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Micr ological tests and measurements in the asses ent of harmful substances and pollution

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Typesetted in the Editorial Office of the Finnish Zoological and Botanical Publishing Board.
Printed in Vammalan Kirjapaino Oy, P.O. Box 92, FIN-38201 Vammala, Finland.

MONOGRAPHS OF THE BOREAL ENVIRONMENT RESEARCH

22

Jukka Ahtiainen

Microbiological tests and measurements in the assessment of harmful substances and pollution

Yhteenveto: Mikrobiologiset testit ja mittaukset kemikaalien ja ympäristön saastumisen riskin arvioinnissa

FINNISH ENVIRONMENT INSTITUTE, FINLAND
Helsinki 2002

The publication is available in the internet:
http://www.ymparisto.fi/eng/orginfo/publica/electro/mb22/mb22.htm

ISSN 1239-1875
ISBN 952-11-1205-0
ISBN 952-11-1206-9 (PDF)
Vammalan Kirjapaino Oy
Vammala 2002, Finland

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I Ahtiainen, J., T. Nakari, M. Ruoppa, M. Verta and E. Talka. 2000. Toxicity screening of novel pulp mill wastewaters in Finnish pulp mills. In: Persoone et al. (eds). *New Microbiotests for Routine Toxicity Screening and Biomonitoring*. Kluwer Academic. New York.pp. 307–317.

II Ahtiainen, J. and K. Mattsson. 2002. The long term effects of novel pulp bleaching effluents on aquatic microbiological processes in a mesocosm study. In: Struthridge et al. (eds.) *Aquatic Impacts of Pulp and Paper Effluents*. SETAC Books (*In press*).

III Laine, M., J. Ahtiainen, N. Wagman, L.G. Öberg and K. Jörgensen. 1997. Fate and Toxicity of Chlorophenols, Polychlorinated Dibenzo-p-dioxins, and Dibenzofurans during Composting of Contaminated Sawmill Soil. *Environ. Sci. Tech*. 31: 3244–3250.

IV Ahtiainen J., M. Järvinen, A. Joutti. and R. Valo 2002. Microbial toxicity tests and chemical analysis as monitoring parameters at composting of creosote contaminated soil. *Ecotox. and Environ. Safety. 53 (In press)*

V Ahtiainen J., A. Myllymäki and P. Vanhala 2002. The effects of different plant protection programmes in cereal fields on soil microbes. *Ecotox. and Environ. Safety. (Accepted for publication)*

VI Ahtiainen J., M. Aalto and P. Pessala. 2002. Biodegradation of chemicals in a standardized test and in environmental conditions. *Chemosphere*. *(Manuscript submitted)*

Author's contribution to the papers:
I J. Ahtiainen was responsible for writing the paper, for the most of the toxicity testing and participated in the project planning. The data was jointly managed and analysed by J. Ahtiainen and M. Verta.
II J. Ahtiainen planned, sampled and analysed the microbiological activity measurements in the mesocosms. The data was jointly analysed with K. Mattsson and the paper was written by J. Ahtiainen.
III J. Ahtiainen was responsible for the toxicity testing, toxicity data analysis and for writing on the toxicity assessments jointly with M. Laine.
IV J. Ahtiainen initiated, planned and carried out the study jointly with R. Valo and M. Järvinen. J. Ahtiainen wrote the paper, which was commented by R. Valo.
V J. Ahtiainen planned and performed the study jointly with A. Myllymäki and P. Vanhala. The data was analysed jointly with A. Myllymäki and the paper was written by J. Ahtiainen.
VI J. Ahtiainen planned the study and wrote the paper, and performed the work with the technical assistance of M. Aalto and P. Pessala.

List of abbreviations

AHCs	Aromatic hydrocarbons
AOX	Absorbable organic halogen
APHA	American Public Health Association
ATP	Adenosine 5'-triphospate
BOD	Biological oxygen demand
CEN	European Committee for standardization
COD	Chemical oxygen demand
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturating gradient gel electrophoresis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSM	Deutche Sammlung die Microben
EC50	The concentration of chemical, effluent or soil elutriate that causes defined toxic effect to 50% of the test organisms, or 50% inhibition of measured activity compared to the control
ECF	Elementary Chlorine Free bleaching
EPPO	European and Mediterranean Plant Protection Organisation
EROD	Ethoxyresorufin *o*-de-ethylase
EU	European Union
FE	Fumigation extraction method for microbial biomass
FDA	Fluorescein diacetate
FI	Fumigation incubation method for microbial biomass
GC	Gas chromatography
GLP	Good Laboratory Practise
GMO	Genetically modified organism
HELCOM	Helsinki Commission for Baltic marine environment protection
HPLC	High performance liquid chromatography
IPPC	Integrated Pollution Prevention and Control (directive 96/61/EU)
ISO	International Organisation for Standardization
LOEC	Lowest effective concentration of chemical or effluent in toxicity tests
MAD	Mutual acceptance of data
MCPA	2-Methyl-4-chlorophenoxyacetic acid
MFO	mixed-function oxidase
NOEC	Highest concentration of chemical or effluent which causes no effect in toxicity tests
NADH	Nicotinamide adenine dinucleotide
OECD	Organisation for Economic Co-operation and Development
OSPAR	Oslo and Paris Convention for the Protection of the Marine Environment of the North East Atlantic
PAHs	Polynuclear aromatic hydrocarbons
PCP	Pentachlorophenol
PCR	Polymeric chain reaction
PME	Phosphomonoesterase
PNP	p-nitrophenyl
PNP-P	p-nitrophenyl phosphate
STU	Soil Toxicity Unit is a toxicity value for soils and soil elutriates (STU = 1/EC50 x 1000)
SFS	Finnish Standardization Assosiation
TCA	Trichloroacetic acid
TCF	Totally Chlorine Free bleaching
TEF	Toxicity emission factor
TER	Toxicity emission rate
TGD	Technical Guidance Document for the risk assessment of chemicals
TIE	Toxicity identification and evaluation
TOC	Total organic carbon
TRE	Toxicity reduction evaluation
TU	Toxicity Unit is a toxicity value for effluents (TU = 1/EC50 x 100)
WHC	Water holding capacity of soil

Microbiological tests and measurements in the assessment of harmful substances and pollution

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Ahtiainen, J. 2002. Microbiological tests and measurements in the assessment of harmful substances and pollution, Monographs of the Boreal Environment Research No. 22, 2002

New chemicals are produced in increasing numbers. In Finland every year about 28 000 different products are manufactured or imported which can be classified as harmful. These products contain about 5000 different harmful substances. We also receive harmful compounds in airborne emissions. Substances are further transformed in industrial processes, in waste management and in the environment by human activities and natural processes. However, only rather limited monitoring data is available about the environmental concentrations of these compounds (concerning about 20–40 substances). The environmental risks of harmful substances can be recognized, assessed and managed by : 1) measuring concentrations in the environment, 2) testing the effects on biota in toxicity tests and 3) monitoring ecosystem changes in the environment. Microbes have a key role in the environment as degraders in the carbon and nutrient cycles. They also have the capability to transform and degrade many harmful man-made substances. Microbes have a strong effect on the exposure of other biota to chemical substances. Hence they can be regarded as primary targets for harmful effects of chemicals or active transformers of chemicals in the environment.

The objective of this study was to evaluate the usefulness and applicability of microbial toxicity tests, biodegradation tests and microbial biomass and activity measurements in the environment for the environmental hazard identification and risk assessment of chemicals, effluents and contaminated soils. The study considers both aquatic and soil environments. The effects of modern pulp mill bleaching effluents on biota were assessed by a set of different biotests and by chemical analysis. The effluents and their long term effects on aquatic microbial processes were also studied in outdoor mesocosms. In one part the biodegradation kinetics of carbon-14-labeled model compounds in standard tests and in environmental conditions were measured and compared. The effects of several pesticides on soil microbes were tested and assessed in laboratory tests and in field trials. The applicability of microbial toxicity tests in the assessment of soil pollution and bioremediation processes was also studied in comparison with chemical analysis. The results of the study can support the development and selection of test methods for environmental risk management and regulatory decisions.

Keywords: Microbes, toxicity, biodegradation, tests, chemicals, pesticides, effluents, pollution, soil, sediments, activity, biomass

1 Introduction

1.1 Background

The increasing number and volume of various chemical substances used by industry, agriculture, households and other everyday activities poses a challenge to assess the environmental risks of this chemicalization. About 28 000 chemical preparations which can be classified as harmful to the environment are imported to or produced in Finland. These preparations contain 5000 harmful substances, many of which are used intentionally to satisfy the demands for production. The substances introduced in our society in various goods end up in the environment both during and after use. We also receive persistent compounds in global airborne emissions. These chemicals can cause environmental risks to human or other biota when they reach a critical concentration, which may be very low for certain substances.

Some harmful compounds are produced as by-products or through environmental transformations, often as a result of microbial activity. Microbes can also enhance environmental pollution by transforming substances to more toxic, bio-accumulative and bio-available forms, for example by methylating mercury in lake sediments, water (Matilainen 1995) and soil (Verta *et al.* 1994, Rudd 1995) or by the reduction of arsenate As (V) to more mobile arsenite As (III) (Macur *et al.* 2001). Certain fungi might be potent producers of the atmospheric ozone-layer decreasing chloromethane (CH_3Cl) (Harper *et al.* 2001). However, microbes also have a key role in the environment as degraders of harmful compounds. Hence microbial populations have a remarkable effect on the exposure of other biota to chemical substances. Microbes can be either targets (soil fertility loss, food web malfunctioning) or active biodegraders and transformers when they are exposed to chemicals.

The environmental risks of harmful substances and pollution can be recognized, assessed and managed by measuring concentrations of the compounds in the environment, by testing the effects of chemicals and pollution on biota using toxicity tests, and by monitoring ecosystem changes in the environment (Fig 1).

Chemicals management, risk assessment, labelling and possible restrictions are based on chemicals testing, on measured data about their existence in the environment and on possible recorded detrimental effects in ecosystems. There are continuing international efforts to harmonise these strategies globally at United Nations (UN) level, in the Organisation for Economic Co-operation and Development (OECD) and in a more structured way in the European Union (EU) member countries. OECD provides the test guidelines, strategies and principles for testing the various properties of chemicals (OECD 1993). These internationally developed, harmonised and validated test methods cover the testing of physical and chemical properties, effects on biotic systems, biodegradation and accumulation and human health effects. To rationalise the testing efforts and to avoid unnecessary testing, it has been agreed that if a chemical is tested in any OECD member country according to OECD guidelines in a laboratory with Good Laboratory Practice (GLP) quality assurance status, the test data should generally be accepted in all the member countries (OECD 1981a). This Mutual Acceptance of Data (MAD- principle) sets high demands for quality and ecological relevance to the test guidelines development.

The methods for environmental analysis are harmonised internationally for water, sediment, soil and waste in the International Standardization Organisation (ISO). They include chemical analysis, toxicity and biodegradation testing, microbiological quality analysis and other biological methods for ecological quality monitoring. The use of ISO standard methods and in EU member countries usually the corresponding European standards (EN) is sometimes mandated by EU directives (for example the Water Framework Directive 2000/60/EU) or recommended by international conventions such as the Oslo and Paris Convention for the Protection of the Marine Environment of the North East Atlantic (OSPAR) and the Helsinki Commission for Baltic marine environment protection (HELCOM).

1.2 Toxicity testing within a triad of techniques

The use of toxicity tests as a complementary tool and not just a substitute for chemical analyses and biological surveys has been formalized in studies of aquatic sediments such as the Sediment Quality Triad (Chapman 1986). This strategy allows a combined "weight-of-evidence" interpretation of possible environmental impacts based on all three

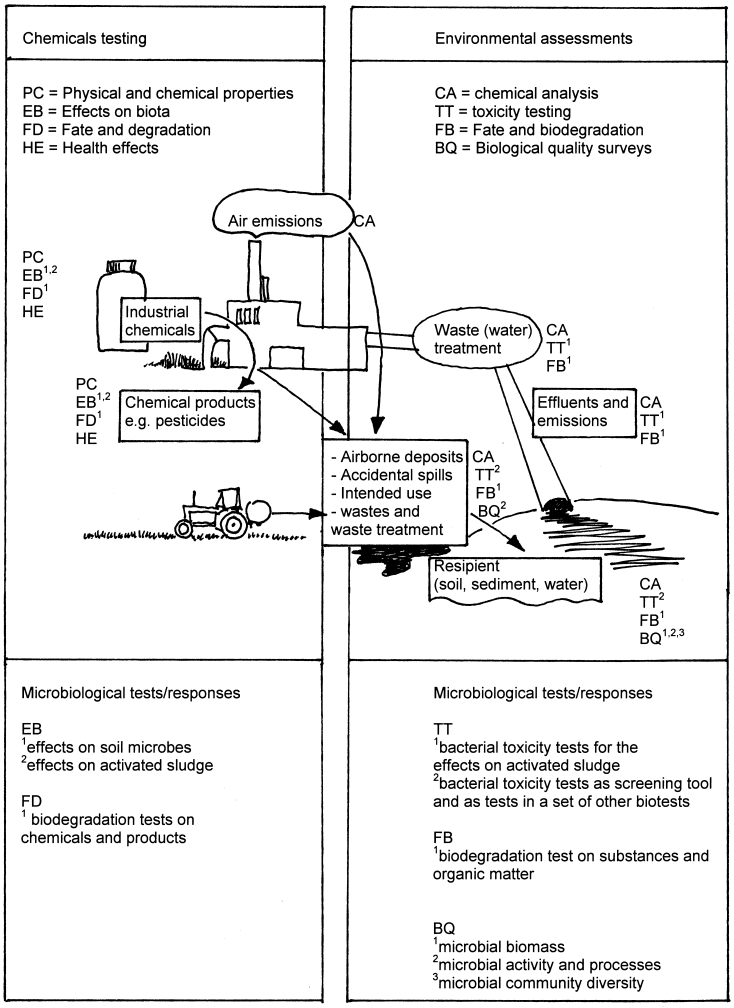

Fig. 1. Conceptual framework and tools for the environmental risk management of harmful substances and the role of microbial tests and responses in the environment.

techniques (Sergy 1987). This could be applied equally and respectively to water, sediment and soil (Chapman 1996, Brohon *et al.* 2001).

1.2.1 Chemical analyses

Using chemical analyses we can analyze certain compounds in the environment or in the effluent which are the probable causes of toxicity. Chemical analysis can predict whether toxic or detrimental effects are likely to occur because of the compounds analyzed, but it cannot provide a proof other of the effects. Chemical analyses can seldom provide information about bioavailability, effects on biota, antagonism or cumulative effects of complex mixtures. They have been widely and traditionally used for regulatory purposes with specified limit values for substances, and they can also be used predictively to calculate expected environmental concentrations. Chemical analyses are also important for environmental monitoring of known harmful substances.

But do we know all the substances we should analyse for and are the analyses technically or economically feasible? The more complex our efflu-

ents are, the more probable is the failure to carry out analyses for the most important substances, if it is not even known what they are. It has been calculated that we might only have identified 10 % of the organic compounds in our surface waters (Galassi *et al.* 1992). The compounds are not stable and they are degraded and transformed by environmental conditions and microbes. The reference to "pure ignorance" about organic substances or more precisely "the ignorance of which we are not even aware"(Blumer 1975), is not without justification. Clearly chemical analyses must be supplemented with other kinds of information in environmental risk assessment.

1.2.2 Toxicity tests

Toxicity tests indicate whether a sample of chemical, waste, effluent, sediment or soil causes a toxic effect on biota. They can provide information about harmful effects of unknown substances.

The advantage of biological toxicity tests over chemical analysis is the direct assessment of the potential biotic impact without the need for extrapolation from the individual impacts of the different chemical constituents. Observed toxicity can be an early warning or even prediction of potential environmental damage. It also takes into account the effects of toxicant mixtures. Toxicity tests can be regulatory if a certain limit of toxicity is set, like the dilution based G- (Giftigkeit) or LID- (Lowest Ineffective Dilution) values in Germany (Steinhäuser 1995).

An important limitation of toxicity tests is that identification of the agent of risk is difficult.

Traditional biotests have been slow and laborious, but technical improvements (automation and scaling down of size) are making them more feasible for routine analysis. They have also been considered to lack the ecological relevance. Only a few species are tested, out of many present in the ecosystems. The other stressors (extreme temperature, water current, predators, disease) are not active in the laboratory tests. Simplistic use of the results might mislead the risk assessment.

However, the exposure of aquatic and soil biota to environmental chemicals, either as individual compounds or as complex effluents, may result in direct (killing, reproduction failure) or indirect effects (food web changes) which cannot be realistically assessed by even very complex model test systems with all possible target species. Often various single species screening tests are employed to detect the possible harmful effects of effluents on biotic systems. Although in the aquatic environment different species and complex food webs are exposed to effluents, the actual toxic impact is confined to generally a few relevant targets at the bio-molecular level. The functional units (respiration, enzyme functions, membrane transport) are often similar in different species. Hence single species tests and rapid *in vitro* screening tests could also be used to screen and classify the potential harmful effects of chemicals and effluents (Wenzel *et al.*1997). In order to simulate the aquatic food chain/web, representatives of different trophic levels (algae, daphnids and fish) are often used as tests organisms.

1.2.3 Biological surveys and environmental assessments

The biological survey of the ecological quality in a given environment is a definitive check on whether an ecosystem has been affected. Biological quality objectives and standards are being set in Europe in the implementation of the EU Water Framework Directive. A biological survey cannot serve in predictive mode (the harm is already done). Sometimes the responsible emission is difficult to identify and verify without toxicity data and chemical analysis of the possible toxicants even if all the other epidemiological principles are applied (Munkittrick and McCarty 1995). All three feet (concentration of the substance, proven toxicity and change in biota) are needed for the table to stand.

1.3 Basic concepts of ecotoxicology

In all environmental toxicity tests three aspects must be considered: the organism exposed, the medium for exposure (e.g. soil, sediment, water) and the measured characteristics (so-called "toxicity endpoint"). The endpoint is assessed or calculated by comparing the effects caused by the tested material to the effects found in control organisms in identical test conditions without the tested substance. In the most common test design the tested material is diluted in the test medium to give a series of concentrations. The degree of toxicity of

the test material can be estimated from the dose-response curve, providing an estimate of the effective concentration (EC). The EC50-value, generally means the dilution or concentration of the tested substance, at which 50 % inhibition of the measured characteristics are observed compared to the unexposed control.

