a consequence, changes in species diversity and biomass occur as ecosystems are "absorbing" nutrient-rich discharges, when high water concentrations of nutrients (be it phosphorus, nitrogen or any other element) occur in the summer time. This situation usually better suits fast

growing, annual filamentous algae than slow growing perennial ones. It may perhaps not be too unrealistic to assume that the same mechanisms also exist for substances of a toxic nature. Systems with rather low diversity but high biomasses might act as buffers for toxic substances in polluted areas. Thus, deleterious effects that would occur in areas with lower biomasses but comparably high diversities would be masked.

By using the model ecosystem technique it is possible to obtain information about several ecologically important processes under polluted conditions: a) Primary production, b) Secondary production, c) Community shift and d) Distribution in and exposure to the system of biologically active compounds (toxic and stimulatory) (Landner 1990).

In the present work, these items are emphasized and the importance of interactive dynamic mechanisms between nutrients and pollution effects are discussed.

4.2 Concentrations and distribution of contaminants

Chemical analyses of total amounts of different substances dosed to the mesocosms showed considerable differences between the effluents (Table 5). This relates to both resin acids, (in particular dehydroabietic acid), steroids, chlorinated phenols and chloroguaiacols. Also the total amount of AOX dosed to the high dosepools varied considerably between mill A and B. However, when specific substances (dehydro-abietic acid, DHA; chlorophenols, CP; chloroguaiacols, CG) were analyzed in different compartments of the mesocosms it was seen that (except for sediment DHA), concentrations of free (non-conjugated) DHA, CP and CG were practically non-detectable. This observation is interesting since at least conjugated RAs were detected in higher levels in the bile of fish exposed to the Au effluent as compared to unexposed fish (Lehtinen et al. 1992). The results indicate that despite a total dosage of 2 g RA with the Au effluent, the organisms modified the RA from a free form to a conjugated one. Alternatively, the substances were absorbed by the *Spirogyra* alga in the pools where this alga occurred, in this case AuHD and BtHD. This possibility is indicated by the fact that the *Spirogyra* pools contained much less organic sediment. Also, the total sediment content of DHA was only doubled in the pool receiving AuHD effluent as compared with the control, despite a high total dosage. On the other hand, the sediment of the mesocosm contaminated with effluent BuHD (without *Spirogyra*) contained as much as 67 % of the total DHA dosed over the experimental period. From the mesocosm exposed to BtHD, with lower biomass of *Spirogyra*, about 9 % of the total dosed DHA was recovered from the sediment. One exception was fond in the AtHD group, where no increased DHA sediment content at all was noticed, despite lack of *Spirogyra* and low animal biomasses in general and a higher total dose of DHA than for example in the case with BtHD. Obviously the substance was processed through the system via other pathways. In this instance it may be noted that the bile concentrations of rainbow trout in this particular dose group (AtHD) were twice as high as the control and other exposed groups (Lehtinen et al. 1992). It may

thus be speculated that a higher proportion of resin acids, for unknown reasons, were subjected to metabolic reactions in this mesocosm.

The EOX concentration in sediment, molluscs, crustaceans and fish increased in mesocosms exposed to untreated and treated effluent from mill A (Table 6). Regarding mill B, exposure to untreated and treated effluent caused increases in EOX concentrations in sediment and *Cardium* only. The concentrations of EOX in the fat of fish were somewhat lower both regarding background concentrations in control material and in those groups showing increased concentrations (AuHD; AtHD) than in a previous mesocosm experiment (Axelsson 1988). In the experiment of Axelsson (1988) one of the effluents tested originated from a mill producing bleached hardwood kraft pulp with roughly the same bleaching sequence as mill A (about 90 % D in the D+C step, no oxygen pre-delignification). The AOX emission per tonne of pulp of the present mill (A) was somewhat higher than that of the previously tested one (Axelsson 1988). The concentration of EOX in fish fat was about 3.5 times higher than the background in fish exposed in the earlier mesocosm experiment in 1986 (Axelsson 1988; Hemming and Lehtinen 1988). In the present work the EOX increased by a factor 5. Regarding the chemical composition of the chlorine containing material, no analysis was performed this time. However, in a previous work, molecular fractionation showed that the bulk of the chlorinated material in fish fat was found in the "fatty acid" and "triglyceride" fraction (Hemming and Lehtinen 1988).

Wesén et al. (1990) characterized the EOX-fraction found in sediment and biota from the field. They found the EOX-fraction in fish to be neutral, but 60-80 % were hydrolyzed by lipase enzymes. 30 % of the cod liver EOX and 80 % of the sediment material was recovered as acidic material, i.e. a considerable fraction of the unidentified, chlorinated substances was bound in esters. Wesén et al. (1990) interpreted their results as indicating that a major fraction of these esters may be a combination between fatty acids and chlorophenolics or between glycerol and chlorinated carboxylic acids. Therefore, EOX could be expected to have a tendency to concentrate through the food chains. This interpretation does not seem to be very plausible for several reasons: a) if there would be a tendency of chlorophenolics to bind to fatty acids their accumulation in fish tissue would be much higher and the efficiency to excrete these as conjugates lower than observed (Oikari and Holmbom 1986), b) the levels of EOX do not increase in fish at controlled exposure via water, even during long exposure periods to BKME containing relatively high concentrations of chlorophenolics (Lehtinen 1990), c) chlorinated substances other than those mentioned above, but of relatively trivial nature, might serve as chlorine donors binding to the oxygen double-bonds of unsaturated fatty acids according to the mechanism demonstrated for tetrachloromethane (Link et al. 1984). If such mechanisms also would occur outside the fish and other organisms such as crustaceans or molluscs is not known presently.

Supporting results that chlorophenolics would not be contributors to the increased EOX-content in organisms in the present work , are the data from chemical analysis of both the effluent contents of these compounds, as well as the non-deviating levels from background of chlorophenolics, found in sediments and biota in the mesocosms. Moreover, the results in Table 6 do not reflect any tendency towards biomagnification but merely the place in the system where the organisms are dwelling. If there would

have been tendencies towards biomagnification it would have been expected that the sticklebacks would have contained relatively more EOX than crustaceans.

An interesting observation is also that the total EOX in the systems seems to be bound into the sediment and less in the biomass (Table 7). Moreover, only a fraction of the total AOX dosed to the mesocosms was bound in the sediment as EOX, and the rest is either leaving the system or broken down. The fraction of the AOX dosed, bound to the sediment as EOX, was fairly constant for mill A, in the high doses, 0.3 and 0.4 %, respectively. On the other hand, relatively more EOX was trapped in the sediment in mesocosms exposed to mill B effluents (1 and 0.6 % respectively) in relation to the total dosage. In this regard it should be remembered that the numbers in table 7 are influenced by the amount of total organic material or total biomass. Thus, lower amounts of organic material and high total amounts of EOX in the sediment are indications that the substances present have a high affinity for particles.

Assuming that about 1% of the AOX in the effluents may be defined as EOX, the sediment in the high dose pools contained between 30 (AuHD) and 86 (BtHD) % of the total EOX dosed over the experimental period.

The present data do not give any information about the bioavailability of the sedimented material and, thus, the role of the food route for the uptake of chlorinated organic material. The mollusc *Cardium sp.* is a water filtrating species, filtering particles from just above the sediment surface. The figures in Table 6 indicate that this species would be effective in absorbing chlorinated (as well as non-chlorinated) material.

In a recent study by Lehtinen et al.(manuscript) the role of the food as uptake route for chlorinated organics was investigated. Contaminated sediment from the immediate discharge area outside a bleached pulp mill was collected, the organisms killed off by freezing and the sediment recolonized with *Tubifex spp*. After two weeks the *Tubifex* were fed to rainbow trout for 8 weeks, whereupon liver and bile was collected for EOX- and (conjugated) chlorophenol-analysis. The results showed no uptake of chlorinated organic compounds in neither *Tubifex* nor fish. In another experiment where fish were fed a diet contaminated with the solid fraction of a pulp mill effluent trapped by sieving theeffluent through a 70 μm mesh cloth, no increased concentrations of liver EOX were obtained (Lehtinen 1990). It thus seems that chlorinated organic material in sediment would not be easily available for organisms. It is also noteworthy that the only experiments where an uptake of EOX successfully has been demonstrated, are those in which effluents from production of bleached hardwood kraft pulp was used as pollutant, i.e. in the work by Hemming and Lehtinen (1988) and in the present work. Possible active compounds are unknown and underlying mechanisms are presently far from being understood. Indications that hardwood effluents contain compounds probably not occurring in effluents from softwood bleaching have been obtained. Another possibility is that certain compounds occur in much higher concentrations in bleached hardwood effluents than in effluents from bleached softwood production.

4.3 Effects on invertebrate fauna

The invertebrate fauna exposed to the effluents tested may be affected in two ways, i.e. directly by substances present in the effluents and indirectly by interspecific competitive mechanisms (Rosemarin et al. 1990, Petersen and Petersen 1989). In previous experiments, one main indirect factor affecting the invertebrate fauna in BKME exposed mesocosms was the inibitive effects on bladder-wrack of chlorate present in pulp mill effluents (Lehtinen et al. 1990). In the present work no obvious effects of chlorate on the bladder-wrack were noted. It was clearly shown by the N/P-quotients that the bladder-wrack was nitrogen deficient and that the nutrients present in the effluents were utilized mainly by the periphyton or planktic algae. This is a further confirmation that increased nitrogen levels in the water of the Baltic Sea favour annual algal growth, which in the long run negatively might affect the existence of bladder-wrack.

The effects noted on the *Gammarus spp.* were in all cases, except for BuLD, strongly stimulatory both regarding total number and growth. The reduced biomass in BuLD was mainly due to a high proportion of juveniles.

The stimulatory effects do not seem to be strongly correlated with eutrophication since the growth of the gammarids was equally much stimulated in AtHD with the lowest levels of nitrogen added. On the other hand, the total bladder-wrack biomass decreased most in this group, which might have been an effect of the gammarids grazing upon this alga. This possible increase of available food might have had growth stimulating effects. Observations made by Haage (1975) are speaking against such an interpretation, however. He noted that a big population of *Gammarus spp.* frequently caused a decrease of the *Idotea*-population. Haage (1975) further concluded that this competition was not food dependent but due to space and shelter, since food was never a limiting factor in an undamaged bladder-wrack community. This leads to the conclusion that food always would be present in surplus for gammarid crustaceans and that increased nutrient levels would not necessarily induce growth stimulation.

The stimulatory effect on *Gammarus spp.* in combination with decreased bladder-wrack biomass resulted in significant crowding in all exposed mesocosms except for Au both doses, and BuLD. Such effects have been noted in previous mesocosm experiments where significant effects of chlorate occurred (Rosemarin et al. 1990). In this instance, it is noteworty that the crowding effect was induced despite that deleterious effects of chlorate did not occur. This indicates that a shift of competition within the animal community was initiated. Whether this is a direct effect of substances present in the effluents or indirect effects of ecological character is hard to say.

Regarding the whole mesocosm community, two possible mechanisms acting synergistically or separately may be speculated upon:

The occurrence of *Spirogyra* in some mesocosms affected the nutrient flow, but at the same time reduced active substances present in the effluents, thus causing a relative stimulation of both animal abundance and biomass (Fig. 24). Even if the presence of *Spirogyra* in unpolluted mesocosms reduced animal production, the

presence of *Spirogyra* in polluted pools generally caused a stimulation. The *Spirogyra* invasion would thus exemplify how a randomly occurring component of an ecosystem could modify a toxic/inhibitive response in the animal component. The inhibitive reaction is in turn shown in the mesocosms polluted by effluent At. These mesocosms were not invaded by *Spirogyra* and showed strongly reduced total animal abundance and biomass in both doses. In this perspective it is not possible to directly compare neither between mesocosms exposed to treated or untreated effluents nor between treated effluent A and B, since the B pools were invaded by *Spirogyra*. Thus, a similar response in Bt as in At cannot be exluded in the case *Spirogyra* was absent.

