Interactions between Pesticides and Microorganisms in Freshwater Sediments

Toxic Effects and Implications for Bioavailability

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2005

Acta Universitatis Agriculturae Sueciae
2005: 23

ISSN 1652-6880
ISBN 91-576-7022-6

Tryck: SLU Service/Repro, Uppsala 2005

Abstract

Widenfalk, A. 2005. Interactions between pesticides and microorganisms in freshwater sediments – Toxic effects and implications for bioavailability.
Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7022-6.

In aquatic ecosystems sediment microbial communities provide many important functions, such as organic matter decomposition and by constituting a major food source for organisms at higher trophic levels. Sediments are also sites were pesticides have been frequently detected. In this thesis, laboratory microcosm experiments on the interactions between pesticides and microorganisms in freshwater sediments were performed.

Natural microbial communities were exposed to both environmentally relevant and high concentrations of different pesticides. In short-term exposures, pesticides decreased overall microbial activity at concentrations that are predicted to be environmentally safe. Surprisingly, short-term exposure to the high pesticide concentrations did not always affect the microbial activity. Long-term exposures (one month), were observed to induce different shifts in the microbial community composition, detected by using molecular methods, depending both on the type of pesticide and the concentrations applied. Hence, toxic effects of pesticides in microorganisms are not always straightforward and easy to interpret.

The microbial community of an artificial sediment used in toxicity tests was shown to have a low microbial activity, biomass, diversity as well as a different community composition compared to natural sediments. This could have implications for the fate of the test compound and the outcome of the toxicity tests, which may need to be considered when interpreting the toxicity test results.

Sediment bacteria and their extracellular polymeric substances (EPS) in biofilms were observed to increase the uptake and bioaccumulation of the hydrophobic pesticide chlorpyrifos to midge larvae, *Chironomus riparius*. This indicates that microorganisms and EPS may increase the bioavailability and be important vectors for the uptake of sediment-associated contaminants in aquatic food webs. Hence, the quality of the sediment organic carbon may need to be taken in consideration in toxicity tests and risk assessments of these pollutants.

This thesis shows that sediment microorganisms can be affected by pesticide exposure and that they can affect pesticide bioavailability. Therefore, the role of microbial processes should be regarded in risk assessments of pesticides.

Keywords: microbial communities, PLFA, T-RFLP, biofilms, EPS, toxicity tests, artificial sediment, hydrophobic contaminants, benthic invertebrates

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Appendix

Papers I - IV

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

I. Widenfalk, A., Svensson, J.M., Goedkoop, W. 2004. Effects of the pesticides captan, deltamethrin, isoproturon, and pirimicarb on the microbial community of a freshwater sediment. *Environmental Toxicology and Chemistry 23*, 1920-1927.

II. Widenfalk, A., Bertilsson, S., Sundh, I., and Goedkoop, W. Effects of agricultural pesticides on community composition and activity of sediment microorganisms – Responses at various levels of microbial organization. (Submitted manuscript)

III. Goedkoop, W., Widenfalk, A., Haglund, A., Steger, K., and Bertilsson, S. Microbial characterization of artificial sediment and comparisons with natural sediment – Implications for toxicity testing. *Environmental Toxicology and Chemistry* (Accepted)

IV. Widenfalk, A., Lundqvist, A., and Goedkoop, W. Sediment bacteria and microbial biofilms mediate the uptake and bioaccumulation of chlorpyrifos in *Chironomus riparius* (Chironomidae, Diptera). (Manuscript)