Depending on the test duration, conditions and organisms the tests can be considered as acute, sub-chronic or chronic. Chronic tests usually cover 10 % or more of the organism's life span (Sergy 1987). Death, growth, reproduction, enzyme activity, mobility or other activities are used as endpoints in the toxicity tests. There are test designs based on a single strain of one organism and also tests which apply to whole communities. The selection of a single strain or a community has a major effect on the sensitivity of the test, especially when using microorganisms. For example in the growth tests the community diversity changes can sometimes mask the toxicity which could possibly be observed by using only one sensitive microbial strain of at a time.

The bioavailability and actual concentration of the tested compound or material in the test systems is of crucial importance. The test medium (water, sediment or soil) and its characteristics determine the bioavailability and sometimes also the toxicity of the tested material. For example the hardness, buffering capacity and pH of water can have a strong effect on the toxicity of a substance. In the soil studies the organic carbon content or clay content can affect the bioavailability of many substances. In the chronic test with longer duration various, flow-through or semistatic test designs must be applied. The possible biodegradation or adsorption to the test vessel or to solid particles must also be considered and often the actual measured concentration of the tested substance can differ remarkably from the nominal concentration used in the test.

As with any analysis, quality assurance in the test design is important. It is most important to validate the test system every time with positive control such as reference toxicant and materials and also to use negative controls in order to be able to check the normal functioning of the test organism.

1.4 Microbiological toxicity tests

Here, microbiological toxicity tests are regarded as test designs using defined bacterial strains, mixed populations of environmental bacteria and to some extent unicellular algae. Bacteria and yeasts are also used widely as test organisms for genotoxicity and mutatoxicity testing. Some protozoan tests and tests using organelles (e.g. mitochondrial particles) resemble microbial tests but these tests are not reviewed here.

Various bacterial bioassays have been developed for toxicity screening of water, wastewater, sediment elutriates and soil extracts (Table 1). In some recent tests solid samples (sediment, soil) can also be tested directly without any extraction (Table 2). Three microbial tests have been developed and harmonized by OECD for testing the effects of chemicals, especially pesticides (Table 3). Most of the tests are based on measurements of growth inhibition, respiration, mineralisation or metabolism, enzyme activity and viability of bacterial cells. In the growth inhibition tests the cell multiplication is usually monitored by optical density measurements (ISO 10721, 1995 and ISO 15522, 1999) or by cell counting or fluorometry in algal tests (ISO 8692, 1986 and ISO 10253, 1998). The respiration tests are based on carbon dioxide production or oxygen consumption during the tests (ISO 8192, 1986). The mineralisation of added material containing organic nitrogen and production of metabolites is used for example in tests measuring nitrification (ISO 9509, 1989 and OECD TG 216, 2000). Several microbial enzyme activities can be measured directly by luminescence measurements (ISO 11348, 1998) or by specific substrate utilization and colour development (Reinharz *et al*. 1987). In one case the active movement of flagellated bacteria is used as an assessed characteristic (Bowdre and Krieg 1974). In anaerobic tests the production of gases is measured simply as pressure changes during the test (ISO 13641, 2001).

The cell walls of bacteria and other organelles differ to some extent from those of higher organisms (Bitton and Koopman 1992). Hence in some cases the ecological relevance of extrapolating the effects to other organisms might be limited. Especially the exposure routes and metabolism of harmful substances are sometimes unique to bacteria. The difference between gram positive and gram negative bacteria should also be considered.

Table 1. Microbial tests used to assess the toxicity of water, wastewater and extracts or elutriates of sediments, soils and wastes.

Test	Organism(s)	Principle	Standardization	Applications	Reference
Luminescent bacteria test	*Vibrio fischeri*	Inhibition of luminescence	ISO SFS-EN 11348	water, wastewater, effluents, elutriates	ISO 1998
Escherichia coli luminescence inhibition test	GMO *E.coli*	Inhibition of luminescence in GMO *E.coli*		water, wastewater	Lampinen *et al*. 1992
Bacillus subtilis luminescence inhibition test	GMO *B. subtilis*	Inhibition of eukariotic luminescence in GMO *B. subtilis*		water, wastewater	Lampinen *et al*. 1992
ATP-TOX test	*Pseudomonas fluorescence Salmonella typhimurium E. coli*	Inhibition of growth and luminescence		water, wastewater sediment extracts	Xu & Dutka 1987
Rapid automated bacterial impedance test	mixed population	Inhibition of production of ionizising compounds by bacteria		water, wastewater soil extracts	Haig & Rennie 1994
Spirillum flagella test	*Spirillum volutans*	Inhibition of flagellated bacteria movement		water samples	Bowdre & Krieg 1974
Activated sludge respiration inhibition test	activated sludge	Oxygen consumption inhibition in activated sludge	ISO 8192	water, wastewater, water constituents	ISO 1986
Polytox respiration inhibition test	mixed culture inhibition	Oxygen consumption		wastewater	Elnabawary *et al.*1988
Activated sludge nitrification inhibition	activated sludge	Inhibition of nitrate production from ammonium salts by activated sludge	ISO 9509	water, wastewater, water constituents	ISO 1989
Dehydrogenase activity inhibition test (ECHA)	*E. coli*	Inhibition of dehydro-genase activity		water, wastewater	Dutka &Gorrie 1986
β-galactosidase activity inhibition tests: MetPAD, MetPLATE	*E. coli*	Inhibition of β-galactosidase activity		water, wastewater	Bitton *et al*. 1992 Bitton *et al*. 1994
FDA hydrolysis test		Inhibition of the hydrolysis of fluoreseine-di-acetate by several enzymes		soil elutriates	Haig & Rennie 1994
β-galactosidase induction inhibition test (Toxi-Chromo)	*E.coli*	Inhibition of production of β-galactosidase		water, wastewater	Reinharz *et al*. 1987
α-galactosidase induction inhibition test	*Bacillus licheneformis*	Inhibition of production of α-galactosidase		water, wastewater	Dutton *et al.* 1990a
Inhibition of activity of anaerobic bacteria (high biomass)	anaerobic digested sludge	Inhibition of anaerobic gas production (pressure measurement)	ISO 13641-1	wastewater, water constituents	ISO 2001
Inhibition of activity of anaerobic bacteria (low biomass)	anaerobic environment bacteria	Inhibition of anaerobic gas production (pressure measurement)	ISO 13641-2	wastewater, water constituents	ISO 2002
Pseudomonas putida growth inhibition test	*P. putida*	Cell multiplication inhibition (biomass)	ISO 10712	water, wastewater, effluents, elutriates	ISO 1995
Pseudomonas fluorescens growth rate inhibition test	*P. fluorescence*	Cell multiplication inhibition (rate)		water, wastewater	Paran *et al*. 1990
Activated sludge growth inhibition test	activated sludge	Cell multiplication inhibition of activated sludge organisms	ISO 15522	wastewater	ISO 1999 constituents

Table 2. Solid phase microbial toxicity tests used for sediment and soil testing.

Test	Organism	Principle	Standardization	Applications	Reference
Microtox Solid Phase	*Vibrio fischeri*	Inhibion of luminescence after exposure and filtration		soil, sediment	Tung *et al*. 1990
Direct contact luminescence bacteria test	*Vibrio fischeri*	Inhibition of luminescence after exposure and centrifugation		sediment	Brouwer *et al.* 1995
Luminescent bacteria flash test	*Vibrio fischeri*	Kinetic measurement of luminescence inhibition during exposure		soil, sediment, wastes	Lappalainen *et al*. 2001
Toxi-Chromo Pad test	*Escherichia coli*	β-galactosidase synthesis Inhibition after/during exposure		sediment, soil	Kwan 1995
B. cereus contact test	*Bacillus cereus*	Inhibition of dehydrogenase activity		sediment, soil	Rönnpagel *et al*. 1995

Table 3. Internationally harmonised tests used for testing the effects of chemicals on microbes.

Test	Organism(s)	Principle	Standardization	Applications	Reference
Carbon mineralization test in soil	Indigenous microbes in soil	The degradation of added organic material in soil	OECD TG217	pesticide and chemical testing	OECD 2000
Nitrogen mineralization test in soil	Indigenous microbes in soil	The degradation and nitrogen mineralisation from added organic material in soil	OECD TG216 ISO 14238	pesticide testing chemical testing	OECD 2000 ISO 1997
Activated sludge respiration inhibition	Activated sludge	Oxygen consumption inhibition in activated sludge	OECD TG209	chemicals	OECD 1984

The outer membrane of gram negative bacteria is an effective barrier to hydrophobic substances (Bitton and Koopman 1992). The permeability of the outer membrane can be enhanced by lyophilisation or by other means (Dutton *et al.* 1990)

Because of the small size, short multiplication times and easily measurable reactions the development of microbial tests is towards microscale and automated testing, on-line systems and field measurements (Blaise *et al*. 1998).

1.4.1 Tests for waters, effluents and aqueous solutions

Microorganisms, particularly bacteria, are attractive for use in wastewater toxicity testing because microbial tests are simple, rapid, sensitive and inexpensive (Bitton and Dutka 1986). By using bacterial toxicity tests it is also possible to estimate the toxic impacts of different, often fluctuating, untreated wastewaters on activated sludge wastewa-

ter treatment processes. Most modern wastewater treatments rely on microorganisms, and therefore their activity is of primary importance to the proper functioning of the treatment process.

Today the most common microbiological test in effluent toxicity assessments is the luminescent bacteria test (ISO 11348, 1998). Since the first studies on the use of luminescent bacteria for toxicity assessment of air pollutants by Serat *et al.* (1965), several test systems for routine use have been developed. The inhibition of light production by *Vibrio fischeri* bioluminescent bacteria indicates disturbance of the energy metabolism. The luminescent measurement assesses the metabolic status of this bacterium (Hastings 1978) and the change in bacterial bioluminescence after exposure to wastewater samples can be used as an indicator of potential toxicity (Bulich *et al.* 1981). A limitation of the use of marine bacteria such as *V. fischeri*, is the required high NaCl concentration (2 %) which is claimed to have effects on the bioavailability of certain compounds (Ribo and Kaiser 1987). One possible solution could be to use other solutes such as saccharose to balance the osmotic pressure during the test (Hinwood and McGormick 1987). Lampinen *et al.* (1990, 1992) developed genetically modified luminescent bacterium from *Escherichia coli* and *Bacillus subtilis* to overcome certain problems with natural luminescent bacteria used in toxicity testing. Luminescent bacteria test kits are also commercially available (for example Microtox ^{TM}, Microbics Co., Carlsbad, CA, USA; Lumistox^{TM}, Dr. Lange GmbH, Berlin, Germany or Biotox^{TM}, Aboatox co., Turku, Finland).

Another standardized test for aqueous solutions is the *Pseudomonas putida* growth inhibition test (ISO 10712, 1995). The bacterium *P. putida* is a common aquatic heterotrophic microorganism. When cultured under specified conditions, in a defined medium with different concentrations of wastewater over several generations, toxic substances present in the wastewater sample can inhibit the cell multiplication of this bacterial species (Bringmann and Kühn 1977). Inhibition of the growth of activated sludge microorganisms is also used as a measured characteristic in a standardized test (ISO 15522, 1999). Growth inhibition tests of *Pseudomonas fluorecence* has been published by Paran *et al.* (1990).

In the activated sludge respiration inhibition test (ISO 8192, 1986), the oxygen consumption of the activated sludge supplemented with glucose is measured for four hours with an oxygen probe (King & Painter 1986). The nitrification potential of the activated sludge microorganisms can be assessed and inhibition of nitrate production from added ammonium salts can be measured (ISO 9509, 1989). The only standardized anaerobic microbial toxicity test (ISO 13641, 2001) is based on simple pressure measurements (anaerobic gas production) in sealed vessels.

The inhibition of enzyme activity or the inhibition of enzyme induction and *de novo* synthesis is assayed in some test procedures. The inhibition of *Escherichia coli* dehydrogenase activity is measured in a rapid ECHA screening test (Dutka and Gorrie 1989) and the inhibition of β-galactosidase activity in MetPAD (Bitton *et al.* 1992) and MetPLATE (Bitton *et al.* 1994). Inhibition of the hydrolysis of fluorescein diacetate (FDA) by several enzymes (proteases, lipases and esterases) and the measurement of fluorescence is applied in a test procedure presented by Haig and Rennie (1994). The inhibition of enzyme induction and *de novo* synthesis are considered to be more sensitive phenomena than the inhibition of enzyme activity (Dutton *et al.* 1988) or other metabolic activity in bacterial cells (Reinhartz *et al.* 1987). The Toxi-Chromo- test is based on inhibition of the activity of the lac-operon and of synthesis of β-galactosidase enzyme (Reinhartz *et al.*1987).

Inhibition of the induction of α-glucosidase in *Bacillus licheneformis* has also been reported to be especially sensitive to hydrophobic substances (Dutton *et al.* 1990). The greater sensitivity is caused by the difference of the cell wall of this gram-positive bacterium compared to *E. coli*.

The growth of unicellular micro-algae has commonly been used in toxicity testing of wastewaters, sediment elutriates and chemicals. These test designs can be regarded as microbial tests and the growth kinetics mainly follow the microbial exponential growth models. When exponentially growing cultures of green alga are exposed (for several generations) to various dilutions of a wastewater sample, the substances in the wastewater can either inhibit or stimulate the algal growth. Standardized test protocols exist for effluent testing with the freshwater species *Pseudokirchinella subcapitata* (former *Selenastrum capricornutum*) in ISO standard 8692 (1989) and with marine species *Sleletonema costatum* and *Phaeodactylum tricornutum* in the standard ISO 10253 (1995). The inhibition of

growth rate and biomass production are the measured characteristics. The tests are based on direct cell counts by automated or microscopic counting, or on measurements of chlorophyl production by spectrometry or direct fluorometry on microwell plates (Thellen *et al.* 1989, Mayer *et al*. 1997). Test designs using a set of algal species have also been published (Blanck *et al*. 1984).

1.4.2 Tests for solid samples

Often the toxicity assessment of solid samples (soil, waste or sediment) is based on the testing of aqueous elutriates or other extracts. Different solvents such as dimethyl sulfoxide (DMSO), methanol, ethanol and acetone can be used for extraction of organic contaminants (Kwan and Dutka 1990). One problem when using solvents is the possible toxicity of the solvent itself. Hence the maximum allowable concentration of the extract solvent in the test must first be measured (Brouwer *et al*. 1990) and the possible synergistic interactions between contaminants must also be considered (Gunkel *et al.* 1993). On the other hand aqueous extraction could underestimate the bioavailability of contaminants and hence their toxicity (Harkey *et al*. 1994). Aqueous extraction could be selective in multiple compound contamination in solid samples, due to the different solubilities of especially organic compounds in water (Ongley *et al*. 1988). The use of assays employing direct contact of test organisms with the sample could better assess the bioavailability and toxicity of the contaminants in solid samples (Rönnpagel *et al*. 1995). During the past decade several bacterial contact procedures have been developed to test the toxicities of soil, sediment and waste samples. The luminescent bacteria test could be performed either with soil water elutriates or with fresh homogenized soil samples with solid phase modification of the standard test (Brouwer *et al*. 1990). In this procedure the bacterial cells were exposed to solid samples and the solid particles were separated from the exposed bacteria by centrifugation before the luminescence measurement. The commercial Microtox ^{TM} Solid Phase (Microbics co., Carlsbad, CA, USA) used filtration as the separation technique of solids from exposed cells prior to luminescence measurement (Tung *et al*. 1990).

A novel possibility could be a kinetic modification of the luminescent bacteria test that could be used to assess soil or sediment samples (Lappalainen *et al*. 1999). In this method a kinetic measurement of the luminescence signal was started at the same time as the bacteria were added and mixed to the eluated sediment or soil sample. Luminescence was measured throughout the 30 s exposure and the possible luminescence inhibition was compared to the initial peak luminescence at the start of the test. This should also help to avoid some of the colour, turbidity or adsorption interference.

Kwan and Dutka (1992) applied the β-galactosidase enzyme synthesis inhibition test with *E.coli* (Toxi-ChromoPad test) to solid sediment samples. The direct contact application proved to be more sensitive than testing the sediment extracts.

The toxicities of soils and sediments could be tested directly and with high sensitivity with the procedure presented by Rönnpagel *et al*. (1995). They used the bacterium *Bacillus cereus* as test organism assessing the inhibition of dehydrogenase activity measured by rezasurin reduction (Liu and Strachan 1981*). B. cereus* is a common soil bacterium, has high affinity to soil particles and as a gram-positive organism its cell wall might be more permeable to hydrophobic organic compounds than the cell wall of the gram-negative bacteria used in most tests.

1.4.3 Testing of the effects of chemicals on microbes

The effects of chemicals and especially of pesticides on soil microorganisms have been studied for risk assessment because of the importance of microbes in soil processes. These studies mainly comprise laboratory studies such as respiration and mineralization inhibition tests. International harmonisation of these methods has only recently been undertaken by the OECD (Table 3).

Harmful effects of a pesticide can often be observed in laboratory studies with soil types which favour high bioavailability of the chemicals. These ‘worst case’ studies are needed for the initial risk assessment of the pesticide. One such ‘worst case’ soil is for example the soil which was selected by an OECD working group for the assessment of effects of chemicals on soil microorganisms (70 % sand, and only 0.5–1.5 % organic carbon). Since in this soil the adsorption of the chemical is minimal

and its bioavailability to microbes is maximal, tests with other soils are generally regarded as unnecessary. There are two harmonised OECD test guidelines for testing the effects of chemicals on soil microbes. Test guideline TG 217 (OECD, 2000) assesses the inhibition of the carbon mineralization of added organic material and TG 216 (OECD, 2000) assesses the inhibition of nitrogen mineralization of added nitrogen-containing organic material (lucerne meal). A more rapid method to screen the effects of chemicals on ammonium oxidation (the first step in the nitrification process) in soil based on chlorate inhibition of further reactions in nitrification was first published by Belser and Mays (1980), further developed for practical testing by Hansson *et al*. (1991) and proposed for harmonisation by Torstensson (1993).