Another mechanism may be examplified by the growth reactions seen in *Gammarus spp.* and the blue mussel, *M. edulis*. A possible response on *Gammarus spp.* via eutrophying mechanisms was discussed above. It is also known that blue mussels are favoured by eutrophication (Wallentinus 1983 and references therein). However, the total amounts of nutrients added to the systems do not, as previously noted, correlate well with the reactions seen in the growth of blue mussel or *Gammarus spp.* (see Figs 15, 16 17, 18 and Table 10). In parallell fish experiments as well as in previous mesocosm experiments, abberrant growth patterns in stickleback brood were observed (Lehtinen et al. 1991; Lehtinen 1989). In one case, one group of substances (mainly beta-sitosterol) was shown to induce identical growth responses in the sticklebacks as did total mill effluents. Beta-sitosterol is much alike the steroid ecdysone, which is the hormone inducing molting and growth in crustaceans (Grant 1978). It is further known that certain turpenoids in wood raw material inhibit metamorphosis in insects. As can be seen from Table 4, the mill A effluents contained high concentrations of extractives including both turpenoids and steroids. A stimulated growth rate might have induced an earlier sexual maturity due to increased number of moltings of the gammarids and as a consequence a higher number of generations and total number. A combined effect of the presence of such compounds in the effluents and a simultaneous lack, or presence, of *Spirogyra* might have influenced several animal species in one direction or the other.

In conclusion, it may be noted that exposure to the different effluents induced several responses both in the plant and the animal subsystems. It was also indicated that the responses largely are influenced by interspecific mechanisms between plants and animals. Moreover, several intricate mechanisms regulating both nutrients and possibly also toxic compounds, as well as compounds acting as chemical doubles to hormones, were indicated. We beleive that by gaining more knowledge of such mechanisms, better predictions of the environmental impact of complex effluents such as pulp mill effluents can be made. It is also obvious that use of multi-species testing methods is necessary in order to gain this knowledge.

5 CONCLUSIONS

In the present work, stimulatory as well as inhibitory responses in different mesocosm subsystems were observed. The direction of these responses are assumed to be

regulated by biological mechanisms. This assumption is based upon the results from pools containing *Spirogyra*, where the invertebrate fauna was stimulated despite the fact that some of these pools received more potentially effective substances than pools without *Spirogyra*, in which inhibitory effects were seen. Thus, *Spirogyra* occurring randomly showed how a biological component might mask an otherwise inhibitory response of pulp mill effluents on the faunal component.

The exposure mostly induced a stimulation of periphyton growth, indicating that nutrients added to exposed mesocosms through effluent exposure favoured fast growing annual algae.

The bladder-wrack was simultaneously nitrogen deficient, showing that this alga is unable to compete successfully for nutrients during the growth season. It is reasonable to assume that the long-term survival of this alga will be impaired in nature under similar water exchange conditions.

The consequences in fresh water are hard to forecast, but in this instance, phosphorus most probably would be the regulating component instead of nitrogen.

The stimulatory effects seen on *Gammarus spp.* might be due to the presence of hormone-like substances of natural wood origin, which possibly influence the frequency of moulting. Other contributing factors could be increased availability of food due to increased periphyton production. The rather specific stimulatory response in this genus of crustaceans (*Idotea spp.* was probably inhibited) might have negative consequeneces through competion for other species dwelling in the bladder-wrack.

Regarding chlorinated phenolics, resin acids and AOX/EOX, no correlations were observed between concentrations and responses.

However, an increased content of EOX was seen in different mesocosm compartments analyzed. There were no indications of biomagnification, however. In addition, the correlation was poor between levels of EOX in organisms and total amount of AOX introduced into the mesocosms, regardless of treatment or no treatment of effluents.

Of the total amount of AOX dosed, only a minor fraction (rougly 1%) was trapped (as EOX) in sediment or the biota analyzed. Of specific compounds only DHA increased, but only in the sediment. The recovery in relation to the amounts dosed was comparably high.

GLOSSARY - ORDLISTA - SANASTO

Abundance	= antal individer av en population = populaation yksilömäärä
Apical	= topp, översta delen av i detta fall blåstång = kärki, rakkolevän ylin osa
Autotrophy	= självproducerande, dvs. solenergibindande växter = tuottaja, ts. auringonenergiaa sitova kasvi
Adult	= fullvuxen (könsmogen) = täysikasvuinen (sukukypsä)
Annual	= ett årig, årlig = yksivuotinen, vuosittainen
Bladder-wrack	= blåstång, *Fucus vesiculosus* = rakkolevä, *Fucus vesiculosus*
Biomass	= vikten av levande organismer = elävien organismien paino
Biota	= levande organismer = elävät organismit
Biomagnification	= ökande koncentration av en substans vävnaderna hos organismer ju högre upp i näringskedjan organismen befinner sig = siirryttäessä ravintoketjua ylöspäin organismien kudoksissa esiintyvien yhdisteiden lisääntyvä pitois.
Bioavailability	= tillgängligheten för upptag av en substans hos organismer. Beror bla på substansens kemiska egenskaper. = saatavuus,käytettävyys yhdisteiden imeytymiseen, riippuu mm. yhdisteen kemillisista ominaisuuksista
Crustaceans	= kräftdjur = äyriäinen
Community	= i denna rapport i bemärkelsen växt- och djursamhällen = tässä raportissa tarkoittaa kasvi- ja eläinyhteisöä
Diversity	= mångformighet, artrikedom = monimuotoisuus, lajirikkaus
Eutrophic	= näringsrik pga höga närsalthalter = ravinnerikas johtuen suuresta ravinnesuolapitoisuud.
Frond	= skott, årstillväxten hos blåstång = verso, rakkolevän vuosikasvu
Filamentous	= trådig (alg) = rihmamainen (levä)

Heterotrophic	= andra organismer än självproducerande växter, omfattar såväl växtätare (herbivorer) som köttätare (karnivorer) samt allätare (omnivorer) = muut organismit kuin tuottavat kasvit, käsittää kasvissyöjät (herbivorit), lihansyöjät (karnivorit) sekä kaikkiruokaiset (omnivorit)
Invertebrate	= djur som saknar inre ryggrad mots. till vertebrater (inkl. mänska). = selkärangattomat eläimet
Interspecific	= mellan arter (t ex konkurrens) = lajienvälinen (esim kilpailu)
Juvenile	= ung, icke-könsmogen = nuori, ei sukukypsä
Littoral	= strand(zon) = ranta(vyöhyke)
Mollusca	= blötdjur (musslor t ex) = nilviäiset (simpukat esim)
Macroscopic	= t ex djur större än 1 mm = esim eläimet suuremmat kuin 1 mm
Macrofauna	= se ovan = ks. edellinen
Metabolism	= organismers ämnesomsättning = organismien aineenvaihdunta
Periphyton	= påväxande vegetation (alger) = päällyskasvusto (leviä)
Primary production	= växternas produktion av biomassa = kasvien biomassatuotanto
Perennial	= flerårig, övervintrande = monivuotinen, talvehtiva
Planktic	= svävande = keijuva
Stickleback	= storspigg, *Gasterosteus aculeatus* = kolmipiikki, *Gasterosteus aculeatus*
Sub-system	= delsystem av t ex strandzonen (littoralen), kan bestå av t ex fiskpopulationerna eller sedimentlevande organismer = esim rantavuöhykkeen osasysteemi, voi koostua esim kalapopulaatioista tai sedimentissä elävistä organismeista
Sub-lethal	= ej akut dödande = ei akuutisti tappava
Secondary production	= produktion av biomassa hos djur = eläinten biomassatuotanto
Taxonomy	= det systematiserade artsystemet av växter och djur = kasvien ja eläinten systemaattinen lajijärjestelmä

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APPENDIX 1/1

Chemical – analytical methods

Water analysis

Chlorinated phenolics analysis by in-situ acetylation and GC-ECD.

5 -10 mL industrial waste water samples (with added 2,6-dibromo phenol as internal standard) were buffered to pH 9 by adding a K_2CO_3/$NaHCO_3$ solution, acetylated with acetic anhydride and extracted with 1 mL hexane. The hexane extract was collected, concentrated under a gentle stream of nitrogen, and a 0.5 - 1.0 µL volume injected on-column for gas chromatographic analysis with electron capture detection (ECD). The column was a slightly polar 25 m capillary DB-5.

Resin and fatty acids (and sterols) analysis by solvent extraction and GC-FID.

50 -100 mL industrial waste water samples (with heptadecanoic acid as internal standard) were buffered to pH 9 by adding a K_2CO_3/$NaHCO_3$ solution and extracted twice with nearly equal volumes of MTBE (methyl *tert*-buthyl ether). The combined MTBE extract was concentrated to near dryness in a rotary vacuum evaporator, dissolved in a few mL diethyl ether/ methanol (9:1), methylated with diazo methane. The solvent was removed under a stream of nitrogen and the residue silylated by adding 50 - 100 µL BSTFA (bis-trimethylsilyl-trifluroacetamide) and heating at 70 °C for one hour. 0.7 µL was injected on GC using a splitless injection technique and the analytes detected with a flame ionization detector (FID). The column was an unpolar 25 m capillary DB-1.

Fish bile analysis

Total chlorinated phenolics, recoverable after alkaline hydrolysis.

600 µL bile samples were freeze dried in open test tubes, and wet and dry weight recorded. The dry residue was hydrolyzed by adding 2 mL of 0.5 M KOH in 90 % ethanol and heating for two hours at 70 °C. The solution was cooled, diluted with 3 mL distilled water, acidified (pH 2) by adding dilute sulfuric acid, and extracted three times with 3 mL diethyl ether. 2,6-dibromophenol and heptadecanoic acid was added as internal standards. The combined ether extracts were divided into two equal parts (one part for resin/fatty acid analysis).

The ether extract was concentrated under nitrogen to less than one mL (i.e. a water-rich residue) and 4 mL carbonate buffer added. Hexane was added and the mixture acetylated by addition of acetic anhydride. The hexane phase was analyzed by GC-ECD as above.

Total resin/fatty acids, recoverable after alkaline hydrolysis.

Hydrolysis and ether extraction as above. The ether extract was concentrated under nitrogen to less than one mL. Buffer solution (5 mL, pH 9) was added and the mixture extracted twice with 4 mL of MTBE. The MTBE was removed by a stream of nitrogen and the extract methylated and analysed by GC-FID as above.

APPENDIX 1/2

Sediment analysis

Total chlorinated phenolics and resin/fatty acids, recoverable after alkaline hydrolysis.

Sediments were freze dried and wet and dry weight recorded. 200 -300 mg dry sediment was weighted into a test tube, a small amount of ascorbic acid added, and hydrolysis performed by adding 2 mL of 2.5 M KOH in 50 % ethanol with simultaneous bubbling of nitrogen through the solution. Tubes were capped and heated at 70 °C for 16 -18 h. The solutions were cooled, diluted with distilled water, acidified by adding concentrated sulfuric acid (cooling), and extracted with diethyl ether and analyzed as for bile samples.

Extractable organic halogen, EOX.

A few grams of wet sediment was shaken (45 min) three times with 10 mL of a cyclohexane-isopropanol (1:1) mixture. The combined extract was washed by shaking three times with acidified $NaNO_3$ (0.25 M, pH 2), and then dried over $NaSO_4$. The volume of the final extract was recorded and a 5 mL aliquote taken for halogen determination with combustion/micro-coulometric halogen titration. The solvent was removed under a stream of nitrogen and a small volume of hexane/iso-oktane was added prior to injection into the furnace of a TOX apparatus.

Total organic material, determined as loss on ignition.

Dry sediment was heated for 3 h in 500 °C, and the loss of weight recorded.

Biological material analysis

Total chlorinated phenolics and resin/fatty acids, recoverable after alkaline hydrolysis.

The analytical procedure was the same as for sediments except that wet sample material was used, and a mechanical homogenization was performed after addition of the alkaline ethanol-water solution.