Introduction

Pesticides

In the world today, an ever-increasing amount and number of chemical substances are used in for example products, buildings, drugs and in agriculture. Only in the USA, more than 72,000 different chemicals are in commerce and each year nearly 3 trillion kg of organic chemicals are manufactured or imported (http://www.informinc.org/xsum_tox95.php, 22-Jan- 2005). Many of these chemicals are accidentally released into the environment, where they could harm organisms, ecosystems, as well as human health. Pesticides are a group of chemicals that are designed to exert toxic effects on their target organisms and that are deliberately spread in the environment to control various pests. Worldwide, it has been estimated that 2.3 billion kg of 1,600 different pesticides are applied yearly (Pimentel, 1995). In Sweden, almost 10 million kg of pesticides were sold in 2003 (KemI, 2003). A major concern with the vast use of pesticides is that they also may harm non-target organisms, primarily at the application site, for example soil organisms and birds. However, since pesticides unintentionally reach other ecosystems, in adjacent and remote (e.g. France *et al.*, 1997) areas, many non-target organisms are exposed. The most common unwanted spread for pesticides used in agriculture is to nearby aquatic ecosystems by spray-drift, surface run-off, or leaching (Kreuger *et al.*, 1999). Indeed, pesticides and their degradation products have been detected in both inland waters and their sediments (Miles and Pfeuffer, 1997, Sundin *et al.*, 2002, Ulén *et al.*, 2002) and estuaries (Steen *et al.*, 2001). Often, the environmental hazards of these pollutants have not been thoroughly investigated.

The effects pesticide exposure on the organisms of aquatic ecosystem by may be acute or chronic, as well as direct or indirect. Acute effects, for example death of organisms, are often easily detected in toxicity tests during assessment of the pesticide. Chronic effects, on the other hand, such as disturbances in behavior, decreased reproduction success, and changes in community structure are far more difficult to detect and are often promoted by long-term pesticide exposures at low concentrations. Both acute and chronic effects may result in altered abundances and taxon richness which may have serious implications for ecosystem health. A direct effect of pesticides on aquatic organisms is for example mortality and disappearance of zooplankton following insecticide exposure. This could lead to an indirect positive effect on phytoplankton due to a decreased grazing pressure. This in turn, may indirectly decrease the growth of macrophytes that experience increased shading. These relationships were nicely demonstrated in a couple of studies by Wendt-Rasch and coauthors (2003). They also concluded that these pesticide effects on aquatic ecosystems are often diffuse and confusingly similar to eutrophication effects and that these two types of stressors may interact. Thus, the complex biological interactions that exist in an aquatic ecosystem make it very difficult to predict the ecological consequences that could be caused by pesticide exposure.

The majority of pesticides used are organic molecules with hydrophobic properties. These hydrophobic properties cause a rapid sorption of the pesticide to soil particles that can be washed into the water. If the pesticides reach aquatic ecosystems in dissolved form, they quickly associate with organic matter in suspension or in the sediment. Sorbed contaminants tend to be less degradable than their dissolved counterparts, since they are less accessible to the degrading action of UV-light, dissolved oxidative chemicals, and microorganisms (Ying and Williams, 2000, Schwarzenbach *et al.*, 1993). This increased persistence following sorption and sedimentation, coupled to the extensive use of pesticides, may cause an accumulation of these compounds in sediments of freshwaters and estuaries. At the same time, sediments provide habitat for many organisms that play key roles in aquatic ecosystems, and which are inevitably exposed to these sediment-associated contaminants.

Pesticides and microbial communities

Sediment microbial communities, including benthic algae, bacteria, fungi, and protozoans, form the base of aquatic food webs and mediate important ecosystem functions like nutrient turnover, primary production, and decomposition (Palmer *et al.*, 2000). Some types of aquatic microorganisms are also capable of degrading organic contaminants and using them as an energy source (Larsson *et al.*, 1988, Allan *et al.*, 2004). Therefore, microbes might promote the remediation of polluted soils and sediments. Conversely, microorganisms have been used in inexpensive toxicity tests to assess the risks of pesticides and other contaminants. For example, tests have been performed with individual bacterial strains, e.g. Microtox™ (Kahru *et al.*, 1996), or by studying sumparameters of microbial communities, for example microbial biomass. These tests, however, may fail to detect changes that occur in the microbial composition, since tolerant microbes could compensate for the loss of functions associated with more sensitive groups (van Beelen and Doelman, 1997). Therefore, toxicity testing with single species or using sumparameters does not provide sufficient information regarding complex interactions between microorganisms.