In order to assess the effects of chemicals on activated sludge microorganisms and their respiration, a short term test TG 209 was developed by the OECD (OECD 1984). The OECD algae test guideline 201 (OECD 1984) is used for testing the effects of chemicals on freshwater algae. This guideline is currently under revision to improve the test procedure and to include more species from different taxonomic groups of algae.

1.5 Micro- and mesocosms to assess effects on microbiological activity

Most aquatic ecotoxicological research on the assessment of effluents or chemicals is concentrated on single-species laboratory testing, or measurements of the effects on certain communities (fish, benthic fauna, algae) of the exposed ecosystems. Extrapolation from single laboratory toxicity tests to natural ecosystems is often uncertain. Conversely, studies on exposed natural ecosystems include much natural variation, both in terms of the sensitivity and exposure of the species and physical-chemical conditions, which complicates the interpretation of observed data. A possible compromise between single species laboratory tests and natural ecosystem tests could be a multi-species mesocosm that simulates the structural and functional mechanisms of natural ecosystems. These mesocosms are also affected by environmental factors other than the exposure studied, but often these factors can be recorded and hence the possible responses caused by the studied exposure could be better interpreted (Lehtinen *et al*. 1997). In mesocosm studies the ecosystem may develop certain characteristics over time (e.g. biomass increases and community production decreases (Odum 1985)). In effluent-exposed mesocosms or in stressed ecosystems these trends may be arrested, reversed or stimulated. Trends in stressed ecosystems include changes in community structure as well as its functions (nutrient cycling, energetics).

In soil studies harmful chemicals, such as pesticides, have effects on many parts of the food web (from microorganisms to soil invertebrates and plants). In order to understand the overall effects on the interactions between different organisms involved in mineralisation processes, terrestrial microcosm or mesocosm techniques can be used (Bruckner *et al*. 1995). These are usually assembled from different elements (microbes, invertebrates, plants) of the modeled ecosystem. They allow assessment of the effects of a particular variable on the system processes by eliminating or controlling that variable and monitoring the whole system responses (Ingham *et al*. 1986)

1.6 Microbiological measurements in environmental pollution assessment

Measurements of microbial activity or biomass can be used in the biological quality assessment of both aquatic and terrestrial environments. The possible observed changes are often a definitive indication of environmental damage. However, because of the microbial community changes in tolerance and species composition in the stressed environment, the measurable changes in total activity or biomass may be masked and are difficult to prove statistically (Giller *et al*. 1998).

The microbial activity assessments in environmental quality monitoring use turnover rates of certain substrates, enzyme activities and respiration as measured variables. The biomass estimates are much debated and these methods include measurements of ATP content of soil and sediments, soil carbon fumigation methods, different respiration measurements with or without added substrate and also microscopic observations of microbial cells. Developments in biochemistry and in molecular biology have improved the possibilities to assess changes in microbial community structure and functional diversity. These methods are reviewed separately for aquatic environments and soil.

1.6.1 Aquatic environments

Microorganisms of the sediment are important mineralizers of organic matter, which is the main source of energy in benthic food webs. This organic carbon and nutrient source consists of particulate organic matter and high molecular weight, polymeric compounds which first need to be enzymatically cleaved outside the microbial cells into smaller molecules before they are available for cellular uptake and further metabolism. Enzyme reactions by extracellular enzymes produced by algae, fungi and bacteria have been proposed to be "key steps" in aquatic food webs (Hoppe 1992). Possible effects of effluents on the microbial loop of the aquatic food web can be assessed by measuring the microbial biomass or activity in the sediment and in the water column.

Aquatic microbial biomass can be measured by fluorescence microscopy counting with DAPI (4',6-diamidino-2-phenylindole) or acridine orange staining (Kepner and Pratt 1994), or by measuring the extractable ATP content of the sample (Karl and LaRock 1975). Microbial activity is often assessed by measuring enzyme activities (phosphatases, ureases, dehydrogenases) or by measuring the heterotrophic activity for example by methods based on incorporation of radiolabelled thymidine (Riemann and Lindgaard-Jörgensen 1990 or leucine (Chin-Leo and Kirchman 1988).

Field studies have shown that extracellular enzyme activity measurements can be used in the biological quality monitoring of sediments (Lee and Tay 1998). Activity measurements of phosphomonoesterase (PME) can be used as an index of potential organic phosphate mineralization. The activity of this aquatic extracellular enzyme may be potentially increased by eutrophicating effluents, e.g. from sewage (Chappel and Goulder 1994) or fish farms (Carr and Goulder 1990, Davis and Goulder 1993), or inhibited by toxic effluents (Burton and Lanza 1987, Tubbing and Admiraal 1991).

The functional diversity of the sediment microbial population and its extracellular enzyme activities can also be assessed by enzyme activity profiles (Poremba 1994).

1.6.2 Terrestrial environments

In field studies or in monitoring soil fertility and in assessing the effects of anthropogenic activities on microbial populations, measurements of microbial activities related to carbon and nitrogen metabolism are used. These measurements, such as soil respiration (Nordgren 1988) and nitrification (Müller *et al.* 1981), were adopted in the EPPO/Coe pesticide risk assessment guidelines (OEPP/EPPO 1994) to assess long term effects of pesticides on soil fertility.

The generally most accepted estimates of soil microbial biomass are considered to be obtained by the soil fumigation incubation method (FI) (Anderson and Domsch 1978), the fumigation extraction method (FE) (Vance *et al*. 1987) or by the substrate-induced respiration method (SIR) (West *et al.* 1986). Microbial active biomass can also be assessed by measuring the amount of active microbes by ATP measurements (Jenkinson *et al*. 1979, West *et al*. 1986, Arnebrant and Bååth 1991,Vanhala and Ahtiainen 1994).

Fluorescein diacetate (FDA) hydrosis by many enzymes (non-specific esterases, proteases and lipases) has also been used to determine the total microbial activity in soils (Schnürer and Rosswall 1982, Adam and Duncan 2001). The FDA hydrolysis measures the activities of both membrane-bound enzymes and free extracellular enzymes (Stubberfield and Shaw 1990). Several other individual enzyme activities, for example urease (Tabatai 1977), phosphatase (Tabatai and Bremner 1969), dehydrogenase (Aoyama and Nagumo 1995) and glucosidase (Barnhart and Vestal 1983) activity have been proposed as tools to assess soil microbial activity. The determination of bacterial growth rates in soil by measuring incorporation of tritium-labelled thymidine (Bååth 1992) or ^{14}C-labelled acetate to lipids (Barnhart and Vestal 1983) has also been published as estimates of microbial activity in soils.

The above-mentioned sum parameters of microbial activity can indicate a severe drop in total microbial community, but may fail to indicate possible harmful changes in microbial community structure. The effects of a pesticide may be very different on fungi and bacteria (Jones *et al*. 1992, Malkomes 1992). The diversity of microbial consortia in soil can be assessed by methods based on nucleic acids (Torsvik *et al*. 1990, Führ and Kubiak 1994,) and phospholipid fatty acid profiles

(Bååth *et al*. 1992), and the functional diversity can be described by substrate utilisation patterns (Degens and Harris 1997) or by soil enzyme activity profiles (Kandeler and Eder 1993, Bandick and Dick 1999, Vepsäläinen *et al*. 2001). Nannipieri *et al*. (1990) showed that the assessment of enzyme profile could be a better estimate of overall microbial activity than a single enzyme measurement when evaluating responses to pollution.

As a complex matrix the soil sets high demands on the applicability of study methods of any biological activity and also on the interpretation of the results. In field studies, natural soil with clay and a high organic carbon content may have such a high adsorption capacity that no effects of chemicals on biota are detected. In Finland, most of the cereal crop production and the use of pesticides is located in areas with high clay and also organic carbon content of soil. This may have effects on the bioavailabilities of the pesticides used.

The methods for soil biological quality assessments are standardized by the ISO Soil Quality Technical Committee 190. Currently there are only two accepted methods to assess soil microbial activity and biomass. The determination of nitrogen mineralisation and nitrification in soils (ISO 14238, 1997) is based on the measurement of ammonification and nitrification after addition of organic nitrogenous matter. The other method is for the determination of soil microbial biomass by fumigation and extraction (ISO 14240-2, 1997). A rapid screening method for soil nitrification based on ammonium oxidation has been proposed as a soil quality standard by Torsstensson (1993).

1.7 Biodegradation studies on chemicals

The natural carbon cycle in ecosystems operates on the assumption that practically all biosynthetic organic materials are biodegradable. Natural compounds of low degradability and man-made compounds may ultimately be degraded but this may require some time for adaptation. The potential for this adaptation and for radical population changes exists in microbial communities. However, the environment will often be ill prepared to withstand vast and increasing amounts of compounds from human activities.

The estimation of biodegradation rates is an important uncertainty factor in the chemical risk assessment mandated by EU legislation (European Commission 1999). Obviously the rate of biodegradation greatly influences the predicted environmental concentrations (PEC) and thus the possible exposure of biota to the chemical.

The existing OECD tests for ready biodegradability have been developed to devise screening methods to determine whether a chemical has the potential to be easily biodegraded, and not to predict the rate of biodegradation in the environment. In these tests the potential biodegradability is classified usually by evaluating the mineralization of the chemical by measuring the decrease of organic carbon, carbon dioxide evolution or oxygen demand during the test. In some tests only the primary biodegradation is assessed by analyzing the disappearance of the original parent compound tested (not mineralisation). In the OECD regime a compound is regarded as readily biodegradable if it reaches 60–70 % degradation during the 10 day period from the start of the degradation (OECD 1981). However, current risk assessment and future EU legislation require degradation rates. Usually these rates must be judged by experts (default values in the EU technical guidance document for the risk assessment of new and existing substances, EC 1999) or derived by modelling e.g. by the Simple Treat fate model (Struijs *et al*. 1991) for wastewater treatment plants based on the available data from ready biodegradability tests.

The fate, biodegradation and chemical transformation of chemicals such as pesticides in soil environments is usually studied in soil columns and with labeled substances. For testing the aerobic and anaerobic transformation and degradation of chemicals in soil, OECD is currently finalizing the guidelines. Hitherto OECD has been able to harmonize only one guideline, TG 304A for inherent biodegradability in soil (OECD 1984).

The several ISO Water Quality standards for testing the biodegradability of organic chemicals or any organic material (effluents) provide strict technical guidance for testing and for test validity. They do not contain guidance for interpreting the results as substances being “readily biodegradable” as do the OECD guidelines with set criteria. The two existing ISO Soil Quality standards for testing aerobic (ISO 11266, 1994) and anaerobic (ISO 14239, 1997) biodegradation of chemicals in soil provide guidance to perform the testing. These ISO standards are to be considered as methods for assessing the capability of soil to degrade organic chemicals rather than for chemicals testing.

Biodegradability depends not only on the molecular structure of the test compound but also on the microorganisms available and on other environmental conditions. The feature of adaptation of the microbial population in the tests, in the wastewater treatment and in the environment is a key issue in the biodegradability testing and in the interpretation of biodegradability data. The standard tests (OECD Test Guidelines and ISO standards) have many arbitrary features compared to actual environments, in order to improve practicability. An important difference is the concentration of the tested chemical in the standard tests and in the aquatic environment or wastewater treatment plants. The disadvantage of the summary parameters measured in the standard tests (decrease of organic carbon, carbon dioxide evolution, oxygen demand) is that higher concentrations of the tested compounds are required than presumably occur in different environmental compartments.

Concentrations of the chemicals in the treatment plants or in the environment are usually at ng–mg carbon per litre levels compared to 20–400 mg C l^{-1} in most tests. The different concentrations of the chemical/substrate will provide different growth kinetics and hence different biodegradation rates (Ingerslev *et al*. 1998). This correlation between test compound concentrations and biodegradation rates has been reported for many substances, revealing a decrease in rates at lower concentrations (Boethling and Alexander 1979, Alexander 1994).

At high concentrations the chemical, if it is biodegradable, can be degraded as a primary substrate and the competent microorganisms grow exponentially, resulting in an accelerating biodegradation rate (Monod kinetics). At low concentrations in the environment, with natural carbon substrates which are degraded simultaneously, the chemical cannot serve as a primary substrate and thus will not support significant growth of the degraders and the biodegradation follows first order kinetics with a constant biodegradation rate. However, adaptation of the microbial community may eventually take place, mediated by constant exposure to the chemical. This may lead to faster degradation, and hence the rates in different environments (adapted/ non-adapted) may differ considerably.

1.8 Objectives of this study

Toxicity tests based on the responses of different organisms have been used to assess the environmental hazard identification and risk assessment of chemicals, waste waters, contaminated sediments and soils for some decades. There also exist methods to evaluate changes in the ecological quality of the various environments based on the structural or functional changes of the biota. Microbiological methods have been applied among other measurements to achieve these goals (Table 4).

The aim of this study was to evaluate the applicability of different microbial toxicity tests (Papers I–V) and microbiological activity or biomass measurements in the environment (Papers II, V) for the effect assessment of chemicals (Paper V), waste waters (Papers I and II) and soil pollution (Papers III and IV). The usefulness of these microbial methods for the regulation and guidance human economic activities (industry, wastewater treatment, agriculture, soil remediation) was also discussed.

The sensitivity of assessment of risks or environmental changes was also compared with tests using other organisms and biological quality measurements and chemical analyses (Papers I–V).

The biodegradability of a compound is one of the most important factors affecting the exposure of biota in the environment to this compound. Hence the biodegradation tests are used to provide an indication of the fate of the substance in the wastewater treatment or in the environment. The aim of the study in paper VI was to compare the biodegradation of two reference compounds in a standard method and in more ecologically relevant conditions in a simulation test with different sources of microbial inocula.

2 Materials and methods

2.1 Study structure and methods

2.1.1 Assessment of environmental effects of industrial effluents

Toxicity assessment and chemical analysis (Paper I and unpublished data)

In the first phase of the study the toxicity of the wastewaters originating from conventional chlor-

Table 4. The research, articles and elements of data on microbial responses in chemicals pretesting, in toxicity testing and in biological quality measurements in the environment used for the environmental effect studies in different areas in this study. The Roman numerals refer to original papers I–VI and T to this thesis.

Study areas	Chemicals pretesting samples	Toxicity testing of environmental assessments	Meso- and microcosm studies	Environment biological quality
Aquatic environment				
The effects of pulp mill and other industrial effluents		a set of different biotests (I , II and T)	sediment and water biomass and activity (II)	extrapolation from the mesocosm data (II and T)
Terrestrial environment				
Assessment of bioremediation of polluted soils				
A. PAH contamination B. Chlorophenol contamin.		bacterial tests with soil and soil elutriates (III and IV)		
Effects of agrochemicals on soil microbes	toxicity and bio-availability testing of pesticides (V)		soil respiration inhibition (V)	field measurements of activity and biomass (V)
Biodegradation				
Standard tests, simulation and real environment	simulation conditions vs. standard tests (VI)		biodegradation of soil contaminants in pilot composts (III and IV)	

ine (Cl_2) bleaching, novel elementary chlorine free (ECF) and totally chlorine free (TCF) bleaching processes was assessed and compared with their chemical composition. In the second part of the work, more detailed data concerning the acute toxicity of the different wastewater streams of modern Finnish pulp mills were collected in order to identify the sources of toxicity in different wastewater streams in the mills and to assess the toxicity reduction efficiency of secondary treatment. In the third part of the study (previously unpublished data) the toxicity and the toxic loads from Finnish pulp and paper mills were compared to those from some other industrial areas such as the metal and mining, chemical and textile industries.

The two pulp mills chosen for the first phase of the study produced bleached hardwood and softwood kraft pulp (Table 5, Verta *et al.*,1996). One of the mills (Mill A1) produced non-bleached board and bleached and non-bleached kraft pulp and the other (Mill B1) produced bleached kraft pulp on two separate hardwood and softwood lines. Both the bleaching waste waters (1 chlorine bleaching, 4 ECF bleaching and 3 TCF bleaching) and the secondary treated effluents (1 chlorine bleaching, 3 ECF bleaching and 3 TCF bleaching) from these mills were sampled and studied. In order to obtain an understanding of the toxic effects, a set of acute and sub-acute bioassays was applied (*Pseudomonas putida* growth inhibition, *Vibrio fischeri* bioluminescence, *Pseudokirchinella subcapitata* algae growth inhibition, *Daphnia magna* mobility inhibition (ISO 6341, 1989), *Brachydanio rerio* zebra fish embryo and sac-fry stage test on fertilized eggs and hatched larvae (SFS 5501, 1991), see 2.2.1 and Ahtiainen *et al.,* 1996 for details)

In the second phase of the study nine pulp and paper mills, practically all the significant bleached pulp producers in Finland, were chosen for the study (Table 6, Ahtiainen *et al.,* 2002). They produced both bleached hardwood and softwood pulp. The process used was either ECF-, TCF- or combined ECF/TCF-bleaching. The untreated waste-

Table 5. The pulp production and effluent volumes of the two mills in the first phase of this study. (ECF = elementary chlorine free, TCF = totally chlorine free, Bleaching sequences : A = acid, C = chlorine, D = Chlorine dioxide, E = sodium hydroxide, O = oxygen, P = hydrogen peroxide, Q = chelating agents, Z = ozone) Source: Paper I.

Mills	Production			Wastewater and effluent volume (m^3 / day)	
	Wood material	Pulp production (t/ day)	Bleaching (sequence)	Bleaching	Total effluent
Mill A1					
	softwood	800	Chlorine (D/C-EoD1-E2-D2)	16 000	62 400
	softwood	900	ECF (Do-Eo-D1-E2-D2)	19 800	72 900
	hardwood	900	ECF (Do-Eo-D1-E2-D2)	50 400	90 000
	hardwood	900	ECF (Do-Eo-D1-E2-D2)	25 200	57 600
	softwood	700	TCF (O-Q-Z-Eop-Az-Ep-E)	13 300	49 700
	hardwood	600	TCF (O-Q-Z-Eop-Az-Ep-E)	9 600	40 800
Mill B1					
	softwood hardwood	1326	ECF (D-E-D-D)	39 800	59 700
	softwood hardwood	980	TCF (Q-P-P-P)	39 200	56 800

waters were collected from debarking (n = 7), black liquor condensates (n = 4), different bleaching sequences (alkaline (n = 19), acidic (n = 19) and total bleaching waste water (n = 18)), total untreated wastewater (n = 15) of the mills and secondary treated (activated sludge) effluents (n = 10) as pooled samples representing the normal functioning of the factory processes. The acute toxicity and the influence of freezing and storage of the samples on the toxicity of the wastewaters were assessed by 2 simple screening tests (luminescence inhibition test and the mitochondrial particle test, see 2.2.1. and Ahtiainen *et al.,* 2002 for details.