Extractable organic halogen, EOX.

The analytical procedure was the same as for sediments except that wet sample material was used, and a mechanical homogenization was performed after addition of the cyclohexane-isopropanol solution.

SUMMARY

EFFECTS IN MESOCOSMS EXPOSED TO UNTREATED AND TREATED TOTAL MILL EFFLUENTS FROM PRODUCTION OF BLEACHED HARD-WOOD KRAFT PULP

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1 INTRODUCTION

The objective with the present study was, based on ecotoxicologically sound principles, to assess the effects on the shallow, rocky littoral zone of the Baltic Sea of pulp mill industrial effluents. The study was performed in land-based so called model ecosystems in which the plant and animal life of the littoral zone of the Baltic Sea are simulated. The study is a continuation of the systematic research, which started in the beginning of the 1980s within the frame of the Swedish Forest Industries Foundation for Water- and Air Pollution (SSVL). In the beginning effluents from production of bleached softwood kraft pulp were studied. The effects of different process alternatives, different bleaching technologies, with and without external treatment were compared with effects of effluents from the, by that time, coventional bleaching sequence (C95+D5)EHDED.

The results from these experiments have mainly been published under 1989-1990 (Lehtinen 1989; Lehtinen 1990; Lehtinen et al. 1990; Rosemarin et al. 1990).

The test series from 1982-1984 showed that traditional chlorine bleaching caused the most pronounced effects in the model ecosystems (mesocosms). Externally treated effluent from a mill with the sequence O(C85+D15)EDED and untreated effluent from bleaching of softwood kraft pulp with the sequence O(C52+D48)EDED caused least effects. All experiments were performed with two effluent dilutions, 400 and 2000 times, based on a normalized effluent volume of 50 m^3/t pulp.

Regarding production of bleached softwood kraft pulp, a certain correlation between biological effects and emissions of chlorinated organic material seemed to exist down to a level of about 2 kg AOX (Adsorbable Organic Halogen). Preliminary experiments indicated that no correlation existed for effluents originating from production of bleached hardwood kraft pulp, however. These preliminary experiments, using effluents from hardwood kraft pulp production, were not possible to conduct to the same extent as those from production of bleached softwood pulp. Consequently there was a need to increase the knowledge on effects of effluents from bleached hardwood pulp production.

In the present experiment in mesocosms effects, distribution and transformation of organic material from production of bleached hardwood kraft pulp production were studied.

2 EXPERIMENTAL SET-UP

The mesocosm technique used in the experiment was developed in the 1970s and has previously been used at testing oil, oil and dispersants, arsenic and tri-chloroguaiachol. The advantages of this technique are long-term exposure, large volume (8 m^3) pools allowing for subsampling, system reproducibility, controlled exposure, low realistic levels of toxic substances used and that the system are open to a flow-through of raw seawater.

The test-systems consist of out-door land-based 8000 l circular pools (1 m

depth) with polyethylene liner, each containing a 3 cm thick sand base and known figures of transplanted bladder-wrack, (*Fucus vesiculosus*), and associated organisms. In order to enable separate studies physiological reponses in fish, a smaller pool (500 L) for rainbow trout is attached to the out-going water from each mesocosm. Survival, growth and parasite frequency are studied in a second additional pool (150 l) attached to the out-going water from the rainbow trout pool.

Brackish water (2.8 l/min) is continuously pumped from 10 m depth from a bay outside the laboratory located in Nagu, in the Archipelago sea of Turku, Finland. The water is running to a seawater tank, from where it is distributed to the mesocosms by gravity flow. Water flow is regulated with capillary tubes. The experimental set-up is presented in figure 1.

The effluents tested were sampled at the respective mill under normal running conditions. After sampling the effluents were transported to the laboratory in Nagu and stored deep frozen in 35 l polyethylene containers. Required amounts of effluents were successively thawed during the experiment. pH, dissolved oxygen and temperature were semi-continuously measured during the experimental period from the incoming and from the out-going water of the test-systems.

The structuring element of the shallow littoral hard bottoms of the Baltic is the bladder-wrack, *F. vesiculosus* L.. Consequently, this alga is also one of the most important species in the mesocosms.

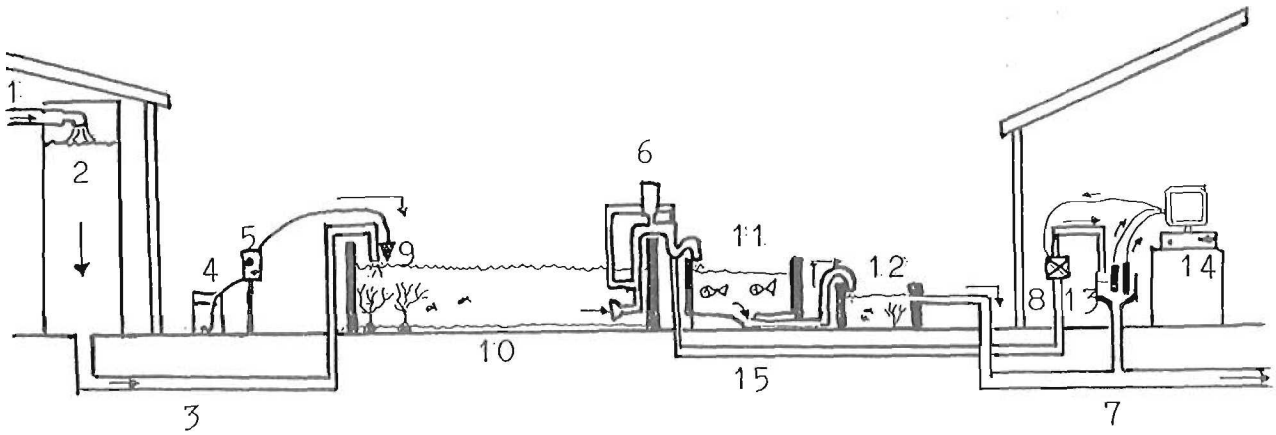

Fig. 1. The mesocosm set-up

1. Incoming water
2. Seawater tank
3. PEH-pipe to the pools
4. Effluent container
5. Membrane pump
6. Siphon for out-going water
7. PEH-pipe for out-going water
8. Electric valve
9. Dosage of effluent water
10. Mesocosm pool
11. Rainbow trout tank
12. Stickleback tank
13. Registration electrodes
14. Computer

Before the experimental start bladder–wrack specimens, attached to their original stone substrata, were collected from a brackish water bay, nearby the laboratory. The associated invertebrate fauna is sample simultaneously when a plastic bag is threaded over the alga. The algae are placed in the same sector in relation to insolation in each pool. The total volume of every specimen is determined by the water displacement technique prior to introduction in the mesocosms. Total volume is also determined at the end of the exposure.

The number of the associated invertebrate fauna is estimated and in case some pool receives a lower number of animals, more animals are added to such pools in order to obtain similar number of animals at the start of the experiment. 100 of young three–spined sticklebacks are also added to act as predators in the systems.

After addition of plants and animals the pools are left to stabilize for at least two weeks before exposure is commenced.

Fishphysiological studies are performed using rainbow trout, (*Oncorhynchus mykiss*), as test organism. Exposure is maintained for totally 8 weeks. Sampling is made after 2 and 8 weeks of exposure. Samples for blood–, bile–, and tissue–analysis are taken in these occasions.

At the beginning of the experimental period two mature stickleback males and three females are placed in 150 L pools in order to produce the fish material used in the experiment on fish populations (survival, growth, tissue structure and parasite frequency). The parent fish are removed after egg hatching.

In the final assessment of the mesocosm work the effects were compared against unpolluted controls by classifying them according to their total impact on the systems based on previously published principles (Lehtinen et al. 1990).

3 EFFLUENTS TESTED

Four effluent were tested in the present work. The effluents are called Au and At (where u stands for untreated and t for treated) and Bu and Bt.

The effluents were tested at two dilutions according to the following codes:

HD = High dose; 400 times dilution
Ld = Low dose; 2000 times dilution

The Au and At effluents are untreated and treated (active sludge) total effluents from production of bleached hardwood pulp according to the sequence (D80+C20)(EOP)DED. Bu and Bt are untreated and treated (pilot plant aerated lagoon) total effluent from production of bleached hardwood pulp according to the sequence O(D27,C68+D5)(EOP)D(EP)D. Production– and processdata are presented in table 1.

Table 1. Production- and processdata from the two mills tested.

	Mill A	Mill B
Production t/d	1 450	945
Effluent volume m^3/t pulp	40	54
Sequence	(D80+C20)(EOP)DED	O(D27,C68+D5)(EOP)(EP)D
ClO_2 in D+C kg/t90	34.7	5.3
Cl_2 in (D+C) %	80	32
Kappa number to bleachery	15	13
Chlorine multiple	0.06	0.08
Active chlorine multiple	0.28	0.12
Total charge active Cl kg/t90	67	35
NaOH E1 kg/t90	19	11.1
NaOH E2 kg/t90	4	4.6
O_2 E1 kg/t90	4.5	4.6
H_2O_2 E1 kg/t90	1.3	1.0
H_2O_2 E2 kg/90	-	1.0
Viscosity	1 075	1 025

4 SUMMARY OF EFFECTS NOTED FOR THE EFFLUENTS TESTED IN 1990

In some of the mesocosms (one control, effl. AuHD, and effl. Bt, both doses) a green alga, *Spirogyra sp.*, occurred spontaneously. In the exposed *Spirogyra*-containing mesocosms the invertebrate fauna was stimulated, despite that some of these mesocosms received higher concentrations of potentially toxic substances than mesocosms without *Spirogyra* in which a decline of the invertebrate fauna was noted. The spontaneous occurrence of *Spirogyra* is an example of how a biological factor possibly may mask and modify an inhibitive effect of an effluent. Ii this instance it is noteworthy that exposed mesocosms containing *Spirogyra* were compared with the control also containing *Spirogyra*.

4.1 Effluent Au, bleaching sequence (D80+C20)(EOP)DED

The effluent flow was 40 m^3/t pulp. The COD was 90 kg/t, AOX 1.6 kg/t, chloaret 5.8 kg/t, chlorinated phenolics 1.8 g/t, resin acids 73 g/t and chlorinated resin acids 4.7 g/t.

After the exposure following effects were noted in the mesocosms:

* Bladder-wrack: In the low dose (2000 times dilution) a lower biomass was

noted as compared with the control. In the high dose the biomass was higher than the control. No deviations of the apical (annual) growth were noted in neither dose.

* Bladder–wrack invertebrate fauna: Lower biomass and abundance in the low dose and contarry to this increased biomass and abundance in the high dose. These effects were mainly due to changes in the mussel populations.

* Sediment invertebrate fauna: Decreased biomass and abundance in the low dose and an increse in the high dose was noted. Dominating species were the blue mussel, *Mytilus edulis*, the heart mussel, *Cardium edulis* and amphipods (Gammaridae).

* Total invertebrate biomass and abundance in the mesocosms: As in the case with the bladder–wrack and sediment associated invertebrates a weak inhibition in the low dose and a stimulation in the high dose of abundance and biomass were noted.

* Growth of three–spined stickleback young: A distinct stimulation after two months ewxposure occurred in both doses. The stimulation was maintained until the end of the experiment.

* The content of conjugated substances in fish bile: A dose–response relationship was noted for both chlorophenolics and resin acids. The concentration of resin acids was clearly higher than that of chlorophenolics. The concentrations were similar after 2 and 8 weeks exposure.

* Hematology: No statistically significant differences as compared with the control were noted.

* Liver metabolism in rainbow trout: Aside from a significantly increased liver–glycogen no differences occurred in the exposed fish.

4.2 Effluent At, bleaching sequence (D80+C20)(EOP)DED, active sludge treatment

The effluent volume was the same as for the untreated effluent Au. COD was 39 kg/t, AOX 1.0 kg/t, chlorate < detection limit, chlorinated phenolics 0.6 g/t, resin acids 4.3 g/t and chlorinated resin acids 2.1 g/t pulp.