If pesticides or other pollutants negatively affect aquatic microbes or alter their interactions, crucial links in nutrient regeneration could be disrupted, which may seriously affect ecosystem function. Analysis of the effects of pesticide exposure on the composition of microbial communities has long been limited by the fact that more than 90% of microorganisms in nature escape cultivation (Ward *et al.*, 1990). The development of culture-independent molecular methods, for example analysis of PLFA composition (Vestal and White, 1989) and genetic fingerprinting (Liu *et al.*, 1997), to analyze microbial communities has brought about new possibilities to also detect pollutant-induced changes in the compositions of natural communities (Rajendran *et al.*, 1994, Pennanen *et al.*, 1996). Despite the frequent occurrence of pesticides in aquatic ecosystems, the interactions between organic contaminants and non-target aquatic microorganisms have thus far been poorly studied (DeLorenzo *et al.*, 2001, Warren *et al.*, 2003). A few studies have addressed the effects of pesticides on aquatic microbes and found

impacts on structural and/or functional response variables (e.g. DeLorenzo *et al.*, 1999, Downing *et al.*, 2004, Petersen *et al.*, 2004).

Bioavailability to benthic invertebrates

Benthic (or sediment-dwelling) macroinvertebrates constitute a link between the base of the food web (microorganisms and detrital material) and higher trophic levels such as fish and waterfowl. Exposure of benthic organisms to pollutants occurs through several different routes, including overlying water, pore water, and ingestion of sediment particles (Fig. 1). Pesticides, especially insecticides, may cause toxic effects also in benthic organisms, since aquatic insect larvae make up a large share of the fauna of benthic communities. However, high concentrations of toxic compounds in the sediment do not necessarily lead to bioaccumulation and adverse effects in the organisms living in the sediment (Burton, 1992). Such a toxic response is dependent on the concentration of a toxic compound in the tissue or at a target receptor inside the organism. The entry of a contaminant into an organism is dependent on its bioavailability, that is the fraction of the total amount of a contaminant in a medium that is available to be taken up and become incorporated into living tissue. This bioavailability is dependent on several chemical, physical, and biological factors, such as the size and hydrophobic properties (often expressed as log K_{ow}) of the compound molecule (e.g. Pruell *et al.*, 2000), pH of the surrounding medium (e.g. Kukkonen, 1991), the quantity (e.g. Goedkoop and Peterson, 2003) and quality (e.g. Gunnarsson *et al.*, 1999) of the organic matter in sediment, as well as food selectivity, ingestion rate (e.g. Leppänen, 1995), and lipid content of the organism (e.g. Landrum and Fisher, 1998).

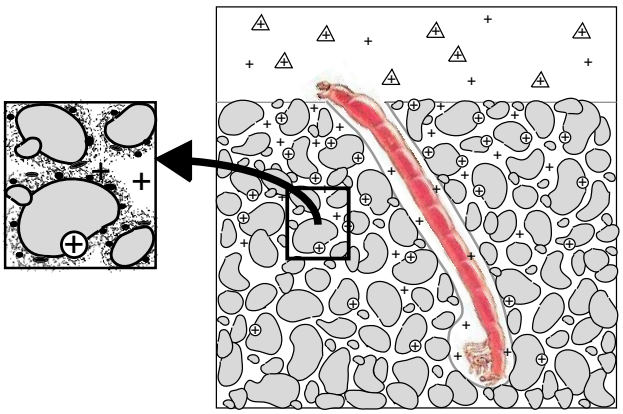

Fig. 1. Schematic overview illustrating the different pathways of exposure of contaminants to deposit-feeding invertebrates (here a *Chironomus* larva); from the overlying water (dissolved + and associated with dissolved organic matter (in triangles)) , from interstitial water (+), and/or sediment particles (circles). The box on the left shows a higher resolution of the contaminant in sediment showing bacteria (black coccs and rods) and the spheres of microbial extracellular polymeric substances (EPS) around the particles to which contaminants can sorb .