In the third part of the study, in which the toxicities (*P. putida* growth inhibition*, V. fischeri* bioluminescence*, P. subcapitata* algae growth inhibition, *D. magna* mobility inhibition) of various industrial effluents were assessed, the pulp- and paper industry was represented by a bleach pulp mill, a non-bleach pulp mill, a non-bleach cardboard mill and a paper mill. Chemical industries were: oil refinery and petrochemical plant, pharmaceuticals production, biotechnical enzyme production and viscose production. Metal industry was represented by stainless steel production, cobalt and zinc production and mining. Textile factories produced leather and yarn. The two effluent samples were collected at each mill as pooled composite samples of one day.

The general chemical characteristics of the effluents; biological oxygen demand (BOD_7), chemical oxygen demand (COD_{Cr}), total organic carbon content (TOC), absorbable organic halogens (AOX), total nitrogen (N_{tot}), total phosphorus (P_{tot}), loss on ignition, suspended solids and colour, were analysed using standardized methods

Table 6. The pulp production and effluent volumes of the mills studied in the second screening phase. (ECF = elementary chlorine free, TCF = totally chlorine free, Bleaching sequences : A = acid, C = chlorine, D = Chlorine dioxide, E = sodium hydroxide, O = oxygen, P = hydrogen peroxide, Q = chelating agents, Z = ozone)
Source: Paper I.

Mills	Production			Wastewater and effluent volume (m^3 / day)	
	Wood material	Pulp production (t/ day)	Bleaching (sequence)	Bleaching	Total effluent
Mill A2					
	softwood	243	ECF (Do-E1-P-D1-E2-D2)	22 000	114 300
	hardwood	1120	ECF (Do-E1-E/O-D1-E2-D2)	24 000	114 300
Mill B2					
	softwood	624	ECF (Do-EoP-D1-EP-D2)	23 500	110 100
	hardwood	775	ECF (Do-EP-D1-EP-D2)	14 600	110 100
Mill C					
	softwood	941	ECF (Q-O-D-E2-D2)	41 600	-
	hardwood	762	ECF (Q-O-D-E2-O2)	37 200	-
Mill D					
	softwood	624	ECF (Q-O/O-p-D1-EP-D2)	30 000	77 300
Mill E					
	hardwood	1140	ECF (O-D-Eo-D-D)	-	57 300
Mill F					
	softwood	1300	ECF (Do-E1-D1-E2-D2)	20 000	80 000
	hardwood	1500	ECF (Do-E1-D1-E2-D2)	20 000	80 000
Mill G					
	softwood	984	ECF (Do-O-D1-E2-D2)	21 600	76 200
Mill H					
	softwood	1000	ECF	18 800	-
	softwood	1930	ECF	47 100	-
	hardwood	900	TCF	12 400	-
Mill I					
	softwood	1044	ECF	30 000	55 200
	hardwood	1259	ECF	40 000	54 100
	softwood	643	TCF	15 000	55 800
	hardwood	1203	TCF	26 000	65 900

(Verta *et al.* 1996). In the first phase of the study 25 major and trace elements, phenolic compounds, fatty acids, resin acids and molecular weight (MW) distribution of the wastewaters were also determined (Verta *et al.* 1996).

Effects on aquatic microbiological processes in mesocosms (Paper II)

The long term effects of untreated and treated ECF and TCF pulp bleaching wastewaters, secondary treated municipal effluent and natural humic water on microbial processes (sediment enzyme activities, see 2.2.3) and on biomass (water column bacterial counts and sediment ATP content, see 2.2.3) were studied in brackish water mesocosms. The effects on other parts of the aquatic food web were also studied (Tana *et al.* 2002) in this one and a half year experiment. For additional background information the toxicities of all samples were assessed by a set of biotests: luminescent bacteria test, algae test and daphnid test. The tested water, wastewater and effluent samples were also characterized by different chemical analysis (Mikkelson and Paasivirta 2002) and the correlation between analysed compounds and mesocosm effects was assessed (Paasivirta and Mikkelson 2002).

The mesocosm ecosystem included macro- and micro algae, invertebrates and juvenile sticklebacks (*Gasterosteus aculeatus*) indigenous to the Baltic sea area. The detailed mesocosm experimental setup is described by Tana *et al.* (2002) and in paper II. The untreated and secondary treated ECF and TCF effluents were studied at two concentrations, and the treated municipal effluent and humus water at the higher concentration. Hence the experiment consisted of two control pools and 10 single mesocosms receiving test waters. All waste water and treated effluent samples were collected as pooled samples and the humus water as a single homogenized sample. The samples were frozen immediately after sampling and stored in a freezer (–18°C) until thawed and diluted to the flow-through system of the mesocosms. The two dilutions, prepared in natural brackish seawater of the tested sample waters, were relatively low (1/400 and 1/2000) and were designed to represent the concentrations in rather large areas in pulp mill effluent recipients in Finland.

2.1.2 Toxicity assessment and bioremediation of polluted soils

Chlorophenol contaminated soil (Paper III)

The degradation of chlorophenols and possible formation of harmful metabolites was studied by chemical analysis and toxicity testing during the pilot-scale composting (6 months) of chlorophenol contaminated sawmill soil and impregnated wood. Four 13 m^3 compost piles were constructed, with nutrient addition, bark chips and pH adjustment by granular lime in the field at the end of May, at Vääksy, Southern Finland (Laine and Jörgensen 1997). One pile was left uninoculated as control, the second was supplemented with straw compost as inoculum and the third and fourth with remediated soil as inoculum.

The composite soil samples were homogenized and sieved through an 8 mm sieve to remove the added bark chips and other coarse particles before the toxicity assessments (see 2.2.2) and chemical analyses (chlorophenols, extractable halogen content (EOX), molecular weight distribution of organic halogen compounds and dioxins and furans, see Paper III).

Creosote oil contaminated soil (Paper IV)

The bioremediation of creosote contaminated soils was studied in two cases of pilot outdoor composting by toxicity assessments and chemical analyses. In both cases the soil from the sawmill area was heavily contaminated with polyaromatic (PAHs) and aromatic (AHCs) hydrocarbons and with metals (As, Cr_2, Cu) from wood preserving activities.

At one site, two pilot composts (5m^3) were constructed in the field near Vilppula in southern Finland. One of the piles was inoculated with *Mycobacterium* inoculant and the other was left uninoculated, thus containing only the "inherent inoculum" of indigenous bacteria. The composts were sampled at the start, after the first summer and after the second summer for chemical analyses and toxicity tests (see 2.2.2). The pooled soil samples were homogenized and sieved through an 8 mm sieve to remove the added bark chips and other coarse particles.

The other site was located at Ilmajoki, in western Finland. A large composting pile (100 m^3) of

soil from an old wood preserving facility was constructed in the field. The soil was screened to remove >50 mm diameter stones and other coarse materials, and then pretreated with 50 % hydrogen peroxide to speed up the breakdown of the recalcitrant, but biodegradable 4–6 ring PAH compounds. After the hydrogen peroxide had completely degraded (tested by a fieldstrip test), a microbial inoculum of PAH degraders, nutrients (N, P and micronutrients) and bark chips was added. The pile was sampled for toxicity tests (see 2.2.2) and chemical analysis of PAH composition at the beginning, after the first week, and then every four weeks during a composting period of 163 days. The compost was thoroughly mixed before sampling and the pooled soil samples (during one summer) were pretreated for analysis as in the first case.

2.1.3 Effects of agrochemicals on soil microbes (Paper V)

The study was designed to assess the effects of two pesticide regimes (conventional full insurance and supervised/reduced pesticide use) and two cultivation techniques (conventional tillage and fertilizing compared to no tillage with less fertilizing) on soil microbes in a field study, and to evaluate these results with laboratory tests. The herbicides used were chlorsulfuron, methyl-chlorophenoxyacetic acid (MCPA) and bentazone; the fungicides carboxin-imazalin and propiconazole; and the insecticides dimethoate and pirimicarb.

Laboratory experiments

The potential harmful effects of pesticides (commercial formulations) on microbes were studied in the laboratory with two bacterial toxicity tests (*P. putida* growth inhibition and *V. fischeri* luminescence tests, see 2.2.1) in aqueous solutions. The soil respiration inhibition potential (see 2.2.3) was measured for five selected pesticides and the bioavailability of three pesticides in the soil was assessed by a solid phase modification of the luminescent bacteria test (see 2.2.2).

Soil basal respiration inhibition by dimethoate, propiconazole, chlorsulfuron, pirimicarb and MCPA- bentazone was measured using an automated respirometer (Nordgren 1988). The same clay soil (clay >70 %, organic carbon content 1.5–2 %) as in the field studies was supplemented with different amounts of pesticides (10, 50, 100, 500 mg kg^{-1} as commercial formulations) and the respiration was followed for two months.

To assess the bioavailability of the pesticides in the soil respiration inhibition tests a solid phase modification of the luminescent bacteria tests was used. The toxicity of each soil sample supplemented with different amounts of dimethoate, chlorsulfuron and propiconazole (10, 50, 100, 500 mg kg^{-1} as commercial formulations) was assessed by the soil-contact modification of the standard luminescent bacteria test.

Field experiments

The effects of chemicals on microbial biota were assessed in the field studies by measuring microbial activities related to carbon and nitrogen metabolism (soil respiration, nitrification potential) and biomass (soil ATP content).The methods for field measurements are described in the analytical methods section 2.2.3.

The field studies were carried out near Jokioinen in southern Finland. The size of the six replicate study fields was 54 m x 120 m. There was some slight heterogeneity between the randomized study fields set in the area in organic matter and clay content, but all the soil in the area would be classified as clay soil. The soil had not been treated with any pesticide during the seven preceding years.

During two growing seasons (1992 and 1993) of barley, different herbicides, fungicides and insecticides were used according to the general guidance calendar (treatment C) or to the actual monitored need for plant protection (treatment D). The herbicides used were chlorsulfuron, MCPA, bentazone, the fungicides were carboxin-imazalin and propiconazole and the insecticides were dimethoate and pirimicarb. In addition, the effects of two different cultivation techniques were studied. These were a normal tillage and fertilizer use (treatment A) and cultivation without tillage and with reduced use of fertilizers (treatment B). Hence the four different treatment compilations were: AC conventional pesticide use, normal tillage; AD reduced pesticide use, normal tillage; BC conventional pesticide use, no tillage, less fertilizers; BD reduced pesticide use, no tillage, less fertilizers.

The sampling was performed before and after pesticide treatments, and the soil was also sampled twice at the end of the growing season in order to assess the longer term effects. From each field, 30 surface soil (0–5 cm) core samples were pooled together. These sieved samples were kept at +4°C for a maximun of four days before analyses of nitrification potential and ATP content. Samples intended for soil respiration measurements were frozen immediately at –20°C.

2.1.4 Biodegradation studies (Paper VI)

Biodegradation of the reference chemicals aniline and 4-chloroaniline was measured and compared in a standard method at standard concentrations (10–20 mg carbon per litre) and in more environmentally realistic conditions at low concentrations (2–26 mg carbon per litre) with ^{14}C- labelled substances and with different sources of inocula.

In the standard test the tested substance was added to a mineral medium as the sole source of carbon, and the sealed vessels with a headspace of air were inoculated with diluted fresh activated sludge from a municipal wastewater treatment plant in Helsinki.

At low concentrations of labelled reference chemicals in the shake batch test (see 2.2.4) the test waters (inocula) were collected from two pristine lakes in southern Finland and from unpolluted Baltic seawater and urban seawater. One of the pristine lakes, Lake Vitsjö in Tenhola, was oligotrophic and the other, Lake Matkunlammi in Hyvinkää was a naturally humic lake with high carbon content. The unpolluted seawater was collected near Tvärminne on the southern coast of Finland and the urban seawater from Helsinki harbour. The waters were stored cool (4°C) and used for the tests on the day after collection. The numbers of heterotrophic bacteria in the natural test waters were enumerated by total aerobic viable counts at 20°C on R2A agar incubated for 3 days. The total organic carbon (TOC) content and some other water quality characteristics (pH, O_2, N_{tot}, P_{tot}) were also analysed.

The biodegradation rates of labelled reference chemicals were also assessed in the standard test conditions (suspended solids 4 mgl^{-1}, see 2.2.4) with activated sludge from the Helsinki municipal wastewater treatment plant. Biomass evolution during the tests was followed by ATP measurements and on the basis of residual ^{14}C- activity in the particulate matter.

2.2 Analytical methods

2.2.1 Microbial toxicity test methods on effluents, soil elutriates and chemicals

Vibrio fischeri luminescent bacteria test

The toxicities of waste waters (*Papers I and II*), soil elutriates (*Papers III,IV and V*) and chemicals (*Paper VI*) were tested according to the standardized luminescent bacteria test (ISO 11348, 1998). In order to test soil elutriates, freshly homogenized soil samples were eluted to deionized water by weighing 5 g of soil to 50 ml of deionized water and shaking them overnight at room temperature. The pH of the elutriates and waste water samples was usually neutralized to pH 7 to give comparable results.

The luminescent inhibition test was accomplished by combining a 500 µl sample with 500 µl luminescent bacterium *Vibrio fischeri* DSM 7151 (same as NRRL B-11177) suspension. The test tubes were then incubated at 15°C in a water bath. Luminescence after 30 min incubation was measured with a luminometer (BioOrbit 1253). The inhibition of luminescence (%) was calculated by comparing sample luminescence to that of the control containing deionized water.

Pseudomonas putida growth inhibition test

Effluent samples (Paper I), soil elutriates (Paper IV) and pure chemicals (pesticides, Paper VI) were tested by automated modification of the standard *Pseudomonas putida* growth inhibition test (ISO 10712, 1995) with the Bioscreen C^R analyzer (Labsystems). In this test, *P. putida* MIGULA (DSM 50026) bacteria were grown at 21°C for 16 hours in a liquid medium (350 µl) in special multiwell plates and the turbidity due to bacterial growth was measured by vertical photometry. The toxicity was assessed as growth inhibition (%) in different dilutions of the samples compared to a deionized water control. The pH of the samples was checked and when needed set at 7. The waste water samples were pretreated by centrifuging (10

min/10 000 rpm) to eliminate coarse particles and by sterile filtration (0.2 μm) to eliminate background bacterial growth.

Pseudokirchinella subcapitata green algae growth inhibition test

The algal growth inhibition testing of the sterile filtered effluent samples (Papers I and II) was performed by the ISO standard method (ISO 8692, 1989) with the alga *Pseudokirchinella subcapitata* (formerly *Selenastrum capricornutum*). Algal growth during 72 h was measured every day without chlorophyl extraction and analysis using whole cell fluorometry (Sequoia-Turner 450 Digital Fluorometer, filters: NB440 (excitation) and SC665 (emission)). The method was calibrated by microscopic counting of algal cells. The toxicity was assessed as growth inhibition (%) in different dilutions of the samples compared with deionized water.

2.2.2 Toxicity test methods on soil

Soil contact test method with luminescent bacteria

To assess the toxicity of soil samples (Papers III and IV) and bioavailability of the pesticides (Paper V) a solid phase modification of the luminescent bacteria tests was used (Brouwer *et al*. 1990). Homogenized soil samples of 5 g were weighed to 50 ml centrifuge tubes and diluted with 16 ml deionised water. This suspension was supplemented with 2 ml 20 % NaCl solution and with a 2 ml inoculum of *Vibrio fischeri* (strain DSM 7151) luminescent bacteria. The tubes were incubated for 15 minutes at 15°C in a water bath. After the incubation the tubes were quickly centrifuged (5 min, 1660 rpm, 300 *g*) to separate the solids from the exposed bacteria. The luminescence of a 1 ml sample of this supernatant was measured with a luminometer (BioOrbit 1253). The sample luminescence was then compared to that of deionized water to calculate inhibition percentage and to estimate EC50 values (50 % inhibition of luminescence).

Toxi-Chromo Pad enzyme synthesis inhibition test

The qualitative or semi-quantitative Toxi-Chromo Pad test (Kwan 1995) with soil samples (Paper IV) was carried out according to the instructions of the kit. Freeze dried *Escherichia coli* bacteria were rehydrated and added to the reaction mixture. After 20 min pre-incubation 0.5 ml of this solution was mixed with 0.5 g of homogenized fresh soil. The mixture was incubated at 37°C for two hours. The mixture was homogenized again and a small drop of incubated soil-bacteria mixture was pipeted onto a chromogenic pad (a glass microfibre filter soaked with blue chromogenic substrate for β-galactosidase). The pads were further incubated at 37°C overnight for the colour development. The intensity of enzyme synthesis was indicated by blue colour and possible toxicity by reduced the enzyme synthesis and weaker colour development.

Soil respiration inhibition test

Soil respiration inhibition of pesticides (Paper VI) was measured by automated respirometry (Nordgren 1988). The same clay soil as in the field studies was supplemented with different amounts of pesticides. The soil respiration was measured on several two-day periods during one month of incubation at 20°C. The soil moisture content was adjusted to 50 % of the water holding capacity in the beginning and after each week. The mean respiration rate in treatments was then compared to that of non-treated similar control soil.

2.2.3 Methods for microbiological activity and biomass measurements

Phosphomonoesterase activity in sediment

The alkaline phosphomonoesterase (PME) activity of the sediment (Paper II) was measured by the modification of soil phosphatase activity method based on a method in which phosphate is released from p-nitrophenyl phosphate (PNP-P) (Tabatai and Bremner 1969). Samples of 2 g fresh, homogenized surface sediment were incubated for 2 h with p-nitrophenyl phosphate (PNP-P) at pH 7.75 (0.1M Tris + 2mM EDTA buffer) at room temperature. The concentrations of p-nitrophenyl

(PNP) were measured spectrophotometrically and the phosphatase activity was calculated from the PNP concentrations compared to blank samples.