Following main effects were noted:

* Effects on bladder–wrack: A clear decrease of the total biomass was seen in both doses as compared with the control. No effects were noted regarding annual apical growth.

* Effects on bladder–wrack invertebrate fauna: A distinct inhibitive effect was noted in both the high and low dose. The total number of animals and their biomass were clearly lower despite that the number and biomass of crustaceans increased.

* Effects on sediment invertebrates: Both the number and biomass were lower than in the control in both doses.

* Effects on total biomass and abundance: Inhibition in both doses.

* Effects on the growth of stickleback young: Stimulation in both doses.

* Concentrations of conjugated substances in fish bile: Increased, dose–dependent concentrations were noted. The concentration of resin acids was higher than

that of chlorophenolics. The concentrations decreased somewhat after 8 weeks exposure as compared with exposure for two weeks.

* Effects on hematology: No statistically significant responses were recorded.

* Effects on liver metabolism: The only statistically significant deviation from the control was an increased liver glycogen level.

4.3 Effluent Bu, bleaching sequence O(D27,C68+D5)(EOP)D(EP)D

The effluent flow was 54 m^3/t pulp. Cod was 29 kg/t, AOX 0.9 kg/t, chlorate 2.0 kg/t, chlorinated phenolics 1.4 g/t, resin acids 9.5 g/t and chlorinated resin acids 0.5 g/t.

Following effects were seen in the mesocosms:

* Effects on the bladder-wrack: A decrease of the total biomass was noted but no effects on the annual, apical growth.

* Effects on bladder-wrack invertebrate fauna: In the low dose a stimulatory effect was noted wheras in the high dose an inhibitive response was seen. Distinct species-related differences as to the responses were seen at exposure to this effluent.

* Effects on sediment invertebrates: In the low dose a stimulatory effect was seen and in the high dose a weak inhibition was obtained.

* Effects on total invertebrate abundance and biomass: In the low dose a weak stimulation occurred, whereas no differences as compared with the control were seen in the high dose.

* Effects on the growth of stickleback young: Growth was stimulated in both doses during the course of the experiment.

* Conjugated substances in fish bile: A dose-dependent increase of chlorinated phenolics and resin acids was seen. The concentration of resin acids was higher than that of chlorophenolics. An increase of the concentration of chlorophenolics was obtained after 8 weeks exposure in comparison with the concentration after 2 weeks exposure.

* Effects on hematology: No statistically significant effects were noted.

* Effects on the liver metabolism: A significantly increased liver-glycogen value was noted in the fish exposed to the low dose.

4.4 Effluent Bt, bleaching sequence O(D27,C+D5)(EOP)D(EP)D, pilot plant aerated lagoon treatment

The effluent flow/ ton pulp was the same as for Bu. COD was 17 kg/t, AOX 0.4 kg/t, chlorate < detektion limit, chlorinated phenolics 0.5 g/t, resin acids 0.5 g/t and chlorinated resin acids 1.6 g/t.

Following effects were recorded:

* Effects on bladder-wrack: A decline of the total biomass was seen in the low dose, but no differences as compared with the control was seen in the high dose. No effects was seen on apical, annual growth.

* Effects on bladder–wrack invertebrate fauna: A clear stimulation of both abundance and biomass in both doses was induced by this effluent.

* Effects on sediment invertebrate fauna: A clear decline of the number of animals was seen in both doses. On the other hand the biomass was clearly stimulated. The low dose exhibited the highest value next to the control of all doses tested.

* Effects on the total invertebrate abundance and biomass: An increase was seen in both doses

* Effects on the growth of stickleback young: Significantly enhanced groth in both doses tested.

* Effects on hematology: No statistically significant differences were noted in neither dose.

* Effects on the liver metabolism in fish: Except for an increased liver glycogen value no statistically significant deviations from the control fish were noted.

4.5 Effects of phytosterols, natural wood compounds

Aside from the effluents tested an additional experiment with three–spined stickleback young and rainbow trout as test organisms was performed using a powder containing phytosteriols, mainly beta–sitosterol, was used as pollutant.

Testing period and test conditions were the same as in the other exposure groups. The reasons for testing the respones of the phytosterols were several: many of the sterols present in the powder are structurally similar to cholesterol. Cholesterol is the basic compound at synthesis of several steroid hormones in organisms and the substance is a necessary compound for maintenance of the fluidity of cell membranes. If interference between steroids present in the wood raw material in fish would occur, this might possibly explain many previously noted responses in fish exposed to pulp mill industrial effluents.

Brood of three–spined stickleback were exposed via the water, initially to a 5 µg/L nominal total steroid concentration. The concentration was increased after two months to 10 µg/L. Rainbow trout were exposed both via water and food. Except for the dose obtained via water the rainbow trout were exposed to a daily food dosage of 26 µg/ind. and during the successive 6 weeks to about 45 µg/ind.

Effects noted:

* Effects on brood of three–spined stickleback: Vacuolization of the liver tissue and stimulated growth. Similar responses were also noted for the fish exposed to the total mill effluents.

* Substances in the bile of rainbow trout: Increased levels of conjugated cholesterols and phytosterol levels. The levels were practically identical with those noted for fish exposed to total mill effluents.

* Effects on the liver metabolism of rainbow trout: An increased liver glycogen value was obtained. The response was identical with that of fish exposed to total mill effluents.

5 SUMMARY OF THE RELATIVE ENVIRONMENTAL IMPACT OF THE EFFLUENTS TESTED

The effluents tested have been classified according to the intensity in change of the response of the parameters studied. Since a relative classification based on a scale from 0–5 (0 = no effect; 5 = highest intensity) the procedure should be regarded as a semiquantitative integration of the results obtained. The method has previously been used for evaluation of the relative environmental impact of previously tested processes (Lehtinen et al. 1991). The method must be considered as rather subjective since the significance of the respones obtained on different biological levels is still not understood. Due to the very extensive material produced it was held important to find a means to summarize the results in a comprehensive way.

The values obtained on effects on bladder–wrack, invertebrates, brood of stickleback and the physiological status of rainbow trout are summarized in table 2 – 5. A total evaluation is given in table 6 in the form of a mean "effect index".

Table 2. Effects on the growth of bladder–wrack.

	Dilution	
Effluent	2000x	400x
Au	2	0
At	3	4
Bu	0	4
Bt	3	0

Table 3. Direct effects on the bladder–wrack invertebrate fauna.

	Biomass		Abundance	
	Dilution			
Effluent	2000x	400x	2000x	400x
Au	3	0	4	0
At	3	4	5	5
Bu	0	1	0	4
Bt	0	0	0	0

Table 4. Effects on mortality, growth, liver histology and parasite frequency of the brood of stickleback.

	Mortality		Growth	
	Dilution			
Effluent	2000x	400x	2000x	400x
Au	0	0	5	5
At	2	1	5	5
Bu	2	0	5	5
Bt	2	0	5	3
	Liver histology		Parasite frequency	
Au	2	3	1	3
At	2	3	3	4
Bu	2	4	4	3
Bt	1	2	5	2

Table 5. Effects on the physiological status (hematology, enzyme activity in liver and metabolism).

	Dilution	
Effluent	2000x	400x
Au	0	1
At	1	1
Bu	0	1
Bt	1	0

Table 6. Calculated effect indexes of the effluents tested.

	Dilution	
Effluent	2000x	400x
Au	2.1	1.5
At	3.0	3.4
Bu	1.6	2.8
Bt	2.1	0.9

The values obtained in table 6 have been obtained by summing the the points given in tables 2–5 for the respective effluent and then dividing them by the number of parameters given in the same tables.

According to table 6 effluent Bt caused the lowest impact. The effects of effluents Au and Bu were at the same level and the highest impact was induced by effluent At. Most prominent regarding effects were the effects on the growth of the stickleback brood and the increased incidence of parasites. Liver structural changes are also noteworthy. Clear correlations between the amounts of AOX dosed, concentrations of conjugated chlorophernolic substances or resin acids in bile of fish were not prevailing, however. Notable in this respect is the high effect index of effluent Bt (active sludge treatment). Reasons behind this might be of indirect biological nature or that this effluent contained other effective substances than other effluents.

The effect indexes obtained in the present work are presented in figure 2. Previously calculated effect indexes from other studies are included in the figure as well. Previously tested processes are presented in table 7.

The effect indexes in figure 2 are plotted against emitted amounts of AOX in kg/t pulp. Regarding production of bleached softwood kraft pulp, it may be noted that some correlation between decreasing AOX and decreasing effect index seems to exist down an AOX–level of about 2 kg/t pulp. At an AOX–level of 2.5 the effect index is lower than that for unbleached softwood pulp production, however. This is indicating that non–chlorinated substances induce effects noted at least at AOX–level below 2–2.5 kg/t pulp. Concerning effluents from production of bleached hardwood kraft pulp there is no correlation between effects and emitted AOX.

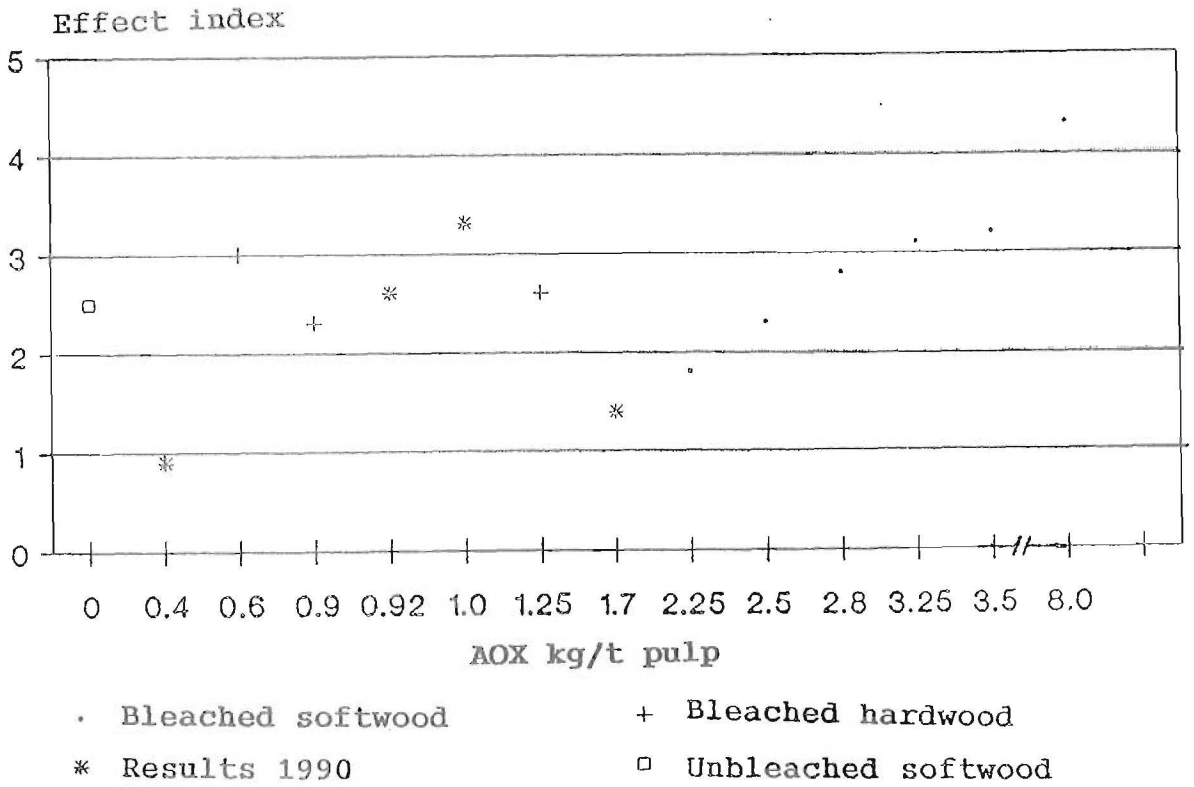

Figure 2. Effect indexes of different total mill effluents tested in mesocosms in the period 1982–1990.