In water-only exposures of fish, the primary route of uptake of dissolved hydrophobic contaminants is mainly through the gills and to a lesser extent through the skin. Therefore, there is a strong relationship between the contaminant concentration in the fish and that in the water (Landrum *et al.*, 1996). For reasons of simplicity, it was assumed that this relationship was valid also for sediment exposures, with only the dissolved fraction in overlying or pore water being available to benthic organisms. Therefore, the risks posed by sediment-associated contaminants have been estimated by using equilibrium partitioning coefficients between sediment and water to calculate the contaminant concentration in pore- and overlying water (Di Toro *et al.*, 1991). However, increasing evidence shows that ingestion of sediment particles may be the major route of uptake for hydrophobic contaminants in benthic invertebrates (Forbes *et al.*, 1998, Kaag *et al.*, 1997, Landrum, 1989). The total organic carbon content (TOC) of sediments has also been considered a major factor governing contaminant bioavailability, being inversely related with TOC (Kukkonen and Oikari, 1991, Spacie, 1994). This has been explained by an increasing sorption capacity with higher organic matter content that would render the contaminants less bioavailable. It has also become evident, however, that not only the quantity, but also the quality of the sediment organic matter is important for the bioavailability of contaminants (Gunnarsson *et al.*, 1999). Refractory organic matter with a high sorption capacity, such as humic substances and soot particles (black carbon), are reported to decrease the bioavailability of organic contaminants (Freidig *et al.*, 1998, Decho and Luoma, 1994, Lamoureux and Brownawell, 2004), whereas more labile carbon sources, e.g. algae and bacteria, can increase their bioavailability (Gunnarsson *et al.*, 1999, Bott and Standley, 2000). The selective feeding by benthic organisms on labile carbon sources, that have a high desorption rate of associated contaminants, is the underlying explanatory process to these observations.

Sediment toxicity tests

The effects of sediment-associated contaminants on benthic organisms can be assessed using sediment toxicity tests. The toxicity tests often aim to define lethal concentrations (e.g. LC_{50}, the concentration at which half of the test individuals die) or no observed effect concentrations (NOEC, the highest concentration before an effect is observed in the test organism) and a number of test concentrations, ranging from zero (i.e. controls) to high, are applied. Based on these results it is then possible to evaluate the toxicity and risks of pesticides to these non-target organisms. Natural sediments are highly complex and differ largely with respect to organic carbon quantity and quality, particle distribution, pH, and biological communities, which could all affect the outcome of toxicity tests (Burton, 1991, Mäenpää *et al.*, 2003). To reduce the variability of test results and facilitate inter-laboratory comparisons in toxicity tests with sediment-living invertebrates, standardized artificial sediments have been developed (e.g. US EPA, 2000). Most of these artificial sediments consist of sand, silt/clay, and organic matter. Peat is the most commonly used organic matter, as recommended in guidelines from for example the Organization for Economic Co-operation and Development (OECD,

1994), but also other sources of organic matter, have been proposed (Ribeiro *et al.*, 1999). Among the benefits of artificial sediments are the absences of indigenous fauna and contaminants, as well as its predefined composition. Despite these positive aspects, standardization of the test sediment also simplifies toxicity tests and may confound the extrapolation of results to field conditions, since artificial sediments may differ substantially from natural sediments in their sorption characteristics, redox conditions, pH, and microbial assemblages.

In toxicity tests with artificial sediment, a 7-10 days conditioning period of the prepared sediment is recommended to stabilize conditions and allow a microbial community to establish (Streloke and Köpp, 1995, OECD, 1994). Despite this recommendation, very few studies have investigated the microbial communities of artificial sediments and compared these with natural sediments. Verrhiest and coworkers (2002) reported that artificial sediments prepared with cellulose had low bacterial abundance and enzyme activities compared with natural sediments. A low microbial biomass and diversity of artificial compared to natural sediments may, for example, result in a slower degradation of test compounds. Therefore, increased knowledge is needed to assess how microbes govern the fate of test compounds in standardized tests, and ultimately toxicity test results.