Sediment enzyme activity profile with the Api Zym- kit

The profile pattern of enzyme activity diversity in mesocosm sediments (Paper II) was assessed semi-quantitatively by the commercial API Zym (BioMerieux, France) enzyme activity kit (Poremba 1994.). A fresh sediment sample of 2 g was homogenized with 8 ml of sterile deionized water. The tubes were centrifuged for 5 min to remove the solid particles. From this supernatant a drop of 65 µl was pipetted to each well of the API Zym strips. The strips were incubated for 16 h at 20°C. The enzyme activities were recorded according to the manufacturer's instructions and expressed as nmol activity per well during incubation time.

Epifluorescence microscopic enumeration of aquatic bacteria

The microbial biomass of the water columns in mesocosms (Paper II) was estimated by DAPI (4',6-diamidino-2-phenylindole) staining and epifluorescence microscopy counting (Leitz Diaplan microscope) (Kepner and Pratt 1994). Water samples of 50 ml were fixed by 5 ml of sterile filtered 10 % glutaraldehyde fixative immediately after sampling. For staining, 4.5 ml of sample and 0.5 ml of dispergant (44.6 g $Na_4P_2O_7$ x $10H_2O$) were homogenized for 30 min in an ultrasound homogenizer. Subsamples of 1 ml were stained with 20 µl DAPI (10mg μl^{-1}) solution in 980 ml deionised water for 7 min and filtered onto black 0.2 µm pore polycarbonate filters (Nuclepore Costar 110656). The visible fluorescent cells were counted from at least 30 fields on filters. The biomass was expressed as viable cells per 1 ml of sample.

Adenosine 5'-triphosphate content in sediment, soil and during biodegradation tests

The active microbial biomass in sediment (Paper II), soil (Paper V) and during biodegradation tests (Paper VI) was assessed by measuring the ATP (adenosine triphosphate) content (Vanhala and Ahtiainen 1994). To extract ATP, samples of 10 g of fresh and homogenized sediment (two replicates, Paper II) or sieved soil (six replicates, Paper V) were placed in 100 ml vials containing 10 ml 20 % trichloroacetic acid and 10 ml 8 mM EDTA. Suspended solids were removed by paper filtration and 0.5 ml aliquots of these filtrates were mixed with 0.1M Tris + 2 mM EDTA buffer in Eppendorf tubes. For the measurement the samples were further diluted 50 times with the same buffer. The ATP content was measured with a BioOrbit luminometric assay kit in a BioOrbit 1251 luminometer (BioOrbit, Turku, Finland). The ATP contents of the samples were then calculated on the basis of the fresh weight of the samples.

To assess the biomass evolution during the biodegradation tests a sub-sample of 0.5 ml was taken from the bottles during each sampling and frozen to –20°C for the ATP analysis. The ATP content of the sample was analyzed according to the protocol of Bio Orbit ATP Kit (BioOrbit, Turku, Finland).

Nitrification potential of soil

Soil nitrification potential (Paper V) was assessed as the capacity of the soil to oxidize ammonium ions to nitrate ions (Müller *et al*. 1981). The moisture contents of six replicate 50 g (dry weight) fresh soil samples were adjusted to 50 % water holding capacity (WHC) and 50 ml of 20 mM $(NH_4)_2SO_4$ solution was added to the soil in 250 ml Erlenmeyer flasks. The flasks were incubated at 20°C for 20 days and the concentration of NO_3^- ions was measured using a nitrate electrode (Orion 920 pH-meter). The nitrification potential (NO_3-N mg g^{-1}) was calculated per dry weight.

Soil basal respiration

The basal soil respiration rate (Paper VI) was measured by weighing six replicate 60 g (dry weight) samples of soil in respirometer cuvettes. The soil moisture content was adjusted to 50 % of the water holding capacity (WHC) and samples were stored for one week at 20°C to stabilize the respiration level. The cuvettes were placed in the respirometer, which captured CO_2 in KOH and measured the change in conductivity (Nordgren 1988). The soil respiration was measured for one week at 20°C.

2.2.4 Biodegradation test methods

Standard headspace CO_2 evolution test

The biodegradation was tested according to the ISO 14593 headspace CO_2 evolution test (1999) at standard concentrations (20 mgC l^{-1}) of the test substance. In this test the tested substance was added to a mineral medium as the sole source of carbon, and the three replicate sealed vessels with a headspace of air were inoculated with diluted fresh activated sludge from a municipal wastewater treatment plant in Helsinki (suspended solids 4 mg l^{-1}). The test was run for 28 d at 20°C with continuous shaking. The biodegradation or mineralization to carbon dioxide was determined by measuring weekly the net increase of total inorganic carbon (TIC) levels over time compared with inoculum or the tested compound in unsupplemented blanks. The inorganic carbon concentrations (TIC) were calculated on the basis of CO_2 concentrations in the headspace after acidification to pH<3 with concentrated (>85 % w/v) H_3PO_4. At this pH the equilibrium constant for the distribution of CO_2 gas and liquid phases in the vessels was 1.0, and hence only the IC concentration in the headspace needed to be analyzed. The extent of biodegradation was expressed as a percentage of the theoretical amount of inorganic carbon based on the amount of the initial addition of the tested compound.

Shake flask batch test

The biodegradation of ^{14}C-radio-labelled reference chemicals was tested with a shake flask batch test ISO/DIS 14592-1 (2000) at different concentration regimes (0.002–26 mg carbon l^{-1} corresponding to 0.004–25.9 mg l^{-1} as chemical) with different inocula.

Testing was carried out in three replicate rubber septum sealed 1l infusion bottles (exact volume 1220 ml) with a liquid volume of 300 ml and a 920 ml headspace. The ^{14}C- labelled test substances were added to obtain a final activity of 50 000 Bq l^{-1} and made up to the tested concentration with unlabelled analytical grade chemical. The tested radio-labelled substances were aniline-C-14-UL 250 Ci/ 0.53 mg and from an other batch aniline-C-14-UL 250 Ci/ 0.6 mg, and 4- chloroaniline-C-14-UL 250 Ci/ 1.5 mg and 4- chloroaniline-C-14-UL 250 Ci/ 3.3 mg. The bottles were incubated at 20°C in near dark or diffuse light with continuous shaking. The maximum test duration was 65 days for 4-chlorophenol and samples were taken weekly.

The biodegradation was monitored by recording the decrease in total organic ^{14}C activity (TOA) by liquid scintillation counting of acidified and air stripped samples. This TOA consists of residual test compound and its metabolites, accumulated microbial biomass and cell fragments. The total activity (TA) is then equal to the sum of above mentioned TOA and inorganic activity (IA), which was stripped off as CO_2 in acidic solution. The degradation rate constant (k) was estimated assuming first order kinetics as the slope of a logarithmic plot of residual activities as a function of time. The half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln(2)\, k^{-1}$.

For the mass balance estimations and for overall check of the test system the activities of different fractions (gas phase, total activity, dissolved residual activity and particulate activity) were also measured by scintillation counting in different trap and scintillation cocktails (see paper III for details).

2.3 Calculation of results and data analysis

Effluent toxicity (Papers I and II) was generally expressed as Toxicity Units (TU). This unit was based on the EC50 dilution at which the effect in the toxicity test was 50 % compared to controls (TU = 1/EC50 x 100).

In order to evaluate the correlation between toxicity and wastewater chemistry, an overall toxicity index (TI) was computed on the basis of the most sensitive tests (luminescent bacteria, algae and zebra fish egg and sac fry test, Verta *et al*. 1996). The original toxicity results of each wastewater or effluent sample in each tests were classified to 5 toxicity classes (TC). Those samples which were not toxic in the test in question had a toxicity class of 0. For the others, values from 1 to 4 were given according to their position in the frequency distribution of the test in question. If the sample toxicity lay within the first fractile it was given a value of 1, if in second fractile, value 2, etc. Finally the toxicity index (TI) was calculated according to the formula:

$$TI = (TC_L + TC_A + TC_{ZH} + TC_{ZS})/16, \quad (1)$$

where TC_L = toxicity class according to the luminescent bacteria test, TC_A = toxicity class in the algae test, TC_{ZH} = toxicity class according to zebra fish hatching and TC_{ZS} = toxicity class based on zebra fish survival.

Effects of sample waters on microbial processes (Paper II) and biomass in the mesocosm studies (water column bacterial counts, sediment biomass and sediment monophosphoesterase activity data) were tested with two way (time and sample water treatment) analysis of variance (ANOVA) with log (x+1) transformation and also with the non-parametric Kruskal-Wallis test because of the distribution of the data. The possible differences observed in ANOVA were tested by the *t*-test and correlations by direct Pearson and by linear regression. The two control mesocosms had to be considered separately because of the different light and temperature conditions.

Soil toxicity (Papers III and IV) was generally expressed as Soil Toxicity Units (STU) or EC50 values of diluted soil elutriates (g soil l^{-1}). The STU was based on the EC50 dilution of soil at which the effect in the toxicity test was 50 % compared to controls (STU = 1/EC50 x 1000).

In pesticide studies the measured field data (Paper V) of soil microbial activity and biomass of six replicate study fields of each pesticide or cultivation treatment on six sampling occasions, separately during the two growing seasons was evaluated by ANOVA (SAS Institute Inc. 1988). The ANOVA was performed for each sampling of each treatment and if any possible significance was found at a risk level of 5 % or lower ($p < 0.05$), pair-wise *t* – tests were performed. Additionally, the possible effects of other environmental factors (soil water content, organic carbon content, NO_3^- and NH_4^+ concentrations) were also statistically evaluated by stepwise least square regression analysis (LSA) (SAS Institute Inc. 1988).

In the study of biodegradation in different conditions (Paper VI), the biodegradation rate and half-life were calculated for the reference chemicals. The degradation rate constant (k) was estimated assuming first order kinetics as the slope of a logarithmic plot of residual activities as a function of time. The half life ($t_{1/2}$) was then calculated as $t_{1/2} = \ln(2)\ k^{-1}$.

3 Results and discussion

3.1 Assessment of environmental effects of industrial effluents

The toxicities of different bleaching wastewaters and treated effluents from the two pulp mills determined by the *Vibrio fischeri* luminescent bacteria, *Pseudomonas putida*, algae and *Daphnia* tests in the first phase of the study are presented in Fig. 2 as Toxicity Units (TU). The ECF and TCF bleaching wastewaters before secondary treatment from

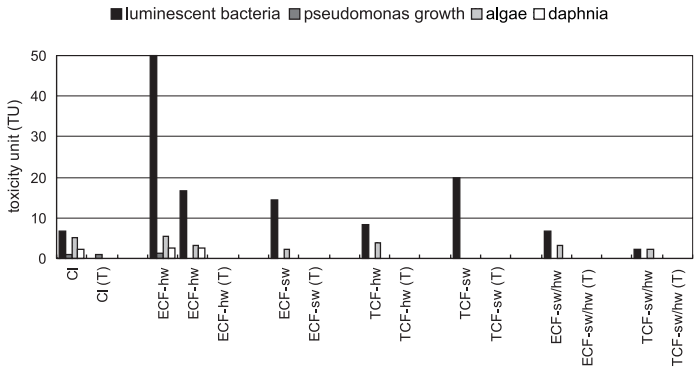

Fig. 2. Toxicity of various bleaching wastewaters (CI = chlorine bleaching, ECF = elementary chlorine free bleaching, TCF = totally chlorine free bleaching, hw = hardwood, sw = softwood) and treated effluents (T) in different biotests.

Mill A1 strongly inhibited the bioluminesce in the *V. fischeri* test. These wastewaters were even more toxic than the tested conventional chlorine bleaching wastewater. The untreated mixed hardwood and softwood bleaching wastewaters from Mill B1 were less toxic than the wastewaters from MillA1. The secondary treated effluents did not exhibit any toxicity to luminescent bacteria.

The bacterial growth inhibition test with *P. putida* showed only slight toxic responses to untreated chlorine bleaching and to one ECF (birch) bleaching wastewater (Fig. 2). Untreated TCF wastewater and all secondary and treated effluents stimulated the growth of the bacteria. Only the untreated bleaching wastewaters inhibited the algal growth (Fig. 2). There were no clear differences in the wastewater toxicity between conventional bleaching and ECF or TCF bleaching. One of the untreated ECF (birch) wastewaters was more toxic than the chlorine bleaching wastewater. Most of the secondary treated effluents stimulated the algal growth. The *Daphnia* were less sensitive than the luminescent bacteria and algae (Fig. 2). However, in the same study the hatching and survival of the fertilized Zebra fish embryos and sac fry stage larvae sometimes appeared to be the most sensitive toxicity test (Paper I, Table 3.).

The toxicities of different wastewater streams (92 samples) from the mills, representing debarking, black liquor condensates, ECF and TCF bleaching sequences (alkaline, acid and total), untreated total effluents and secondary treated effluents were assessed by the luminescent bacteria and the mitochondrial particle tests (Paper I) . The results are expressed as Toxicity Units (TU) (Table 7). The most toxic wastewater seemed to be the debarking process waters and black liquor condensates when assessed with both screening tests. The TCF wastewaters were very toxic in the luminescent bacteria tests. Unfortunately, the number of TCF bleaching effluents assessed in this study was too limited (n = 3) to make valid statements concerning their average toxicity. After the secondary treatment the effluents gave only sporadic and slight toxic responses. Although the data are not shown here, it is worth mentioning that freezing

Table 7. Mean and range of the toxicity units (TU) of the 92 untreated waste water streams (DB = debarking, P = black liqueur condensates, ECF(b) = alkaline ECF bleaching, ECF(a)= acidic ECF bleaching, ECF(t)= total ECF bleaching, TCF(b) = alkaline TCF bleaching, TCF(a) = acidic TCF bleaching, TCF(t) = total TCF bleaching, WW = total waste water) and treated effluents (E) determined by the luminescent bacteria and mitochondria tests, (Source: Paper I)

Sample	Number of	Luminescent bacteria test		Mitochondria test	
	samples	Mean	Range	Mean	Range
DB	7	253	7–1000	52	13–125
P	4	56	7–100	45	4–111
ECF(b)	16	16	3–100	33	6–100
ECF(a)	16	29	3–100	16	2–77
ECF(t)	16	38	2–200	23	3–83
TCF(b)	3	400	100–1000	11*	-
TCF(a)	3	550	100–1000	6*	-
TCF(t)	3	400	100–1000	7*	-
WW	15	52	2–100	16	5–33
E	10	2.5	NT–3	6	2–11

* only one sample

and one month of storage at –18°C did not have a statistically significant effect (*t*-test, $p > 0.05$) on the toxicity of the samples (Ahtiainen *et al*. 2002).

In the first phase of the study a toxicity index (TI) was derived on the basis of luminescent bacteria, algae and zebra fish test results (Paper I and Verta *et al.* 1996). This overall toxicity based on the four most sensitive measured endpoints representing different trophic levels (bacteria, algae, fish) appeared to correlate better with sample organic carbon content than with AOX (Table 8). This rather poor correlation of biological effects and halogenated organic compounds such as AOX has also been documented in an earlier study (Hodson, 1996). For example mixed function oxygenase (MFO) induction in fish has been often strongly associated with pulp mill effluents. However, chlorine is not an essential component of these MFO inducing compounds and thus AOX concentrations in pulp mill effluents and surface waters are unrelated to MFO induction potency. Verta *et al*. (1996) also found some correlation of overall toxicity with the concentrations of total phenols, fatty acids and dichloromethane (DCM) extracts. However, they could only partly explain the predicted toxicity by using phenol, fatty acid and resin acid concentrations in their regression model.

The correlation of the TOC content and luminescent bacteria toxicity was not obvious in the screening study (Fig.3 in Paper I). This could be explained partly by some highly toxic samples from TCF bleaching, which might have contained some peroxide residuals. Unfortunately these could not be chemically analysed. The eukaryotic mitochondrial particle test and luminescent bacteria test were poorly intercorrelated ($r = 0.266$, data not shown).

Table 8. Correlation of the overall toxicity index (TI) of the pulp mill waste waters (n = 15) with chemical variables in the first phase of the study (Verta *et al.* 1996).

Chemical variable	Correlation coefficient	p-value
COD	0.928	0.001
BOD	0.803	0.001
TOC	0.774	0.001
Total phenols	0.755	0.001
DCM extracts	0.743	0.01
Fatty acids	0.693	0.01
AOX	0.605	0.01

The results indicate that the natural substances in the wood, and their transformation and degradation products, could be the bioactive compounds in pulp mill wastewater. Especially wood debarking waters and black liqueur condensates, which contain high amounts of these mostly unknown substances (Axegård *et al*., 1993), appeared to be acutely toxic. The toxicity of TCF and ECF bleaching wastewater appeared to be a result of many bioactive substances rather than a few specific compounds. These and other compounds from the pulp bleaching may be eliminated by suitable biological secondary treatment. This may be the primary reason for the observed reduction in effluent toxicity.

For the above-mentioned reasons it is very difficult to predict the environmental impact of novel pulp mill effluents on the aquatic biota by using only defined chemical analysis. Laboratory toxicity tests will continue to play an important role in directing management decisions regarding the environmental effects of pulp mill process modifications (Kovacs and Megraw 1996). Until we know more about the possibly harmful substances originating from wood, the use of bioassays in the assessing of pulp mill effluents is recommended. A simple set of biotests such as the algae, *Daphnia* and luminescent bacteria assays could be used. The luminescent bacteria test appears to be the best for preliminary screening and classifying effluent toxicity. Furthermore by combining the bioassays and effluent fractioning, some of the problematic compound groups could possibly be identified and the processes could be improved. This kind of toxicity identification and evaluation (TIE) approach can be applied to industrial effluents or to any complex mixture (Kszos *et al*. 1992, Ankley and Burkhard 1992). Furthermore, a similar strategy (toxicity reduction evaluation, TRE) can be used to guide and improve the process and the wastewater treatment for effective removal of toxicity (USEPA 1988).