Table 7. Comprehensive list of effluents from different processes tested in the period 1982–1990.

Process	External treatment	AOX
Softwood pulp (1982–84)		
Unbleached	no	0
(C95+D5)EHDED	no	ca. 8
(C87+D13)EDED	aerated lagoon	2.8
O(C83+D17)EDED	no	3.5
O(C85+D15)EDED	aerated lagoon (partial treatm.)	3.25
O(C85+D15)EDED	(pilot) aerated lagoon	2.25
O(C52+D48)EDED	no	2.5
Hardwood pulp (1986)		
(D92+C8)(ED)D(EP)D	no	0.6
O(C82+D18)EDED	no	1.25
O(C51+D49)EDED	no	0.9
1990–test, hardwood pulp		
(D80+C20)(EOP)DED	no	1.7
"	activated sludge	1.0
O(C27,D68+D5)(EOP)D(EP)D	no	0.9
"	(pilot) aerated lagoon	0.4

6 GENERAL CONCLUSIONS

6.1 The ecosystem

Both stimulatory and inhibitory effects were noted in the exposed mesocosms. The direction of these responses may be assumed to be regulated through biological mechanisms, for instance changes in interspecific competition. This assumption is among other things based on observations made in mesocosms, in which *Spirogyra* occurred. The prevalence of this green alga was presumably due to the chance. In pools containing most *Spirogyra*, the invertebrate fauna was stimulated despite that these pools simultaneously received a higher dose of potentially toxic compounds than pools without *Spirogyra*. In pools without *Spirogyra* the invertebrate fauna was inhibited. The random occurrence of *Spirogyra* is an example on how a biological factor may mask and modify an otherwise inhibitive response of an effluent. In 1991 the experiment with effl. Bt was repeated, now without *Spirogyra*. This time the invertebrate fauna was inhibited, which further supports the assumption of the modifying effect of *Spirogyra* (Lehtinen et al. unpublished).

The exposure mostly induced a stimulated growth of periphytic algae. This is an indication of that substances introduced with the effluents favour growth of rapidly growing annual algal species. At the same time the bladder–wrack exhibited decreasing tissue nitrogen levels, which shows that this perennial species is unable to compete for nutrients with the annual, rapidly growing algae during the vegetation period. This is also indicating that the bladder–wrack, under the present simulated conditions, may

be disfavoured in the Baltic at exposure to pulp mill effluents, but also other effluents containing nutrients, i.e. nitrogen, may act in the same way (Elmgren 1989). The mode of action of the same effluents in fresh water lakes is hard to say explicitly. Presumably phosphorus is the limiting factor in limnic environments, which may mean that nitrogen has a smaller role in fresh water than in marine environments, however.

The stimulatory effects noted on amphipods (*Gammarus spp.*) may be due to hormon-like substances, present in the wood raw material, inducing an increased moulting frequency. The relatively specific response of this group of organisms may cause interspecific changes in competition between the bladder-wrack associated animal species. This was indicated by the decreased abundance of another crustacean (*Idothea spp.*) in the exposed mesocosms.

No clear correlations between levels of AOX, chlorophenolics, resin acids and effects were seen. The EOX-level increased in several cases in different compartments of the mesocosms, however. Despite of this, no signs of biomagnification was observed. In addition the analyzed EOX-levels depended little on the total amount added AOX in the mesocosms. About 1% of the total dose AOX added was traceable in sediment or organisms.

6.2 Fish studies

The experiments performed on fish with ohytosterols and total mill effluents caused very similar responses (growth, liver histology etc.).

The significance of stimulated growth is presently not understood. In field studies a connection between stimulatory effects on growth and impaired gonad development in fish has been made. In the present work possible effective substances have been indicated.

The 8 week long exposure of rainbow trout did not cause any induction on the detoxification enzymes analyzed. The increased levels of liver glycogen and the liver histological changes noted, do indicate some kind of metabolic disorder, however. Furthermore, the increased levels of conjugated cholesterol in fish bile are an indication on that metabolic responses occurred in fish exposed to both steroids and effluents. However, no acute liver damage seemed to prevail, since the ALAT-levels in serum of fish were not elevated.

Exposure to untreated effluents induced somewhat higher levels of chlorinated phenolics and resin acids in the bile of fish than treated effluents. Generally the levels differed only slightly from natural background levels. The analyzed levels of conjugated chlorophenolics and resin acids cannot therefore be connected to the effects obtained in fish.

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YHTEENVETO

ORGAANISEN AINEEN VAIKUTUKSISTA, KULKEUTUMISESTA JA MUUNTUMISESTA LEHTIPUUMASSAN TUOTANNOSTA AIHEUTUVIEN KOKONAISJÄTEVESIEN ALTISTAMASSA MALLIEKOSYSTEEMISSÄ

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Suomen Ympäristötutkijaryhmä

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1 JOHDANTO

Tässä tutkimuksessa on pyritty parhaalla mahdollisella ekotoksikologisella tavalla arvioimaan metsäteollisuuden jätevesien vaikutuksia vesiympäristöön käyttäen maalle pystytettyjä malliekosysteemejä. Tutkimuksen asiantuntemus perustuu aikaisempiin malliekosysteemitutkimuksiin, joita tehtiin Ruotsin metsäteollisuuden rahoittamana vuosina 1980–1986 (SSVL–85). Tällöin arvioitiin erilaisten valkaisuprosessien pitkäaikaisia ympäristövaikutuksia ja verrattiin niitä perinteiseen kloorivalkaisuun (eli (C95+D5)EDED). Tutkimustulokset aikaisemmista tutkimuksista on julkaistu pääasiassa vuonna 1990 (mm. Lehtinen 1989, 1990, Lehtinen et al. 1990, Rosemarin et al. 1990).

Murtoveden rantavyöhykkeen ekosysteemin kaikki tärkeimmät osat on sisällytetty näihin malliekosysteemitutkimuksiin. Tähän kuuluvat aineiden kulkeutuminen ja muuntuminen sekä vaikutukset veteen, sedimenttiin, leviin, selkärangattomiin sekä kaloihin. Altistukset toteutettiin alhaisissa ekologisesti relevanteissa pitoisuuksissa pitemmän ajanjakson kuluessa (5–6 kuukautta). Näitä koejärjestelyjä on Ympäristötutkijaryhmän toimesta käytetty myös erilaisten yhdisteiden kuten arsenikin, triklooriguajakolin, kloraatin sekä öljyn ja jalostamojätevesien ympäristövaikutusten tutkimiseen.

Vuosien 1980–86 kokeissa (SSVL–85) todettiin, että perinteinen valkaisu, jossa klooorikaasun osuus oli 95 % CD–vaiheessa, oli ympäristön kannalta haitallisin. Valkaisujaksolla O(C85+D15)EDED varustettuna ilmastuskäsittelyllä sekä jaksolla O(C52+D48)EDED (käsittelemätön jätevesi) vaikutukset olivat vähäisimmät. Kaikki jätevedet testattiin 400 ja 2000 kertaa laimennettuna perustuen jätevesimäärään 50 m^3/tonni massaa.

Aikaisemmat tutkimukset on tehty pääosin havupuumassan tuotannosta aiheutuvilla jätevesillä ja niiden vesistövaikutukset aktiivilietelaitoskäsittelyä lukuunottamatta tunnetaan suhteellisen hyvin. Todettavien jätevesivaikutusten ja TOCl:n suhteen näyttää olevan tietty riippuvuus TOCl–tasolle 2 kg/t massaa. Tämän tason alapuolella ei voida tieteellisin perustein osoittaa selvää riippuvuutta. Alustavat kokeet ovat osoittaneet, että tällaista riippuvuutta ei olisi lehtipuumassan osalta. Lehtipuumassan tuotannosta aiheutuvista vaikutuksista on kuitenkin ollut selvää tietämyksellistä puutetta.

Nyt raportoitavissa malliekosysteemitutkimuksissa on selvitetty orgaanisen aineen vaikutuksia, kulkeutumista ja muuntumista lehtipuumassa tuotannosta aiheutuvien kokonaisjäteveisen altistamissa malliekosysteemialtaissa. Tutkimukset ovat jatkoa aikaisemmin tehdyille selvityksille ja niistä saadut tulokset on huomioitu tuloksia tarkasteltaessa. Tutkimukset on tehty yhteistyössä Suomen ja Ruotsin metsäteollisuuden kanssa, jotka ovat myös vastanneet tutkimusten rahoituksesta.

2 MALLIEKOSYSTEEMIN KUVAUS

Tutkimuksissa käytetyt malliekosysteemit ovat alunperin nyt Suomen ja Ruotsin Ympäristötutkijaryhmässä toimivien tutkijoiden 1970–luvulla kehittämiä. Niitä on käytetty menestyksekkäästi öljyn ja dispersanttien, arsenikin, kloraatin, triklooriguajakolin sekä metsäteollisuuden jätevesien ekologisten vaikutusten selvittämiseen. Tämän tutkimusmenetelmän etuja ovat pitkä altistumisaika (5 – 6 kuukautta), suuri tilavuus (allas 8 m^3) mahdollistaen osanäytteiden ottamisen, menetelmän toistettavuus, hallittu altistus, alhaiset realistiset myrkyllisten yhdisteiden pitoisuudet sekä se, että systeemi on avoin murto– tai makean veden läpivirtaukselle. Malliekosysteemikokeet omaavat huomattavasti korkeamman ekologisen reaalitason kuin esimerkiksi tavanomaiset lyhytaikaiset annostelupitoisuudeltaan suuret laboratoriotestit. Samanaikaisesti malliekosysteemikokeet tuottavat paremmin toistettavaa aineistoa suhteellisen alhaisin kustannuksin verrattuna kenttätutkimuksiin.

Malliekosysteemit koostuvat ulkona sijaitsevista maalle pystytetyistä 8 000 l:n altaista (syvyys 1 m), jotka on vuorattu polyetyleenistä valmistetulla sisäpussilla. Jokaisen altaan pohjalla on 3–5 cm paksu hiekkakerros sekä tunnetut määrät kasveja ja eläimiä rannan ekosysteemistä, jossa jätevedet yleensä esiintyvät luonnossa väkevimmillään (Itämeressä rakkolevän, *Fucus vesiculosus*, hallitsema vyöhyke ja sisävesissä kasvien hallitsema rantavyöhyke). Kalafysiologisten vaikutusten selvittämiseksi jokaisen malliekosysteemialtaan yhteydessä on erillinen allas, jossa altistetaan kirjolohia. Kalapopulaatio– ja histologia tutkimuksia tehdään kolmipiikillä kolmannessa erillisessä altaassa, johon vedet virtaavat suuresta malliekosysteemialtaasta.

Malliekosysteemialtaisiin on jatkuva murtoveden virtaus (2,8 l/min) ja vesi pumpataan 10 metrin syvyydestä tutkimusaseman edustalla sijaitsevasta lahdesta. Veden viipymä altaissa on 48 tuntia ja jätevesien vaikutuksia tutkittiin kasvukauden ajan kesäkuusta marraskuun loppuun. Koejärjestelyt on esitetty kuvassa 1.

Tutkittavat jätevedet kerättiin tehtaan käydessä normaalisti. Näytteenkeruun jälkeen jätevedet toimitettiin Nauvoon ja säilytettiin pakastettuina polyetyleenitankeissa. Kokeiden aikana aina vaadittava määrä vettä sulatettiin ja pumpattiin altaisiin annostelupumppujen avulla. Murtoveden virtaus altaisiin oli säädelty vakioksi lasikapillaarian avulla, jotka määräajoin puhdistettiin. Jäteveden annostelu tarkistettiin päivittäin.

Koko jätevesialtistuksen ajan seurattiin sisääntulevan veden sekä yksittäisistä malliekosysteemialtaista lähtevän veden pH:ta, happipitoisuutta ja lämpötilaa sekä altaisiin tulevaa valaistuksen määrää. Nämä mittaukset tehtiin automaattisesti joka täystunti ja aineistoa käytettiin ekosysteemin perustuotantokyvyn laskemiseen.