Toxic loads from pulp and paper mill effluents to recipients were compared with effluents from chemical, metal and textile industries (previously unpublished data) . The toxicity (TU) and the toxic

loads of secondary treated effluents are presented in Table 9 as toxicity emission rates (TER). Certain effluents of the metal and textile industries were very toxic especially to algae (highest TU = 50). However, the effluent toxicity and possible environmental effects should always be considered together with the effluent flow and receiving water body. The effluent flow from pulp and paper mills is often great compared to other industries and hence even sporadic toxicity can cause significant effect in the recipient. The sensitivity of different tests varies depending on the toxicant composition in the effluent. As seen in this limited data, effluents from the metal and mining industry (six samples) or textile industry (six samples), possibly containing heavy metals were occasionally especially toxic to algae. The luminescent bacterial test and the algae growth inhibition tests have often been observed to be the most sensitive tests in routine monitoring of the toxicity of industrial effluents (Diehl *et al*. 1995). This has also been stated by Wängberg *et al*. (1995) who studied the toxicities of various industrial waste waters. Especially an algal test battery with different phylogenetic groups of microalgae in multiwell plates increased the sensitivity to detect toxicity compared to standard algae tests.

However, some of the bioactive compounds in pulp mill effluents can have potential long term effects (reproduction and community changes) on aquatic biota. These impacts on community structure and function cannot be assessed by short term laboratory toxicity tests. The zebra fish egg and sac-fry tess give some indication of chronic effects but usually model ecosystem studies (mesocosms) or long scale monitoring data are required to assess community changes.

The long term effects of ECF and TCF bleaching wastewaters, secondary treated effluents, natural humus water and treated municipal effluent on the microbial biomass (total cell number) development in the mesocosm studies (Paper II) is presented in Fig. 3. Compared to the variation of the two control mesocosms no drastic or statistically significant effects (two-way ANOVA) of the waste waters was detected. Bacterial counts varied between 2 x 10^6 and 8 x 10^6 cells per ml. The untreated wastewaters occasionally increased the biomass in the water column compared to controls during the summer periods. This could be due to

Table 9. Effluent flow (Q), toxicity (TU) and loads (TER) of treated effluents from various industries assessed by different biotests (compilation from unpublished data).

Industry (number of plants)	Number of samples	Q ($1000m^3/d$)	*P. putida* TU TER	*V.fischeri* TU TER	Algae TU TER	*Daphnia* TU TER
Pulp and paper (n = 12)	24	22.7–115	nt*–10 0–363	nt–2.1 0–50	nt–2.9 0–188	nt–1.5 0–36
Chemical (n = 4)	8	0.5–21	nt–10 0–6	nt–8 0–75	nt–33 0–110	nt–3 0–22
Metal and mining (n = 3)	6	0.3–29	nt–2.7 0–1	nt–1.4 0–30	nt–33 0–970	nt–3 0–39
Textile (n = 3)	6	0.03–0.15	nt–2.5 0–0.07	nt–1.9 0–0.26	nt–50 0–1.4	nt–2.9 0–0.33

*nt = non-toxic

the nutrients and dissolved carbon in the wastewaters. However, at the end of the growing seasons no statistically significant ($p > 0.05$) differences between mesocosms were detected.

The active microbial biomass as ATP content in sediment and its seasonal fluctuation in mesocosms exposed to 1/400 dilutions of sample water is presented in Fig. 4. The sediment biomass showed elevated fluctuation compared to controls during the one and a half year experiment in all the mesocosms. Occasionally, the biomass of the two control mesocosms differed, but the difference was not statistically significant (*t*-test, $p > 0.05$). The difference between controls might be due to the different light and temperature conditions of these mesocosm basins, as seen in an earlier study (Lehtinen *et al*. 1997).

Alkaline phosphomonoesterase (PME) activities in the surface sediment in mesocosms exposed to 1/400 dilutions of sample water are presented in Fig. 5. There were no overall statistically significant effects ($p > 0{,}05$) of different waste waters on the enzyme activity. However, the same kind of increased oscillation could be detected in treated mesocosms compared to control mesocosms as in the sediment microbial biomass. Often a relationship between sediment surface phosphatase activity and chlorophyll suggests that benthic algae might be a major source of phosphatases (Chappel and Goulder 1994), or that there exists a link between algal activity and bacterial phosphomonoesterases. It has been suggested that the exudated organic carbon from the photosynthetic algae directly enhances the phosphatase activity in the same sediment biofilm (Espeland and Wetzel 2001).

In this study the PME activity did not decrease during the winter, under ice and snow cover. In some mesocosms (ECF HD, ECF LD) the highest peaks in activity (four times higher than controls) were measured on 5th March under the thick ice cover. On this single sampling occasion the ECF LD and ECF HD mesocosms differed significantly from the controls (ANOVA: $p = 0.02$ and $p < 0.01$ respectively). This may be due to a very early diatom algal bloom or to higher bacterial metabolic activity during the winter. The mesocosm exposed to municipal effluent (MUN) reached the highest enzyme activity in spring (three times higher than controls on 6th May statistically significant by *t*-test, $p < 0.05$) but the activity collapsed during the summer. At the same time this mesocosm appeared to pass through nutrients, especially nitrogen (Tana *et al*. 2002). The system lost more ammonium than was introduced and consumed about 80 % more nitrate than controls. This negative ammonium value could be a clear sign of degradation of the biomass produced.

Exposure to the waste waters did not change the qualitative diversity of enzyme activities measured by API Zym strips (data not shown here). Almost the same enzymes gave positive reactions both in the mesocosms exposed to bleaching waste water and in the control mesocosms. Untreated TCF wastewater and naturally humic water gave the highest diversity of positive reactions of different enzymes. A possible explanation for this could be that these waters contained higher amounts of different substrates and carbon sources than were received by the other mesocosms. However, the changes in enzyme profiles between sampling occasions were inconsistent and difficult to interpret. Hence no statistical evaluations could be applied. The inconsistency of the results might be due to the fact that the enzyme activities chosen for this commercial kit originate from clinical microbiology and their relevance to aquatic enzyme processes may be limited. However, the API Zym kit yielded approximately similar results to the quantitative measurements of the PME activity.

The toxic effects of the bleaching wastewaters could be observed in the laboratory tests (Paper I). This toxicity to microbial biota could not be clearly statistically demonstrated in the mesocosm studies (Paper II). The varying environmental conditions and the exposure were together the most important factors affecting the microbial variables in the mesocosms. The methods used assess mainly the changes in total biomass or microbial activity. Hence these measurements will only reveal severe harmful effects, and fail to indicate the possible harmful changes in the structure of the microbial community. The changes in the microbial community structure may result in long term effects on nutrient cycling and on food webs. However, when the two main stress factors caused by waste waters were toxic and eutrophying substances, the effects were sometimes hard to interpret. How it is possible to discriminate between toxic and eutrophying responses? Eutrophication should not be understood as increased growth rates of all biota. A clearly beneficial nutrient or carbon source increase for one species may be detrimental to other organisms.

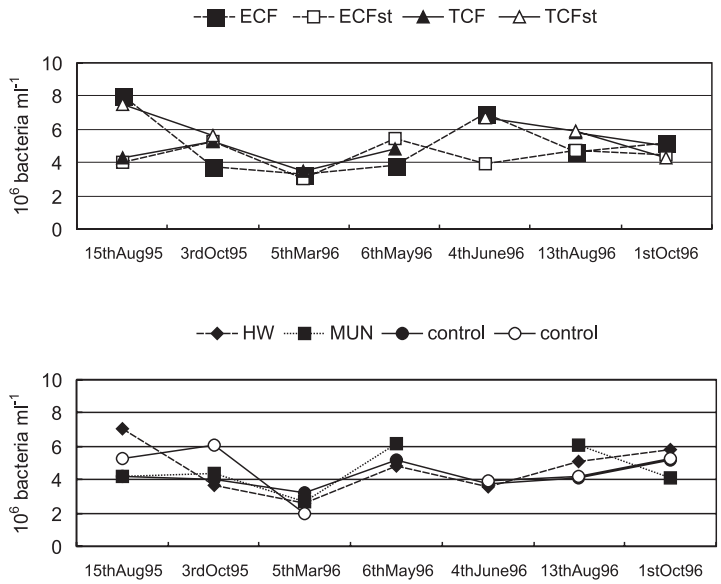

Fig. 3. Microbial biomass (mean of two samples) of the water columns exposed to 1/400 dilutions of sample waters during the mesocosm experiment. (ECF = ECF bleaching wastewater, ECFst = treated ECF effluent, TCF = TCF bleaching wastewater, TCFst = trated TCF effluent, HW = natural humus water, MUN = treated municipal effluent),

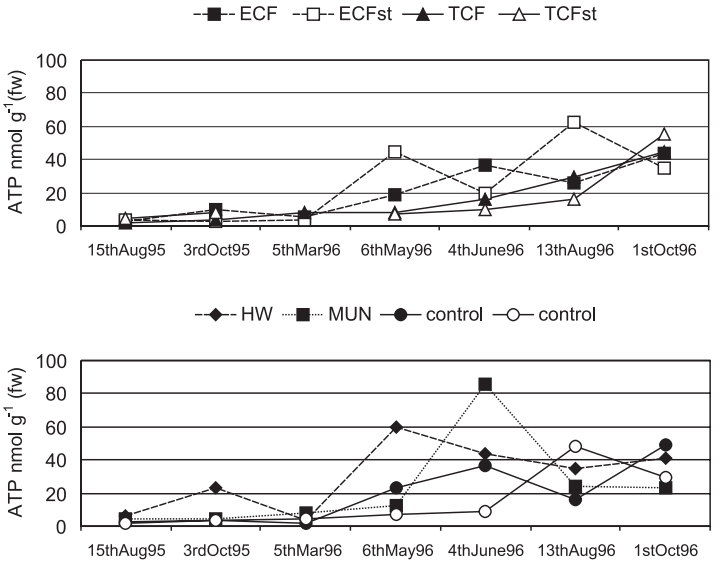

Fig. 4. The mean microbial biomass in the sediment (mean of two samples) in different mesocosms exposed to 1/400 dilutions of the sample waters during the experiment. Symbols of the sample waters as in Fig. 3.

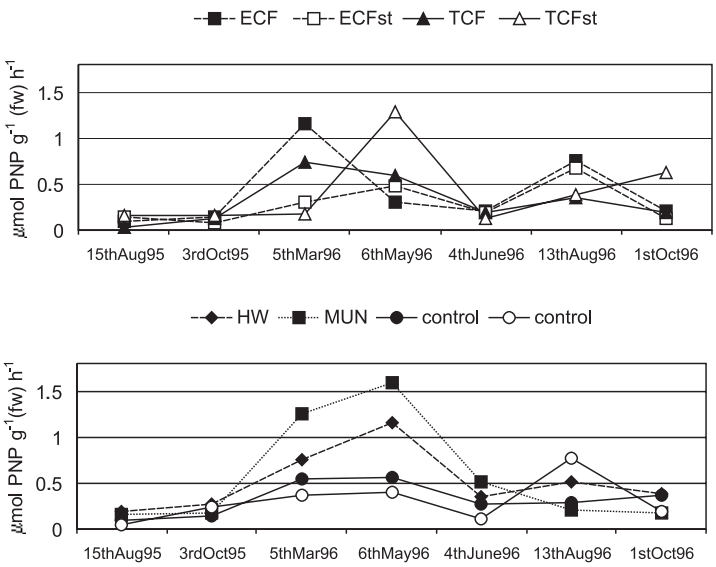

Fig. 5. The alkaline phosphomonoesterase (PME) in the sediment (mean of two samples) in different mesocosms exposed to 1/400 dilutions of the sample waters. Symbols are as in Fig. 3.

After the initial characterization of effluent toxicity and possible effects on the biota by a larger set of bioassays representing different trophic levels, some more simple tests can also be applied for routine toxicity monitoring and generally no acute toxicity should be detected in the secondary treated effluents of modern pulp mills. Biotests, and among them microbial tests can provide a useful tool for the environmental hazard evaluation of mixed industrial effluents possibly containing unknown harmful substances. Microbial tests also have an important role in the identification and evaluation of toxic substances and in guiding toxicity reduction by improving industrial or treatment processes. The biological quality surveys in the recipient or in extensive mesocosms studies such as in this study contain much environmental variation and the possible detrimental effects of pollution are difficult to observe. This environmental variation is meant to be partly controlled and exactly documented in mesocosm studies. However, the experimental design (enough replicates to have statistical power) must be compatible with the available resources and with financial realities.

3.2 Toxicity assessment of polluted soils

The toxicity of chlorophenol contaminated soil and wood chips decreased during composting as assessed by both modifications of the luminescent bacteria tests (Paper III). This decrease of toxicity followed decreasing chlorophenol concentrations in the compost piles (Table 10).

Chlorophenols, as direct un-couplers of oxidative phosphorylation and hence of energy production for luminescence, are usually highly toxic in luminescent bacteria tests. The EC50 concentrations for several chlorophenols are between 2 and 5 mg l^{-1} (Ribo and Kaiser 1987). In this study about 200 mg total chlorophenols per kg dry weight soil appeared to be the threshold value for chlorophenol toxicity in the tests. The reason for this high threshold value for toxicity, is most probably the limited bioavailability of chlorophenols. Strong adsorption to soil organic matter reduced the toxicity of pentachlorophenol in studies by van Gestel and Ma (1988) and probably also of other chlorophenols. This has also been documented in microcosm studies in which the effects of PCP (50 and 500 mg per kg d wt soil) on soil invertebrate

Table 10. The total chlorophenol concentration and the toxicity as Soil Toxicity Units (STU) of soil elutriates in the standard luminescent bacteria test and as luminescence inhibition in the soil contact test during composting of the heavily contaminated pile (source: Paper III).

Sampling time (weeks)	Total chlorophenols (mg/kg dry wt)	Reduction from start (%)	STU of soil elutriates (%)	Reduction from start (%)	Inhibition in soil contact test
0	1800	-	2000	-	na*
3	2400	-	1000	50	na
5	1800	-	2000	-	100
7	1100	38	400	80	75
9	350	81	250	87	55
13	500	72	200	90	45
17	100	94	0	100	0
21	180	90	0	100	0

* na = not assessed

Table 11. Soil toxicity as soil toxicity unit (STU) assessed by the solid phase luminescent bacteria test (LUM Solid), standard luminescent test (LUM Elutr.) and *P. putida* growth inhibition test (PGI Elutr.); and total PAH contents ($mgkg^{-1}$), and their reduction (%) in inoculated and non-inoculated control piles at the beginning of the experiment, after the first summer and at the end of the next summer.

		At start	After 1st summer	After 2nd summer
Inoculated pile				
	LUM Solid	1540	714 (54%)	285 (81%)
	LUM Elutr.	500	154 (69%)	100 (80%)
	PGI Elutr.	3330	222 (93%)	91 (97%)
	Total PAHs	23600	31800 (-)	5500 (77%)
Control pile				
	LUM Solid	1540	286 (81%)	250 (84%)
	LUM Elutr.	500	33 (93%)	100 (80%)
	PGI Elutr.	3330	222 (93%)	100 (80%)
	Total PAHs	23600	10120 (57%)	45600 (81%)

communities and microbial activities were assessed (Salminen *et al*. 1995). Only the high concentration (500 mg kg^{-1}) reduced microbial activities as measured by soil ATP content and soil respiration.

The toxicity and the total PAH content of soil in a two-year period of bioremediation of soil heavily contaminated with polyaromatic hydrocarbons in the smaller pilot pile composting are presented in Table 11 and in Paper IV. In this smaller pilot study the toxicity assessed by the solid phase luminescent bacteria test, the standard luminescent test and the *P. putida* growth inhibition test did not decrease in the inoculated pile as much as in the control pile. The increase of total and some identified PAHs in the inoculated pile during the first summer composting was also noteworthy (Paper IV, Table 2). A probable explanation for this was the heterogeneity of the compost. The tarry creosote was unevenly distributed in the pile, which made representative sampling of the pile difficult. However, increased toxicity was not observed in

the toxicity tests after the first period, although the PAH concentration increased. After the second summer almost complete degradation of PAHs and similar reduction in toxicity was observed in both the pilot piles.

In the larger scale case study fluctuating but generally decreasing toxicity was also recorded during the five months of composting (Fig. 6). The plateau phases in degradation were also detected by all toxicity tests as temporarily increasing toxicity. The explanation could be the increase of individual PAH compounds with higher toxicity, such as benzo(a)pyrene (Table 4 in Paper IV). Certain unknown transformation products, other aromatic hydrocarbons with high toxicity, or degradation to more toxic or more bioavailable individual compounds could also cause increased toxicity. Ultimately the reduction of PAHs was almost 80% and the decrease in toxicity could be seen with both modifications of the luminescent bacteria tests but not so clearly with the *Pseudomonas* growth inhibition test. The toxicity of the soil to *P. putida* was rather low throughout the composting. However, the observed oscillation of toxicity based on STU units correlated well with the other tests. The Toxi-Chromo Pad test indicated similar reduction of toxicity to enzyme synthesis in bacteria (Paper IV, Table 5). After two and a half weeks of composting no inhibition was observed.

The toxicity and chemical data gathered from these case studies on chlorophenol and PAH contaminated soils is rather limited (pooled single samples) and descriptive. Hence strong statistical conclusions cannot be drawn. The microbial toxicity tests, especially the solid phase luminescent bacteria test, proved to be a practical tool for assessing the toxicity during bioremediation of creosote and chlorophenol contaminated soil. Even if the high metal content causes its own basic level of toxicity during composting, it did not mask the toxicity caused by changing concentrations of organic contaminants. The semi-quantitative or more qualitative Toxi-Chromo Pad test also gave indication of reduced toxicity during composting. However, in the final assessment of the environmental risks of contaminated or bioremediated soil, a set of different trophic level biotests should be used (Maxan *et al*. 2000) and other environmental concerns, such as genotoxicity (Belkin *et al*. 1994) should also be evaluated.

As in the case of effluent assessments, the biotests have the value of being applicable to guiding the contaminated soil treatment processes, and they should be used together with chemical analysis in the final evaluation of remediated soil. The soil matrix definitely requires the evaluator of the soil quality to more adequately consider many factors such as the contaminant distribution, homogeneity and bioavailability compared to effluent risk evaluation. However, many similar uncertainty factors of sampling and analysis are involved both in chemical analysis of defined compounds and in the determination of the limit concentrations of substances, as in using biological toxicity tests.