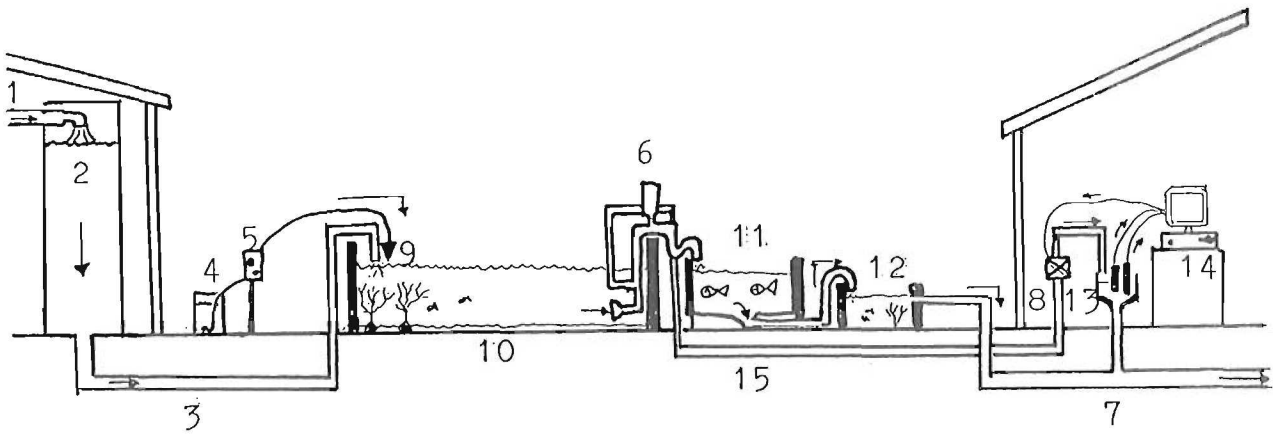
Altaissa olleiden rakkolevien tilavuus määritettiin sekä kokeen alussa että altistuksen lopussa. Rakkolevät sijoitettiin malliekosysteemialtaisiin tasaisesti ja samaan kohtaan kutakin allasta. Rakkolevät kerättiin Nauvon tutkimusaseman läheisten saarten rantavyöhykkeestä. Rakkolevien mukana tulleet eri eliölajien määrät arvioitiin ja mikäli johonkin altaaseen tuli vähemmän eliöitä niitä täydennettiin, jotta kussakin altaassa olisi ollut sama määrä eliöitä altistuksen alussa. Altaisiin laitettiin lisäksi 100

kappaletta vastakuoriutuneita kolmipiikin poikasia. Altaisiin luodut ekosysteemit saivat tasaantua ja vakiintua kahden (2) viikon ajan ennen altistusten aloittamista.

Altistuksen loputtua marraskuun 12–15 pnä. 1990 rakkolevät kerättiin altaista ja niiden tilavuus määritettiin. Rakkolevien joukossa elävävistä selkärangattomista otettiin näytteet, joista määritettiin lajisto ja määrä (biomassa ja runsaus), joka suhteutettiin kasvillisuuden määrään. Tällä tavoin voitiin arvioida malliekosysteemialtaan rakkolevästössä elävien selkärangattomien kokonaismäärä. Sedimentissä elävistä pohjaeläimistä otettiin myös näytteet kustakin altaasta altistuksen loputtua. Pohjaeläimet määritettiin lajilleen ja jaoteltiin kokoluokittain. Tulokset on ilmoitettu kokonaismääränä ja biomassana allasta kohti. Tulosten tarkastelussa on tehty allaskohtaisia vertailuja yhdistämällä rakkolevistä ja sedimentistä kerätty aineisto.

Malliekosysteemialtaiden yhteydessä oli pienemmät 500 litran altaat, joihin johdettiin isosta altaasta ulostuleva vesi ja, joissa tehtiin 8 viikon kalafysiologinen altistus kirjolohella. Altistus alkoi 3. syyskuuta ja päätyi 29. lokakuuta 1990. Kaloista otettiin näytteet 2 ja 8 viikon altistuksen jälkeen. Kaloista otettiin veri-, sappi- ja kudosnäytteet, joista tutkittiin hematologisia ja aineenvaihdunnallisia vasteita sekä maksan histologiaa. Sappinäytteistä määritettiin konjugoituneet orgaaniset klooriyhdisteet ja hartsihapot kalojen altistumisasteen määrittämiseksi.

Varsinaisissa malliekosysteemialtaissa ja niiden yhteydessä olevissa pienemmissä altaissa (ei kirjolohialtaat) tehtiin kalapopulaatiotutkimuksia kolmipiikillä. Näissä tutkimuksissa selvitettiin kalojen kasvua, kuolleisuutta, maksan histologiaa sekä loisten esiintymistä ja kalasairauksia. Kolmipiikit asetettiin altaisiin vastakuoriutuneina poikasina. Emokalat oli pyydetty tutkimusaseman edustalla olevasta lahdesta.



Kuva 1. Kaavakuva malliekosysteemistä.

1. Sisääntuleva vesi
2. Tasausallas
3. PEH-putki altaisiin
4. Jätevesisäiliö
5. Kalvopumppu
6. Veden poistojärjestelmä
7. Poistoputki
8. Magneettiventtiili
9. Jäteveden annostelu
10. Malliekosysteemiallas
11. Kirjolohiallas
12. Kolmipiikkiallas
13. Mittauselektrodit
14. Tietokone

Lopullisessa malliekosysteemitutkimusten tulosten arvioinnissa vaikutukset on suhteutettu allaskohtaisesti verrattuna vertailualtaaseen, johon on johdettu vain puhdasta murtovettä. Tutkittuja jätevesiä on sen jälkeen verrattu toisiinsa luokittelemalla ne kokonaisvaikutusten perusteella.

3 TUTKITUT JÄTEVEDET

Tässä tutkimuksessa tutkittiin kahden eri tehtaan lehtipuujakson käsittelemätöntä ja käsiteltyä kokonaisjätevettä. Tehtaista käytetään nimityksiä A ja B. Käsittelemättömiä jätevesiä kutsutaan Au:ksi ja Bu:ksi, ja käsiteltyjä jätevesiä At:ksi ja Bt:ksi. Jätevesiä tutkittiin kahdessa eri laimennoksessa:

HD = high dose; 400 kertainen laimennos

LD = low dose ; 2000 kertainen laimennos

Tehdas A käyttää massatuotannossaan jatkettua keittoa ja tuottaa täysin valkaistua lehtipuusellua valkaisulla (D80+C20)(EOP)DED. Jätevedet käsitellään aktiivilietelaitoksessa, jossa vesien viipymä on 24 tuntia. Tehdas B tuottaa täysin valkaistua lehtipuumassaa valkaisujaksotuksella O(D27,C68+D5)(EOP)D(EP)D ja sen kokonaisjätevedet käsitellään ilmastetussa lammikossa, jonka viipymä on 8–9 päivää. Lehtipuumassan lisäksi tehtaat tuottavat havupuumassaa, mutta tässä tutkimuksessa tutkittiin nimenomaan lehtipuumassan tuotannosta aiheutuvien jätevesien vaikutuksia. Tehtaiden tuotanto ja prosessitietoja on esitelty taulukossa 1.

Taulukko 1. Kahden tutkitun tehtaan tuotanto, jätevesivirtaamat ja prosessitietoja.

	Tehdas A	Tehdas B
Tuotanto t90/d	1 450	945
Jätevesivirtaama m^3/t	40	54
Valkaisujaksot	(D80+C20)(EOP)DED	O(D27,C68+D5)(EOP)D(EP)D
ClO_2 D+C vaiheessa kg/t90	34,7	5,3
Cl_2 D+C vaiheessa kg/t90	9,8	11,1
ClO_2määrä (%)	80	32
Kappaluku (valkaisuun menevä)	15	13
Kloorimultippeli	0,06	0,08
Aktiivi kloorimultippeli	0,28	0,12
Kokonais ClO_2 kg/t90	67	35
NaOH E1 kg/t90	19	11,1
NaOH E2 kg/t90	4	4,6
Happi E1 kg/t90	4,5	4,6
H_2O_2 E1 kg/t90	1,3	1,0
H_2O_2 E2 kg/t90	–	1,0
Viskositeetti dm^3/kg	1 075	1 025

Koodi	Valkaisuprosessi	Puhdistus
Au	(D80+C20)(EOP)DED	käsittelemätön
At	(D80+C20)(EOP)DED	aktiivilietelaitos
Bu	0(D27,C68+D5)(EOP)D(EP)D	käsittelemätön
Bt	O(D27,C68+D5)(EOP)D(EP)D	ilmastettu lammikko

Yksityiskohtaisempia prosessi- ja päästötietoja varten viitataan tutkimuksista tehtyihin laajempiin raportteihin (Lehtinen ym. 1991 ja Lehtinen ym 1991).

4 YHTEENVETO TUTKITTUJEN JÄTEVESIEN SUHTEELLISESTA LUOKITUKSISTA

Eri tutkitut jätevedet on luokiteltu niiden aiheuttamien vaikutusten perusteella. Tämä on periaatteessa semikvantitatiivinen lähestymistapa integroida eri osista saadut tulokset käyttäen suhteellista pisteytysmenetelmää. Vaikutustaso on sitä alhaisempi mitä pienempi pistemäärä on. Pistemäärä 0 viittaa siihen, että poikkeama vertailusta ei ole merkitsevä. Tätä luokitusta tulee pitää suhteellisen subjektiivisena, koska edelleenkään ei ole tietoa eri biologisilla tasoilla todettujen vaikutusten suhteellisesta tärkeydestä.

Taulukko 2. Vaikutukset rakkolevän biomassaan (*Fucus vesiculosus*).

	Laimennus	
Jätevesi	2000 x	400 x
Au	2	0
At	3	4
Bu	0	4
Bt	3	0

Taulukko 3. Suora vaikutus rakkolevän eliöstöön (makrofauna).

	Biomassa		Lukumäärä	
	Laimennus			
Jätevesi	2000 x	400 x	2000 x	400 x
Au	3	0	4	0
At	3	4	5	5
Bu	0	1	0	4
Bt	0	0	0	0

Taulukko 4. Kolmipiikin kuolleisuus, kasvu ja maksan histologia ja loiset.

	Kuolleisuus		Kasvu	
	Laimennus			
Jätevesi	2000 x	400 x	2000 x	400 x
Au	0	0	5	5
At	2	1	5	5
Bu	2	0	5	5
Bt	2	0	5	3
	Histologia		Loiset	
Au	2	3	1	3
At	2	3	3	4
Bu	2	4	4	3
Bt	1	2	5	2

Taulukko 5. Vaikutukset kirjolohen fysiologiaan (hematologia, entsyymiaktiisuus ja perusaineenvaihdunta).