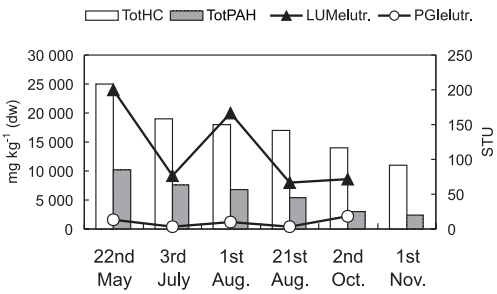

Fig. 6. The total hydrocarbon (TotHC) and total polyaromatic hydrocarbon (TotPAH) content of the pooled soil sample during the five month composting period and the toxicity of soil elutriates in the luminescent bacteria (LUMelutr.) and in the *Pseudomonas* growth inhibition(PGIelutr.) tests as soil toxicity units (STU) in the larger scale pilot composting.

3.3 Effects of agrochemicals on soil microbes

The different pesticide treatments (Paper V) did not have remarkable effects on soil microbes measured by soil respiration, nitrification and soil ATP content in the field studies (Fig. 7 and in Paper V Figs. 1 and 2 for both studied seasons). The microbial activity and biomass mainly followed the weather conditions. The study strategy simulated realistically normal crop production processes, and the sampling program, experimental design and analyses of the field samples were planned to assess the possible acute and long term effects of normal pesticide use. However, because of the large temporal and spatial scale of the field experiment the variability of the environmental

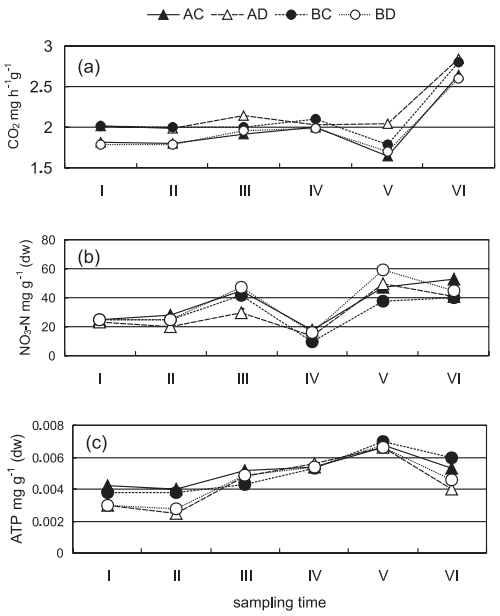

Fig. 7. The seasonal succession of soil respiration (a), nitrification potential (b) and soil ATP content (c) as means of six replicates in four different pesticide and cultivation treatments (AC = conventional cultivation and full insurance pesticides, AD = conventional cultivation and no pesticides, BC = lighter cultivation and full insurance pesticides, BD = lighter cultivation and no pesticides) during the summer of 1993.

factors (aerial soil heterogeneity, weather) did not allow strong statistical interpretation. The observed changes were usually not statistically significant. The only statistically significant difference (ANOVA: $p < 0.02$) was the contemporary elevated nitrification potential after the chlorsulfuron herbicide treatment. Hence it was not possible to conclude that the reduced and supervised pesticide use (AD, BD) was less harmful to soil microbes than the conventional use (AC, BC), as did Jones *et al.* 1992 in a similar study. On the other hand, it must also be understood that the AC and BC treatments with pesticide use represent the accepted and normal (and not extreme) barley production conditions in Finland.

Many pesticides have been assessed for their side-effects on carbon and nitrogen transformation in soils. Anderson (1992) reported studies in which the effects of 68 different pesticides at normal, fivefold and tenfold doses on soil carbon and nitrogen mineralization processes were assessed. Only 5 out of 68 pesticides caused a decrease of 15 % in nitrification potential at the normal dose. Moreover only 15 of the pesticides studied had some effects even at the highest doses. The pesticides were not named and probably none of them were the same as those used in this survey.

In the laboratory soil respiration inhibition tests, some effects of the propiconazole would have been expected (Table 12) on the basis of toxic

Table 12. Toxicity of crop protection chemicals in bacterial toxicity tests (EC50) and in the soil respiration inhibition test (LOEC) in the same soil as in the field studies. (From Paper V).

Pesticide	Use	Toxicity		
		Luminescent bacteria EC50 (mg l^{-1})	*Pseudomonas* growth EC50 (mg l^{-1})	Soil respiration inhibition LOEC (mg l^{-1})
Chlorsulfuron	herbicide	5	60	ne
MCPA	herbicide	1.25	62.5	ne
Propiconazole	fungicide	1.25	125	125
Dimethoate	pesticide	4	200	ne
Pirimicarb	pesticide	25	250	ne

ne = no effects observed

responses in the luminescent bacteria test, in which the EC50 value was 1.25 mg l^{-1}. However, the only effective concentration of propiconazole was 125 mg kg^{-1}. During the field experiment propiconazole was found in the surface soil at a maximum concentration of 2 mg kg^{-1} (data not shown). Hence the lack of clear field effects on soil microbes was reasonable. The fungicide propiconazole selectively affects fungi, and it has been reported that it decreased the length of fungal hyphae in a field test (Emholt 1992). However, agricultural soils tend to have bacterial-based energy channels rather than fungal mechanisms as in forest soils (Moore and Hunt 1988). In one earlier laboratory study, propiconazole also caused dose-response effects on soil respiration only at concentrations higher than those used in the field (Emholt 1992).

In the field studies and in the laboratory assessments (soil respiration, nitrogen and carbon mineralization processes), a natural heterogenic population of soil microbes was exposed to the chemical for various periods of time. If there were sufficient numbers of resistant species or taxonomic groups in the exposed community, the activity of these microbes could camouflage the possible effects on other, more sensitive microbes. This was not the case with single species tests, which could be seen as more sensitive but which are considered to be ecologically less relevant. In agricultural soils the key microbial processes in nutrient circulation should be assessed. It should also be understood that one intention in cultivation is to control the diversity of the microbial community for more favorable crop production conditions, for example avoiding denitrification and fungal diseases.

The high clay (>70 %) and organic matter (2.6–4.1 %) contents of the studied soil appeared to have an effect on the bioavailability of the chemicals, hence reducing the exposure of organisms to the pesticides studied. The rapid reduction of soil toxicity to luminescent bacteria could be seen in the bioavailability assays (Fig. 8). The rapid toxicity reduction during one day could not be explained by the degradation of the chemicals and it was probably due to rapid adsorption to soil particles and hence reduced bioavailability. This kind of limited bioavailability of dimethoate has also been observed in studies on soil fauna (Martikainen *et al*. 1998). In the same study dimethoate was also documented to degrade rather rapidly .

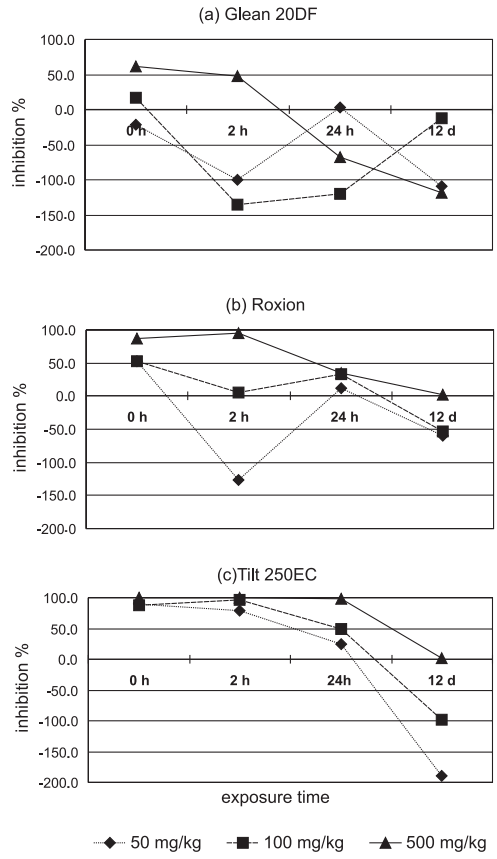

Fig. 8. The toxicity of soil 0 h, 2 h, 24 h and 12 days after three different pesticide treatments with Glean 20 DF herbicide, Roxion insecticide and Tilt 250 EC fungicide in different concentration of the product.

The Finnish boreal environmental conditions and the poor bioavailability of chemicals in soil may lead to poor biological degradation of substances and possible accumulation of pesticide residues in soil. Whether this is a risk, especially when a field is turned to forest or pasture (changes in pH, organic carbon content and soil structure), should also be carefully assessed. The overall use of pesticides has mainly been decreasing during the past twenty years in Finland (Hynninen and Blomqvist 2001). Today, new pesticides are very

efficient. For example only 4 g of chlorsulfuron herbicide is needed for an area of one hectare. This sets high demands for the chemical analysis of trace amounts of modern pesticides and their residues in soil.

The field assessments of the effects of chemicals on soil microbes can be evaluated by combining the chemistry, biovailability data, soil and substance toxicity and observed biological responses. The study design is very important in field experimental work and the varying environmental conditions can interfere with treatment responses. In the case of a severe contamination detrimental effects on soil microbial biomass and activity have been seen, as in the case of airborne heavy metal contamination measured by similar methods to those used in this study (Vanhala and Ahtiainen 1994). However, the initial risk assessment of pesticides will also mainly be based on the results of harmonized laboratory studies and toxicity tests in future chemicals policy. The field assessments have their value in more definitive ecological research as an effort to combine and understand the effects observed on various parts of the ecosystem food webs.

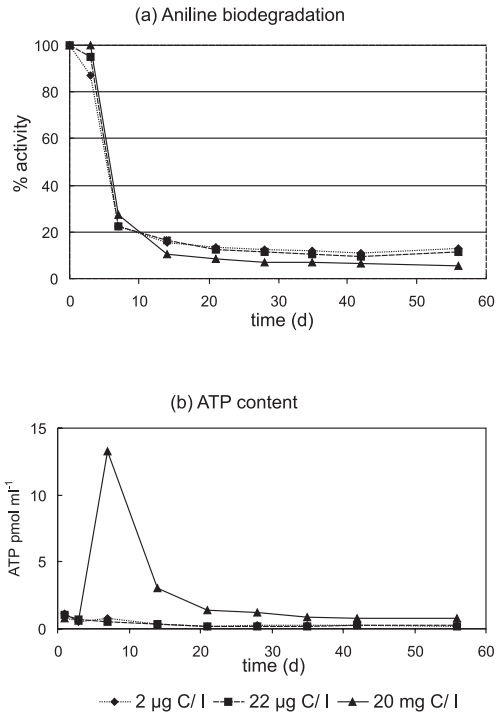

Fig. 9. Biodegradation curves (% of added activity remaining) of aniline in different concentrations in the activated sludge in standard test conditions and development of biomass measured as test solution ATP content.

3.4 Biodegradation of chemicals

The results of this study partly support the theory that low concentrations provide different biodegradation kinetics than concentrations used in the standard tests (Paper IV, Table 2). The degradation of the studied reference chemical aniline at different concentrations was always rapid in the standard test conditions (Fig. 9) compared to rather slow degradation of chloroaniline (Fig. 10). The high concentration (25.9 mg l^{-1}) of biodegradable aniline in standard test conditions with activated sludge appears to support exponential growth of degraders (Fig. 9). Unfortunately, the biomass was not monitored in this particular experiment by ATP measurements during chloroaniline biodegradation, but it usually remains rather low (data not shown). However, the source of the inoculum appeared to be an even more important factor affecting biodegradation rates than the concentration of the tested chemical. A broad range of biodegradation rates (Table 2 in Paper VI) and half-lives (Fig. 11) was observed for both tested reference substances at different concentrations with various sources of the inocula.

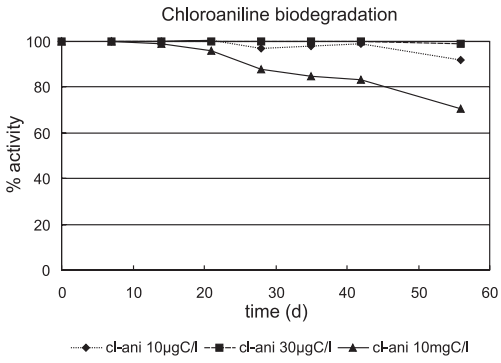

Fig. 10. Biodegradation curves (% of added activity remaining) of chloroaniline in different concentrations in the activated sludge in standard test conditions.

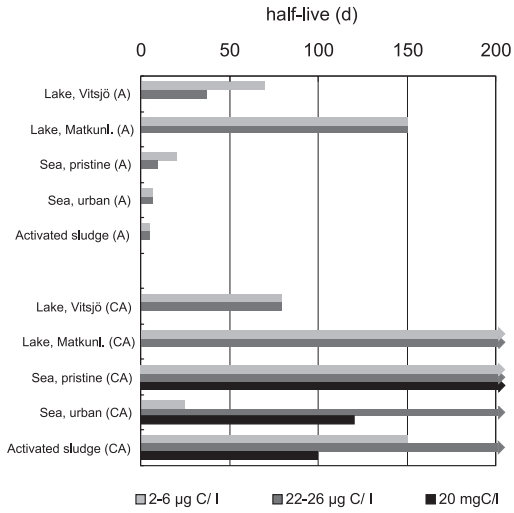

Fig. 11. The half-lives of aniline (A) and chloroaniline (CA) in different waters tested in various concentrations.

Aniline is well known as a readily biodegradable substance in standard test conditions (Painter 1995) and almost total degradation was also observed observed in a eutrophic lake (Subba-Rao *et al*. 1982). In this study aniline degraded rapidly in the ISO standard test conditions and in urban sea water, also at low concentrations of 0.002 mg l^{-1} (half lives 5–8 days). Aniline satisfied the criteria for ready biodegradability reaching 60 % mineralization of the theoretical carbon dioxide production. In activated sludge and in urban harbour sea water the aniline degraders appear to be present in rather high numbers. However, degradation was clearly slower in unpolluted sea water (half lives 10–20 days) and the high concentration of aniline (25.9 mg l^{-1}) yielded a broad range of rather inconsistent half lives of 40–150 days. The higher concentration might already have been close to toxic levels for some microbes. In natural lake waters degradation was generally much slower. In oligotrophic lake water the half lives at low concentrations varied from 35 to 70 days. In a humic lake with high organic carbon content the aniline degraded poorly. The reason could be that the microbes are adapted to degrade only the natural carbon pool present in the lake and not aniline-like compounds.

Chloroaniline degraded very slowly in all natural waters and often the degradation even failed, e.g. in pristine sea water and in humic lake water. The fastest degradation was observed in urban harbour water with half lives between 15 and 120 days. This range was wide and the results were rather inconsistent. Similar inconsistency in biodegradation half-live, with more recalcitrant compounds was earlier observed by Ingerslev and Nyholm (2000). In standard biodegradation test conditions the lag phases have also been long and sometimes the biodegradation has even failed.

The biodegradation rates of the chemicals tested in Finnish natural waters appeared to be lower (near zero especially with 4- chloroaniline) than the rates documented in some other countries (Nyholm and Toräng 1999). An important factor for degradation in natural environments is the number of potentially adapted degrading microbes present. If the discharge of a certain compound is continuous the microbial population will develop the capability to mineralize these molecules. In pristine environments the variety of biodegradation potential for man-made substances is probably more limited. This could clearly be seen in this work when comparing the pristine (Tvärminne) and urban sea (Helsinki harbour) areas. In wastewater treatment the most important factor in the establishment of an adapted population, in addition to continuous discharge of the compound, is the sludge retention time (van Ginkel *et al*. 1995). This time defines the age of the activated sludge and how often it will be renewed.

The source of the inoculum appeared to be more important factor influencing the rate of biodegradation than the concentration of the tested compounds in this study. However, the data were very limited, and especially in standard test conditions with activated sludge, when the biomass was also assessed by ATP analysis, different growth kinetics of the microbes were observed depending on the tested concentrations (Fig. 9). The concentration normally used in the standard tests (20 mgC l^{-1}) caused rapid and exponential growth of microbial biomass. Aniline, a readily degradable substance, degraded well in activated sludge regardless of the concentration. In pristine natural water the difference in degradation rates between 0.002 and 0.022 mg carbon contents of the tested compound per litre could be indicated. 4-Chloroaniline appeared to be such a poorly degradable compound in Finnish conditions that the effect of con-

centration on the degradation rates could not be clearly established.

Technically, the these modern biodegradation testing methods functioned well. The ISO 14593 headspace carbon evaluation test (Battersby 1997) provides a modern, practical "ready biodegradability" screening method with rather stringent conditions with which volatiles and poorly soluble substances can also be tested. The shake flask batch test with radio-labelled compounds (Nyholm *et al.* 1996, Ingerslev *et al.* 1998) can produce data simulating many environmental compartments such as natural surface waters, but also activated sludge at environmentally relevant concentrations.

Biodegradation testing is an important tool in estimating the exposure of biota in various environments to chemicals for the risk assessment. It has great value in conjunction with the chemical analysis and toxicity tests in effluent studies. If an effluent gives a toxic response, due mainly to unknown organic substances, biodegradation information on this mixture of substances could provide guidance to treatment improvements, or help to estimate the exposure of biota in the environment receiving the effluent.

Because of the importance of biodegradation data to exposure estimations in risk assessment schemes, basic research on biodegradation kinetics in different environments is greatly needed. This should direct the harmonized test guideline development towards more environmentally relevant conditions to provide better estimates of biodegradation rate constants.

3.5 Applicability of microbial tests and measurements in environmental assessments

Microbial tests applied in toxicity assessment of industrial wastewaters (3.1 and Paper I), polluted soils (3.2 and Papers III and IV) and agricultural chemicals (3.3 and Paper V) yielded useful and relevant information (combined with the chemical analysis) on the possible environmental effects of complex mixtures of harmful substances and chemicals. The industrial wastewaters could be ranked for their toxicity using microbial tests. These tests appeared to be rather simple and convenient tools for toxicity screening of different in-mill wastewater streams. The bioremediation of chlorophenol and PAH polluted soils could be monitored and evaluated using the two modifications of the luminescent bacteria test. The potential microbial effects of pesticides used in the field studies could be screened more sensitively using single species toxicity tests than in indigenous microbial community tests such as the respiration inhibition test. The solid phase modification of the luminescent bacteria test appeared to be especially useful in bioavailability assessment of pesticides in soil.