	Laimennus	
	2000 x	400 x
Au	0	1
At	1	1
Bu	0	1
Bt	1	0

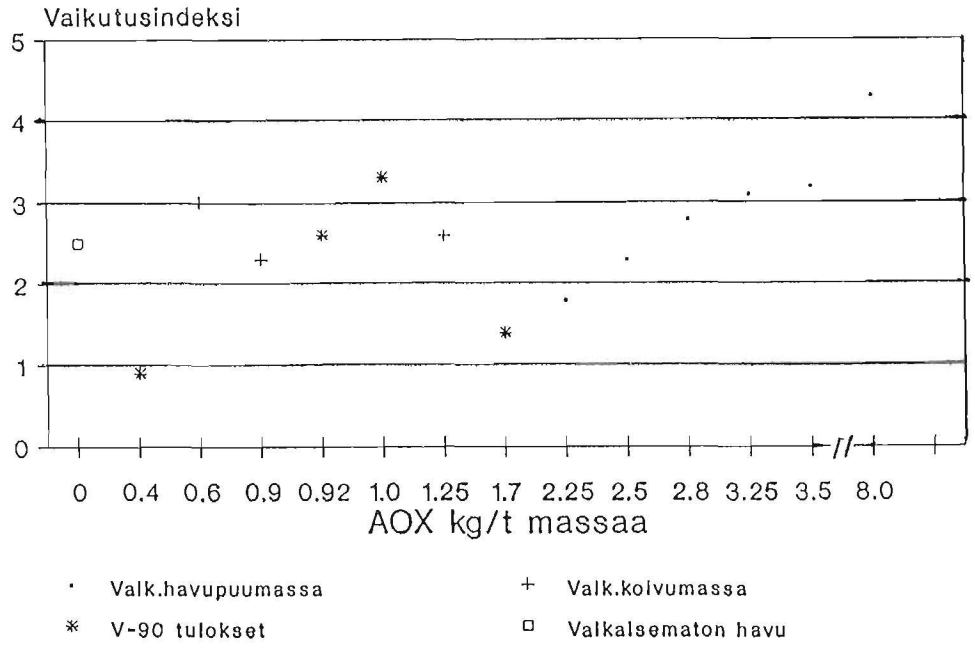
Taulukko 6. Summavaikutus malliekosysteemiin (vaikutusindeksi).

	Laimennus	
	2000 x	400 x
Au	2,1	1,5
At	3,0	3,4
Bu	1,6	2,8
Bt	2,1	0,9

Taulukon 6 summavaikutusluvut on saatu laskemalla ko. jäteveden saamat arvot taulukoissa 2–5 yhteen ja jakamalla nämä luvut tutkittujen suureiden määrällä.

Taulukon 6 mukaan vähäisimmät vaikutukset todettiin jätevedellä Bt. Jätevesien Au ja Bu vaikutukset ovat olleet samaa suuruusluokkaa ja suurimmat vaikutukset todettiin jätevedellä At. Merkittävimmät muutokset voitiin todeta kalojen kasvussa (kasvun lisääntyminen) ja loisten lisääntyneessä määrässä jätevesille altistetuissa kaloissa. Kalojen maksan histologiassa oli myös todettavissa muutoksia altisteuissa kaloissa. Altaisiin kohdistuneiden AOX–päästöjen tai kalojen sappinesteestä mitattujen yhdistepitoisuuksien sekä havaittujen vaikutusten välillä ei voitu osoittaa selvää riippuvuutta. Merkille pantavaa on jäteveden At (aktiivilietelaitos käsitelty) saama korkein vaikutusindeksi. Tämä saattaa olla seurausta epäsuorista biologisista vaikutuksista tai, että jätevesi on sisältänyt merkittävästi muita vaikutuksia aiheuttavia yhdisteitä.

Kuvassa 2 on esitetty vuoden 1990 malliekosysteemikokeista saadut vaikutusindeksiluvut sekä verrattu niitä aikaisemmissa tutkimuksissa saatuihin vastaaviin lukuihin (taulukko 7). Havupuumassan tuotannosta aiheutuvien jätevesien sisältämän AOX–pitoisuuden ja vaikutusindeksin suhteen voidaan todeta selvä riippuvuus tasolle 2 kg AOX/t. Vaikutukset pienenevät AOX–pitoisuuden pienentyessä. AOX–tason 2,5 kg/t massaa alapuolella vaikutusindeksi on jo pienempi kuin valkaisemattoman massan vaikutusindeksi. Tämä osoittaa, että muut kuin AOX:llä mitattavat aineosat ovat vastuussa todettavista vaikutuksista. Lehtipuumassan tuotannosta aiheutuvien jätevesien AOX:n ja vaikutusindeksin välillä ei voida todeta vastaavanlaista riippuvuutta kuin havupuumassalla.



Kuva 2. Malliekosysteemikokeista saadut vaikutusindeksiluvut ajalta 1982–1990.

Taulukko 7. Vuosina 1982–1990 tutkittujen prosessien jätevesien ominaisuudet.

Prosessi	Ulkoinen puhdistus	AOX
Havupuu (1982–1984)		
Valkaisematon sulfaatti		
havupuumassa	Ei	0
(C95+D5)EHDED	Ei	n. 8
(C87+D13)EDED	Ilmastuslammikko	2,8
O(C83+D17)EDED	Ei	3,5
O(C85+D15)EDED	Ilm.lammikko (osittain)	3,25
O(C84+D16)EDED	Ilm.lammikko (pilotti)	2,25
O(C52+D48)EDED	Ei	2,5
Lehtipuu (1986)		
(D92+C8)(E+D)D(E+P)D	Ei	0,6
O(C82+D18)EDED	Ei	1,25
O(C51+D49)EDED	Ei	0,9
Vuonna 1990 tutkitut lehtipuumassat		
(C20+D80)(EOP)DED	Ei	1,7
"	Aktiivilietelaitos	1,0
O(D27,C68+D5)(EOP)D(EP)D	Ei	0,9
"	Ilm.lammikko (pilotti)	0,4

5 TUTKITTUJEN KOKONAISJÄTEVESIEN VAIKUTUSTEN YKSITYISKOHTIA

5.1 Käsittelemätön kokonaisjätevesi (D80+C20)(EOP)DED (Au)

Tämä jätevesi on lehtipuumassan tuotannosta aiheutuva kokonaisjätevesi ja jätevesivirtaus näytteenottohetkellä oli 43 m^3/tonni massaa. Jäteveden COD oli 90,3 kg/t, AOX 1,6 kg/t, kloraatti 5,8 kg/t, kloorifenoliset yhdisteet 1,8 g/t, hartsihapot 73,1 g/t ja kloorautuneet hartsihapot 4,7 g/t.

Jätevedellä todettiin seuraavia vaikutuksia malliekosysteemissä.

* Rakkolevä: suuremmassa laimennoksessa todettiin levän kokonais–määrän vähentymistä, ja pienemmässä laimennoksessa päinvastainen kokonaismäärän lisääntyminen vertailualtaisiin verrattuna. Rakkolevän vuosikasvussa ei todettu eroavuuksia.

* Rakkolevässä elävät selkärangattomat: suuremmassa laimennoksessa eläinten määrän ja kasvun pienentymistä ja pienemmässä laimennoksessa selvä eläinten määrän ja biomassan lisääntyminen. Nämä vaikutukset johtuvat pääasiassa simpukoissa todetuista muutoksista.

* Sedimentin pohjaeläimet: suuremmassa laimennoksessa eläinten määrän ja biomassan pienentymistä ja pienemmässä laimennoksessa päinvastainen eläinmäärän ja biomassan kasvu. Hallitsevina lajeina sinisimpukka, sydänsimpukka ja katkat.

* Selkärangattomien kokonaismäärä: edellisten perusteella suuremmassa laimennoksessa lievä inhiboiva vaikutus ja pienemmässä laimennoksessa stimuloiva

vaikutus kontrolliin verrattuna.

* Kolmipiikin kasvu: selvä kasvun lisääntyminen altistuksen jatkuttua 2 kuukautta kummassakin laimennoksessa.

* Konjugoituneiden yhdisteiden pitoisuudet kirjolohen sappi-nesteessä: annosvasteisuus todettavissa sekä kloorifenoleiden ja hartsihappojen osalta. Hartsihappojen määrä selvästi suurempi kuin kloorifenoleiden. Pitoisuudet yhtäsuuria kahden ja kahdeksan viikon altistuksen jälkeen.

* Veriparametrit: ei tilastollisesti merkitseviä eroja vertailuryhmään eikä annosvasteisuutta altistuksen aikana.

* Kirjolohen maksan aineenvaihdunta: maksan glykogeenipitoisuuden nousua lukuunottamatta ei muutoksia vertailukaloihin verrattuna.

5.2 Kokonaisjätevesi (C20+D80)(EOP)DED + aktiivilietelaitos (At)

Tämä jätevesi on lehtipuumassan tuotannosta aiheutuva jätevesi, joka on käsitelty aktiivilietelaitoksessa. Jätevesivirtaus oli näytteenottuhetkellä 43 m^3/tonni massaa ja jäteveden COD oli 39 kg/t, AOX 1,0 kg/t. kloraatti -/t, kloorifenoliset yhdisteet 0,6 g/t, hartsihapot 4,3 g/t ja kloorautuneet hartsihapot 2,1 g/t.

Jätevesi testattiin 400 (high dose) ja 2000 (low dose) kertaisina laimennoksina ja seuraavat vaikutukset todettiin:

* Rakkolevä: määrän selvä pienentyminen (> 20 %) kontrolliin verrattuna. Vuosikasvussa ei todettu vaikutuksia.

* Rakkolevässä elävät selkärangattomat: selvästi inhibitiivinen vaikutus sekä suuremmassa että pienemmässä laimennuksessa. Eläinten määrä ja biomassa olivat pienentyneet kokonaisuudessaan huolimatta siitä, että katkojen lukumäärä ja biomassa olivat lisääntyneet erityisesti pienemmässä laimennuksessa.

*Sedimentin pohjaeläimet: selvä inhibitiivinen vaikutus pohjaeläinten määrään ja biomassaan. Suuremmassa laimennuksessa (LD) oli kaikkien malliekosysteemialtaiden pienin pohjaeläimistö.

* Selkärangattomien kokonaismäärä: inhibitiivinen vaikutus.

* Kolmipiikin kasvu: selvä kasvun lisääntyminen altistuksen jatkuessa.

* Konjugoituneiden yhdisteiden pitoisuudet kirjolohen sappinesteessä: annosvasteisuus todettavissa sekä kloorifenoleiden että hartsihappojen osalta. Hartsihappopitoisuudet suurempia kuin kloorifenolipitoisuudet. Pitoisuudet pienentyneet jonkin verran 8 viikon altistuksen jälkeen.

* Veriparametrit: kypsymättömien punasolujen lievä lisääntyminen vertailuryhmän kaloihin verrattuna. Tilastollisesti merkittäviä eroja ei kuitenkaan esiintynyt kuten ei myöskään annosvasteisuutta.

* Kirjolohen maksan aineenvaihdunta: maksan glykogeenipitoisuuden nousua lukuunottamatta ei merkittäviä muutoksia vertailukaloihin verrattuna.

5.3 Käsittelemätön kokonaisjätevesi O(D27,C68+D5)(EOP)D(EP)D (Bu)

Tämä jätevesi on lehtipuumassan tuotannosta aiheutuva kokonaisjätevesi ennen ulkoista puhdistusta ja jätevesivirtaama näytteenottohetkellä oli 54 m^3/tonni massaa. Jäteveden COD oli 29,2 kg/t, AOX 0,90 kg/t, kloraatti 2,0 kg/t, kloorifenoliset yhdisteet 1,4 g/t, hartsihapot 9,5 g/t ja klooratut hartsihapot 0,5 g/t.

Jätevedellä todettiin seuraavia vaikutuksia malliekosysteemissä:

* Rakkolevä: määrän pienentyminen kummassakin laimennuksessa kontrolliin verrattuna. Vuosikasvussa ei todettu vaikutuksia.

* Rakkolevässä elävät selkärangattomat: vertailuryhmään verrattuna suuremmassa laimennuksessa lukumäärän ja biomassan lisääntymistä ja pienemmässä laimennuksessa vähentymistä. Eri lajien välillä huomattavia eroja.

* Sedimentin pohjaeläimet: suuremmassa laimennoksessa (HD) stimuloiva vaikutus ja pienemmässä laimennuksessa ei eroa vertailuryhmään.

* Selkärangattomien kokonaismäärä: suuremmalla laimennuksella lievästi stimuloiva vaikutus kun taas pienemmän laimennuksen vaiku-tus lievästi inhibi-tiivinen.

* Kolmipiikin kasvu: selvä kasvun lisääntyminen altistuksen jatkuessa.

* Konjugoituneiden yhdisteiden pitoisuudet kirjolohen sappinesteessä: annos-vasteisuus todettavissa sekä kloorifenoleiden että hartsihappojen osalta. Hartsihappo-pitoisuudet suurempia kuin kloorifenolipitoisuudet. Klooriguajakolien määrä lisääntynyt 8 viikon altistuksen jälkeen.