Because of the above-mentioned observations the microbiological toxicity tests could be of use in the toxicity identification and evaluation (TIE) and toxicity reduction and evaluation (TRE) schemes applied in aquatic and terrestrial environments. The microbiological tests also have a role in the regulatory context, such as in pollution permits and in regulatory routine monitoring.

The microbiological single species (or strain) test methods often appeared to be the most sensitive tests compared with tests with other organisms in the wastewater studies (3.1 Paper I). The responses in microbial tests had some correlation with other tests and with the analysed chemical variables, and hence they could be used as surrogate analyses for screening purposes. However, the bacterial and algal tests had their intrinsic value in the set of biotests, as both groups of organisms have important functions in the ecosystems.

Microbiological biomass and activity measurements were applied for evaluating the effects of pulp mill wastewaters in aquatic mesocosm studies (3.1 and Paper II), and of pesticides in terrestrial field and microcosm studies (3.3 and Paper V), on microbial communities. The used measurements proved to be applicable and usually adequately sensitive to observe the changes, but the environmental variation and limitations in the experimental design did not enable strong statistical conclusions in either of the studies.

The study on biodegradation kinetics (3.4 and Paper VI) with the two reference compounds revealed some important differences between the standard biodegradation test method, routinely used in the biodegradation assessments, and the simulation test method. The rate of biodegradation depends clearly on the source of the inocula used in the tests, and also on the tested concentration of the compound in the test. This should be considered when estimating the half-lives of chemicals in various environments on the basis of the current data provided by the standard tests.

4 Summary and conclusions

The microbiological toxicity tests proved to be rather good indicators of effluent toxicity. The luminescent bacteria test and algae test were usually the most sensitive tests, was also reported in other studies. The use of biotests together with chemical analysis is to be recommended in evaluating and monitoring complex mixtures (such as pulp and paper mill effluents) containing unknown substances. The toxicity of pulp mill wastewaters correlated well with the total organic carbon content of the samples (TOC, COD, BOD) but less strongly with the AOX content. After secondary treatment, in which most of the carbon pool and toxic substances appeared to be degraded, the effluents gave only sporadic and weak toxic responses. The treated effluents from chemical, metal and textile industries were occasionally toxic. However, the effluent toxicity should always be considered together with the effluent flow and the hydrological characteristics of the receiving water body. The data on the wastewater or effluent toxicity can be used to guide the industrial processes or the wastewater treatment in toxicity reduction evaluation (TRE) schemes. By combining toxicity testing, sample fractioning and manipulation and chemistry the potentially toxic substances could be identified in complex mixtures as in toxicity identification and evaluation (TIE) strategy.

Unfortunately, the use of biotests in routine and regulatory monitoring has been rather limited in Finland. Most of the monitoring and regulatory permits for effluents are based on chemical analyses. The new Water Framework Directive 2000/60/EU sets demands for analysing certain harmful priority substances from the effluents and from the environment and for assessing the ecological quality in the environment. It does not directly support biological testing of effluents, although the biological effects on biota (ecological quality) is the main objective of the directive. Similarly, the Integrated Pollution Prevention and Control (IPPC) directive (96/61/EU) is based on chemical variables.

As in effluent studies, microbial tests (either on soil elutriates or directly in contact with soil) provide data for the final evaluation of remediated contaminated soil. The biotest can also guide the selection of the best available remediation techniques. If the soil is contaminated for example with known heavy metals it is rather safe to evaluate the environmental risk on the basis of chemical analyses. However, if the soil is contaminated by several organic substances and metals, which is often the case, the use of simple in the characterization and mapping of the contamination can be useful. In the case of pure heavy metal contaminations, biotests can be applied in the actual bioavailability testing in order to refine the exposure estimations.

Microbial measurements have an important role in the assessment of biological quality of aquatic or soil environments. Because of the other varying environmental factors such as weather, the minor effects on microbial communities are difficult to prove statistically. Microbial communities have a strong and sometimes rapid potential for community changes, which can mask the possible effects on sensitive microbes. If even slight significant effects are observed in the level of the whole community biomass or activity, these evidently affect the overall ecosystem functions, as seen in areas with strong heavy metal pollution. One practical option to assess community changes could be the measurement of functional diversity e.g. by enzyme activity profiles.

The effects on microbes should also always be considered in the risk assessment of chemicals. This is already the case for pesticides but should be broadened to include other industrial and household chemicals. In practice, biodegradation data is sometimes used in chemicals risk assessment as source of information on toxicity to microbes.

The biodegradation of chemicals is an important factor affecting the exposure of biota to chemicals. This fact has been noted in the EU technical guidance document for the risk assessment of chemicals. The scientific development in biodegradation testing has been rather slow despite the urgent needs. Often the interest of both industry and regulators has been the desire to keep the obligatory biodegradation testing as cheap and simple as possible. Hence, most of the existing OECD test guidelines have been developed to screen whether the chemical has the potential to be easily degraded or not. These test guidelines are not meant to predict the rate of biodegradation in the environmental compartments. The efforts to create a sound scientific basis for modelling of fate and behaviour of chemicals have often been rejected with the arguments that either the research project would last too long, whereas environmental legislation could not wait, or the results of the project would be too complex for practical use.

However, both basic science and test method development must in the future better meet the requirements of chemicals policy in biodegradation testing. As seen in this study and in other studies earlier, biodegradation of chemicals in the standard tests and in various environments differs sometimes significantly. The reasons for this, in addition to many environmental abiotic factors, are the high concentrations of the tested chemical in the standard tests compared to those in the environment, and the variability of inocula from different sources. Whether we can accept these differences should be considered, and the results observed in this and other studies should direct the testing strategy and test guidelines development towards simulation of more environmentally relevant conditions.

The microbial reactions in toxicity tests and in the environment do have some correlation with the reactions of other biota. In certain cases, especially in the screening or monitoring of the possible changes, for example in effluent quality or in soil remediation processes, they can serve as surrogate tests for toxicity to other biota. However, the microbial reactions have a value of their own. The microbes are needed in the environment to sustain the food webs and in various mineralisation processes.

In most cases environmental risk evaluation requires chemical analysis of the suspected contaminants, toxicity testing of the contamination (chemical, effluent, contaminated soil or sediment) and survey of the biological quality (activity, biomass, diversity). In this contex, when the importance of microbial processes and of the microbial link in the food webs in various environments is established, the microbial toxicity tests and microbial measurements in the environment should be included in the risk assessments of various environmental pollution problems.

Yhteenveto

Ympäristömme kemikalisoituu jatkuvasti. Suomeen tuodaan, tai täällä valmistetaan noin 28 000 vaaralliseksi luokiteltua valmistetta, jotka sisältävät yli 5000 vaarallisiksi luokiteltavaa yhdistettä. Lisäksi aineita kaukokulkeutuu maahamme ja aineet muuntuvat edelleen teollisuusprosesseissa, jätteiden käsittelyssä ja päästyään ympäristöömme, niin ihmistoiminnan kuin luonnon omienkin prosessien toimesta. Vain muutamien kymmenien aineiden päästöistä, kulkeutumismääristä tai pitoisuuksista ympäristössämme on tietoa. Haitallisten aineiden aiheuttamia ympäristöriskejä voidaan tunnistaa, arvioida ja hallita 1) mittaamalla aineiden pitoisuuksia ja hajoavuutta, 2) testaamalla aineiden vaikutuksia eliöihin myrkyllisyystesteissä tai 3) havainnoimalla niiden aiheuttamia muutoksia ekosysteemeissä. Näillä kaikilla menetelmillä on yksittäisinä puutteensa ja etunsa.

Mikrobeilla on tärkeä rooli aineiden kiertokulussa. Ne muuntavat ja hajottavat myös haitallisia aineita vaikuttaen näin merkittävästi muiden eliöiden altistumiseen. Ympäristön mikrobisto ja ekosysteemeille välttämättömät aineidenkierron prosessit ovat myös herkkiä kemikaalien ja haitta-aineiden vaikutuksille. Mikrobit voivat toimia paitsi aktiivisina haitta-aineiden hajottajina ja muuntajina, myös huomioitavina kemikaalien haittavaikutusten kohteina. Tutkimuksen tarkoituksena oli arvioida mikrobiologisten myrkyllisyystestien, ympäristöstä tehtävien mikrobiologisten mittausten sekä hajoavuustestien soveltuvuutta ja hyödyllisyyttä kemikaalien, jätevesien ja saastuneiden maiden ympäristöriskin arvioinnissa.

Jätevesien ympäristöriskejä on perinteisesti arvioitu ja pyritty hallitsemaan jäteveden sisältämien aineiden pitoisuuksien avulla. Kemiallinen analytiikka kykenee kuitenkin havaitsemaan ainoastaan tiettyjä kulloinkin analysoitavia aineita. Myrkyllisyystestien avulla voidaan pyrkiä arvioimaan myös tuntemattomien haitta-aineiden vaikutuksia ja aineiden yhteisvaikutuksia. Mikrobiologisten myrkyllisyystestien ja muiden biotestien soveltuvuutta erityisesti lukemattomia orgaanisia yhdisteitä sisältävien sellutehtaiden jätevesien tarkkailuun tutkittiin kemiallisen analytiikan rinnalla. Modernin sellutehtaan puhdistetun jäteveden ei tulisi olla akuutisti myrkyllistä, ja tähän myrkyttömyyden tarkkailuun yksinkertaisetkin biologiset myrkyllisyystestit näyttäisivät sopivan hyvin. Jäteveden sisältämillä aineilla voi olla myös pitkäaikaisvaikutuksia ja vaikutuksia, jotka välittyvät ravintoverkon tai aineiden kierron muutosten kautta. Näitä vaikutuksia pyrittiin selvittämään puolentoista vuoden malliekosysteemikokeilla. Malliekosysteemejä altistettiin jäteveden laimennoksin ja vaikutuksia tutkittiin ravintoverkon eri osiin mm. veden ja sedimentin mikrobibiomassaan ja -aktiivisuuksiin. Kuitenkaan tilastollisesti merkittäviä jätevesien haittavaikutuksia ei näissä kokeissa voitu havaita. Jätevesien kuormitus näkyi sekä rehe-

vöitymisenä, että vuosittaisten aktiivisuuksien ja biomassan vaihteluvälien (oskillaation) voimistumisena.

Mikrobiologisia myrkyllisyystestejä, mm. valobakteeritestin eri sovelluksia, käytettiin myös arvioitaessa kloorifenoleilla ja polyaromaattisilla hiilivedyillä saastuneiden maiden biologisen kunnostuksen onnistumista. Kompostoinnin aikana myrkyllisyys väheni yleensä samaa tahtia mitattujen haitta-aineiden pitoisuuksien vähenemisen myötä.

Viljanviljelyssä yleisesti käytettyjen torjunta-aineiden vaikutuksia maan mikrobistoon tutkittiin sekä laajoin kenttäkokein että laboratoriossa myrkyllisyystestein. Kenttäkokeissa ei pystytty havaitsemaan normaalilla torjunta-aineiden käytöllä tilastollisesti merkittäviä vaikutuksia maan mikrobibiomassaan tai aktiivisuuteen. Laboratoriokokein voitiin osoittaa eräiden torjunta-aineiden myrkyllisyys bakteeritesteissä, ja myös todeta muutaman valitun kemikaalin nopea sitoutuminen maa-ainekseen ja siitä johtuva myrkyllisyyden väheneminen.

Biologisen hajoavuuden standardisoitujen testien olosuhteet eroavat huomattavasti ympäristön olosuhteista. Nämä OECD:ssä ja ISO:ssa harmonisoidut testit on tarkoitettu aineiden luokitteluun helposti tai huonosti hajoaviksi, eikä niistä suoraan saa tietoa aineiden pysyvyydestä eri ympäristöissä. Nykyinen kemikaalien riskinarviointi tarvitsisi kuitenkin juuri tietoa aineiden hajoamisnopeuksista ympäristössä. Tässä työssä vertailtiin radioaktiivisesti leimatun kahden malliaineen, aniliinin ja kloroaniliinin, hajoamisnopeuksia standarditestiolosuhteissa ja eri vesiympäristöjen simulointitesteissä. Malliaineiden hajoamisnopeudet vaihtelivat riippuen ympäristön kuormitustasosta ja historiasta, sekä tutkittavan aineen pitoisuudesta ko. testissä.

Myrkyllisyystestit, ja niiden joukossa mikrobimyrkyllisyystestit sopivat hyvin jätevesien ja saastuneiden maiden tutkimiseen ja mahdollisen ympäristöriskin arviointiin kemiallisen analytiikan tukena. Yhdistämällä myrkyllisyyden testaus, näytteen hallittu manipulointi ja fraktiointi on mahdollista pyrkiä myös tunnistamaan myrkyllisyyden aiheuttajia sekä ohjata näin puhdistusprosesseja. Näitä periaatteita voi soveltaa niin jätevesiin, kuin haitta-aineilla likaantuneisiin sedimentteihin ja maihin. Arvioitaessa ympäristön ekologista laatua, kuten esim. EU:n vesipolitiikan puitedirektiivi edellyttää, mikrobibiomassan tai -aktiivisuuden mittaukset tulisi sisällyttää metodeihin niin vesikuin maaympäristössäkin, koska mikrobit ovat eri ekosysteemeissä tärkeä ravintoverkon toiminnallinen osa.

Kemikaalien riskinarvioinnissa joudutaan joskus käyttämään tietoa aineen biologisesta hajoavuudesta sen mikrobivaikutusten kertojana. Varsinaisia kemikaalien vaikutuksia esim. maan mikrobeihin velvoitetaan tutkittavaksi yleensä vain torjunta-aineiden kohdalla. Johtuen edellä mainitusta mikrobien tärkeästä roolista ekosysteemissä, mikrobivaikutuksiin tulisi kiinnittää entistä enemmän huomiota kaikkien kemikaalien riskinarvioinnissa ja ympäristövaaran luokittelussa.

Biohajoavuus itsessään on erittäin tärkeä kemikaalien tutkittava ominaisuus. Se vaikuttaa paljon ympäristön eliöiden altistumiseen kemikaaleille. Siksi riskinarvioinnissa tarvittaisiin tuloksia aineiden hajoavuusnopeuksista eri ympäristöissä. Näitä tuloksia ei saada suoraan nykyisistä harmonisoiduista hajoavuustesteistä, joiden olosuhteet on suunniteltu ainoastaan kemikaalien luokitteluun helposti, huonosti tai ei ollenkaan biologisesti hajoaviksi. Tulevaisuudessa menetelmien tulisikin antaa tietoa hajoavuusnopeuksista ympäristön eri olosuhteissa.

Yleensä haitta-aineiden aiheuttaman ympäristöriskin kattava arviointi edellyttää siis epäiltyjen aineiden kemiallista analytiikkaa, kuormituksen tai saastuneen alueen myrkyllisyyden testausta ja ympäristön biologisen tilan arviointia. Ymmärrettäessä mikrobien tärkeä rooli eri ekosysteemeissä mikrobimyrkyllisyystestit ja mikrobiologiset mittaukset ympäristössä tulisikin sisällyttää mukaan riskinarviointiin erilaisissa ympäristön kuormitus- ja saastumistilanteissa

Acknowledgements

This work was mainly carried out at the Research Department of the Finnish Environment Institute and most of the experimental work in the Laboratory of Finnish Environment Institute. I would like to thank Director general Lea Kauppi for providing good working facilities and support. The work has been financed by the Finnish Environment Institute, Ministry of the Environment and the Academy of Finland.

My warmest thanks to Dr. Maarit Niemi, the supervisor of this work. The encouragement and energetic and caring attitude of Dr. Maarit Niemi has been the backbone and driving force through-

out the study. She had the patience for gathering up my threads of thought and formulating them to ideas. Thank's for your style of guiding and stimulating without pushing! Prof. Seppo Niemelä originally gave me the enthusiasm to environmental microbiology together with Dr. Maarit Niemi. They both encouraged me to use microbiological methods in the fields and waters of ecotoxicology. I also thank all my co-workers and colleagues in our laboratory, in whole Finnish Environment Institute and in other institutes and research laboratories. Many people came close colleagues in these multidisciplinary research projects. Colleagues from Finnish Environmental Research Group (MFG), Agricultural Research Centre, in Universities of Helsinki, Jyväskylä and Joensuu and from Regional Environment Centres deserve my deepest thanks for the inspiring spirit in the projects. The international discussions in the ISO and OECD working groups have been important for opening my eyes to the gaps between science and environmental management.

Especially, BSc. Miia Aalto is thanked for skilful technical assistance and most of all for the patience towards all of my "great and not always so practical" innovations. Prof. Mirja Salkinoja-Salonen is warmly thanked for encouragement and pragmatic support throughout the writing of this work. The summary was greatly improved due to the comments of the reviewers Prof. Rauni Strömmer and Prof. Jussi Kukkonen. I am thankful to Michael Bailey for the educating way of revising the English. Siiri Kainulainen and Raili Malinen provided valuable help in finishing the layout for printing.

The familiar forest surrounding the laboratory in Hakuninmaa gave relaxing moments with birds, berries and mushrooms during the writing processes. Of course, the sea, fish, moments repairing and renovating greasy machines in our dusty and oily garage during the freetimes helped in the process. Most of all thanks to my parents for teaching the stamina to try to work hard and properly when needed, and sense to relax when possible. I thank also Elina for her love and patience to my absented mind on several occasions.

To my parents

Helsinki, May 2002 Jukka Ahtiainen

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Books

Tikkanen T. 1986. *Kasviplanktonopas* [Phytoplankton guide], Gaudeamus, Helsinki, 210 pp. [In Finnish].

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Cosby B.J., Homberger G.M., Wright R.F. & Galloway J.N. 1986. Modeling the effects of acid deposition: control of long term sulfate dynamics by soil sulfate adsorption. *Water Resour. Res.* 22: 1238--1291.

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ISSN 1239-1875
ISBN 952-11-1205-0
ISBN 952-11-1206-9 (PDF)