* Veriparametrit: veriparametreissa ei eroa vertailuryhmään verrattuna.

* Kirjolohen maksan aineenvaihdunta: maksan glykogeenipitoisuus kohonnut pienemmässä laimennuksessa. Muissa parametreissa ei todettu eroja vertailuryhmään.

5.4 Kokonaisjätevesi O(D27,C68+D5)(EOP)D(EP)D + ilmastettu lammikko (Bt)

Tämä jätevesi on lehtipuumassan tuotannosta aiheutuva jätevesi, joka on käsitelty ilmastetussa lammikossa. Jätevesivirtaus oli näytteenottohetkellä 54 m^3/tonni massaa ja jäteveden COD oli 17,3 kg/t, AOX 0,4 kg/t, kloraatti –/kg/t, kloorifenoliset yhdisteet 0,5 g/t, hartsihapot 0,5 g/t ja kloorautuneet hartsihapot 1,6 g/t.

Jätevesi testattiin 400 (high dose) ja 2000 (low dose) kertaisina laimennoksina ja seuraavat vaikutukset todettiin:

* Rakkolevä: määrän selvä pienentyminen suuremmassa laimennuksessa (LD), mutta pienemmässä laimennoksessa ei todettu muutoksia vertailualtaisiin. Vuosikas-vussa ei todettu vaikutuksia.

* Rakkolevässä elävät selkärangattomat: kummassakin laimennuksessa lievä stimulaatio sekä eläinten määrään että biomassaan.

* Sedimentin pohjaeläimet: eläinten määrässä todettavissa inhibitiivinen vaikutus,

mutta biomassaan selvä stimulatiivinen vaiku-tus. Pienemmässä laimennuksessa biomassan lisäys kontrolliin verrattuna oli suurin kaikista altisteuista mallieko-systeemialtaista.

* Selkärangattomien kokonaismäärä: stimuloiva vaikutus sekä eläin-ten lukumäärään ja biomassaan.

* Kolmipiikin kasvu: stimuloiva vaikutus altistuksen jatkuttua kaksi kuukautta.

* Konjugoituneiden yhdisteiden pitoisuudet kirjolohen sappinesteessä: annos-vasteisuus todettavissa sekä kloorifenoleiden että hartsihappojen osalta. Kloorifenolei-den ja hartsihappojen pitoisuudet samaa suuruusluokkaa ja yhtä suuria 2:n ja 8:n altistusviikon jälkeen.

* Veriparametrit: ei tilastollisesti merkittäviä eroja vertailukaloihin eikä annosvasteisuutta altistuksen aikana.

* Kirjolohen maksan aineenvaihdunta: maksan glykogeenipitoisuuden nousua lukuunottamatta ei merkittäviä muutoksia vertailukaloihin verrattuna.

5.5 Puuraaka-aineessa esiintyvät steroidit (mm. sitosteroli)

Tutkittujen jätevesien lisäksi suoritettiin kolmipiikki- ja kirjolohialtistus puuraaka-aineessa esiintyvällä steroidilla. Kolmipiikkialtistus kesti 5 kuukautta ja kirjolohialtistus 8 viikkoa. Altistuskokeen suorittamiseen oli useita syitä: testatut steroidit ovat hyvin kolesterolin kaltainen. Kolesteroli puolestaan on perusmolekyyli useiden steroidisten hormoonien synteesissä ja se on myös tarpeellinen solukalvon normaalille toiminnalle. Mikäli nyt tutkitun steroidin ja kolesterolin välillä tapahtuisi sekaannusta, nämä voisivat selittää jätevesien vaikutuksesta kaloissa todettuja vasteita.

Kolmipiikkejä altistettiin veden kautta altistuspitoisuuden ollessa aluksi 5 µg/l ja kahden altistuskuukauden jälkeen 10 µg/l. Kirjolohia altistettiin ravinnon kautta ja niille syötetty steroidi määrä oli kahden ensimmäisen viikon aikana 26 µg/yksilö ja seuraavien kuuden viikon aikana noin 45 µg/yksilö.

* Kolmipiikin kasvu: selvä stimuloiva vaikutus.

* Kolmipiikin histologia: maksan solujen rakkuloitumista. Vastaavaa todettiin tutkituille jätevesille altistetuissa kolmipiikeissä.

* Kirjolohen sappineste: kohonneet kolesteroli- ja puuperäisten steroidien pitoisuudet altistetuissa kaloissa.

* Veriparametrit: kypsymättömien punasolujen lievä lisääntyminen altistetussa ryhmässä kontrollikaloihin verratuna. Muiden veriparametrien kohdalle ei todettu merkitseviä eroja.

* Kirjolohen maksan aineenvaihdunta: maksan glykogeenipitoisuuden nousua lukuunottamatta ei merkittäviä muutoksia kontrollikaloihin verrattuna.

6 YLEISIÄ JOHTOPÄÄTÖKSIÄ

6.1 Ekosysteemi

Malliekosysteemin eri osissa todettiin sekä stimuloivia että inhiboivia vasteita. Näitä vasteita ja niiden suuntaa oletetaan säädeltävän biologisin mekanismein. Tämä olettamus perustuu osaksi havaintoihin altaista, joissa esiintyi runsaammin *Spirogyra* viherlevää. Tämän levän esiintyminen eri altaissa oli sattumanvaraista. Runsaammin viherlevää sisältävissä altaissa todettiin selkärangattomia eläimiä stimuloiva vaikutus huolimatta näiden altaiden altistumisesta suuremmalle määrälle potentiaalisesti haitallisia aineita verrattuna altaisiin, joissa viherlevää ei esiintynyt, mutta vaikutus selkärangattomiin oli inhibitiivinen. Sattumanvaraisesti esiintyvä *Spirogyra* viherlevä on siten osoitus biologisesta tekijästä, joka voi peittää ja naamioida sellujätevesien eliöstöön kohdistuvan inhibitiivisen vasteen.

Jätevesialtistukset aiheuttivat enimmäkseen levien kasvun lisääntymistä. Tämä osoittaa, että jätevesien mukana malliekosysteemeihin lisätyt ravinteet suosivat nopeasti kasvavia yksivuotisia leviä. Samanaikaisesti typpipitoisuus oli pienentynyt rakkolevissä, jotka siten eivät pysty kilpailemaan ravinteista kasvukauden aikana. Tämän perusteella voidaan olettaa, että nyt simuloiduissa olosuhteissa rakkolevän säilymiskyky murtovedessä on heikentynyt. Makeassa vedessä tapahtuvia vaikutuksia on vaikea ennustaa, mutta todennäköisesti fosfori on sisävesissä typen sijasta rajoittava tekijä.

Katkoissa todetut stimuloivat vaikutukset voivat johtua puuraaka-aineessa esiintyvistä hormooneja muistuttavista yhdisteistä, jotka ovat lisänneet muodonvaihdosten taajuutta. Muina kasvua lisäävinä tekijöinä voivat olla levätuotannon mukanaan tuoma käytettävissä olevan ravinnon määrä. Katkojen melko omaleimaisella vasteella saattaa olla vaikutusta muille rakkolevän seassa eläville eliöille. Esimerkiksi *Idotea*n kohdistui todennäköisesti inhiboiva vaikutus altistetuissa malliekosysteemeissä.

Kloorifenoli-, hartsihappo- ja AOX/EOX-pitoisuuksien ja todettujen vasteiden välillä ei havaittu riippuvuutta. EOX-määrä kuitenkin lisääntyi analysoiduissa malliekosysteemin eri osissa. Eliöiden kudoksista analysoitujen vieraiden yhdisteiden lisääntyvää pitoisuutta ravintoketjussa (biomagnifikaatio) ei havaittu. Lisäksi eliöistä mitattujen EOX-tasojen ja malliekosysteemeihin annostellun kokonais-AOX:n välinen riippuvuus oli vähäistä riippumatta siitä oliko annosteltava jätevesi käsittelemätöntä tai ulkoisesti puhdistettua. Annostellusta AOX:stä voitiin jäljittää vain vähäinen osa (noin 1 %) sedimentistä tai malliekosysteemin eliöstöstä.

Jätevesien altistus aiheutti erilaisia vasteita sekä kasveissa että eläimissä, ja näihin vasteisiin vaikuttivat suuresti lajien väliset vuorovaikutukset. Lisäksi saatiin viitteitä monimutkaisista mekanismeista, jotka säätelevät ravinteiden, mahdollisten myrkyllisten yhdisteiden kulkeutumista ja muuttumista. Tällaisten mekanismien tuntemuksen avulla voidaan paremmin ennustaa ja ennakoida sellujätevesien ympäristövaikutuksia. On myös ilmeistä, että monilajisten testimenetelmien käyttö on tarpeellista näiden tietojen hankkimiseksi.

6.2 Kalatutkimukset

Valkaistun lehtipuumassan tuotannosta aiheutuville jätevesille ja kasvisteroideille altistetuissa kolmipiikeissä todettiin vaikutuksia kasvussa, maksan histologiassa ja kaloissa esiintyvien loisten määrässä. Toistaiseksi on tuntematonta mikä merkitys kasvuun kohdistuvalla vaikutuksella on. Kenttätutkimuksissa todettuja lisääntymiseen liittyviä häiriöitä ja niiden toistettavuutta ei nyt tehdyillä tutkimuksilla ole voitu varmentaa. Näiden seikkojen selvittäminen ja varmentaminen vaatii vielä pitempi-aikaisia koko elämänkierron sisältäviä altistuksia.

Kahdeksan (8) viikon altistuksen jälkeen ei kirjolohen hematologisissa vasteissa eikä myöskään maksan MFO-entsyymeissa todettu eroja vertailuryhmän kaloihin verrattuna. Altistettujen kalojen aineenvaihdunnan häiriintymistä osoitti maksan glykogeenipitoisuuksien kohoaminen yhdessä maksakudoksen solujen rakkuloitumisen kanssa. Jätevesien samoin kuin steroidien kalojen ainenvaihduntaan kohdistuvia vaikutuksia osoitti myös sitoutuneen kolesterolin ja muiden steroidien lisääntyneet tasot sappinesteessä. Maksakudoksen akuutista vaurioitumisesta ei kuitenkaan ollut kyse, koska seerumista analysoitujen maksaentsyymien (ALAT) taso ei poikennut vertailukalojen vastaavasta tasosta.

Käsittelemättömien ja ulkoisesti puhdistettujen jätevesien sekä steroidien kaloihin aiheuttamien vaikutusten välillä oli hyvin pieniä eroja tai niitä ei esiintynyt lainkaan. Tämä osoittaa, että eri altistetuilla kalaryhmillä on yhtenevät vastemekanismit.

Käsittelemättömät jätevedet altistivat kaloja hieman enemmän kuin käsitellyt (ilmastettu lammikko, aktiivilietelaitos) jätevedet, kun altistuminen arvioitiin sappinesteessä esiintyvien konjugoituneiden kloorifenoli- ja hartsihappopitoisuuksien perusteella. Kaikista altistusryhmistä mitatut konjugaattipitoisuudet olivat kuitenkin vain vähän luonnosta tavattavia tausta-arvoja suurempia. Kloorifenoli- ja hartsihappo-konjugaattien ja todettujen vaikutusten välistä riippuvuutta ei siten voida esittää.

Jätevesien AOX-pitoisuuden ja todettujen vaikutusten välillä ei ollut riippuvuutta.

Koska tutkitut jätevedet ja steroidit aiheuttuivat samanlaisia vaikutuksia kalojen maksan histologiaan, sapen steroidipitoisuuksiin sekä kalojen kasvuun, pidämme tärkeänä, että tulevissa tutkimuksissa paneudutaan steroidien sekä niiden kaltaisten kloorautumattomien puuraaka-aineessa esiintyvien luonnollisten yhdisteiden vaikutusten arvioimiseen.

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