Toxicity tests results have been used to calculate environmentally safe concentrations of organic contaminants and pesticides in an effort to estimate their hazards and set aquatic quality criteria for policy making. In the Netherlands, Crommentuijn *et al.* (2000) used toxicity test results, mainly NOECs, from the literature to calculate maximum permissible concentrations (MPCs). According to Crommentuijn *et al.* (2000) MPC is the concentration of a substance in the environment above which the risk of adverse effects to ecosystems is considered unacceptable. Likewise, concentrations of substances in the environment below which the occurrence of adverse effects are considered negligible, NCs, are defined as 1% of the MPC. The NC should also account for possible additive or synergistic effects of organic contaminants to aquatic organisms. In general, Crommentuijn *et al.* (2000) concluded that there is a great demand for ecotoxicological data to establish more reliable estimates of MPCs. This was particularly true for sediment, where most of the MPCs were based on toxicity data from water exposures using the equilibrium partitioning method, resulting in MPCs with great uncertainties. It is therefore highly unclear if the estimated MPCs for sediment exposures presented by Crommentuijn and coworkers (2000) in fact are environmentally safe.

Aquatic biofilms

In aquatic environments, the majority of bacteria are found growing in biofilms on surfaces of submerged substrata or sediments (Jackson *et al.*, 2001). Biofilms are complex communities that, besides bacteria, are composed of algae, protozoa, and fungi embedded in a matrix of extracellular polymeric substances (EPS), mainly polysaccharides (Lock *et al.*, 1984). The formation of biofilms gives the microbial

cells many advantages compared to free living cells, such as protection against environmental changes (pH, desiccation) and toxic substances, an efficient trapping of nutrients (Decho, 1990), and an increased capability to degrade refractory compounds (James *et al.*, 1995). EPS is also a labile food source which is consumed by deposit-feeding invertebrates (Baird and Thistle, 1986). Additionally, since EPS has an efficient sorption, but yet weak binding, of various compounds, these may rapidly desorb during gut passage (Decho and Lopez, 1993), resulting in an increased bioavailability. An increased uptake of EPS-associated dissolved organic matter (Sherr, 1988) and heavy metals (Schlekat *et al.*, 2000, Selck *et al.*, 1999) through ingestion of EPS by benthic invertebrates has been observed, but their role in the bioaccumulation of organic contaminants has been poorly investigated. However, Wolfaardt *et al.* (1994) observed an increased bioaccumulation of the herbicide diclofop when associated to EPS, indicating that biofilms/EPS can play an important role for the introduction, bioaccumulation and subsequent trophic transfer of hydrophobic contaminants in the aquatic food web (see the left box of Fig. 1).

Interactions between pesticides and sediment microorganisms

The interactions between pesticides and sediment microorganisms that are considered in this thesis are summarized in figure 2. The main process that drives these interactions is the partitioning of a hydrophobic pesticide to the organic compartments in the aquatic system, for example sediment, organism tissue, dissolved organic matter, and microbes and EPS in biofilms. This process is an equilibrium partitioning of the contaminant both between the surrounding water and the organic compartments, as well as among the compartments. Thus, in a sediment-water system, there will be a constant sorption and desorption of the contaminant, depending on the binding capacity and contaminant concentration in each compartment. In the equilibrium partitioning theory, the bioavailability is assumed to be inversely related to the sediment's total organic carbon content (Lake *et al.*, 1990). Thus, different sorption characteristics depending on the quality of the organic carbon are not considered. To simplify figure 2 only the sorption/desorption between organic compartments and the overlying water is illustrated. Once the pesticide has partitioned to the organic compartments it can affect the organisms. The first type of interaction is the pesticide's toxic mechanisms, which could affect both function and composition of sediment microorganisms, and possibly impair the ability of the community to recycle nutrients and perform other ecosystem functions. Secondly, the microbial community may degrade the pesticide, thus reducing the sediment pollution. On the other hand, microbial transformations could produce metabolites that are even more toxic than the mother compound. These two interactions could occur both in communities of free-living microbial cells and those in biofilm formations. Another important interaction considered in this thesis, is the high sorption capacity of pesticides by microorganisms and EPS. Since benthic organisms feed on these sediment components, biofilms may be an important vector for the uptake and introduction of contaminants in the aquatic food web.

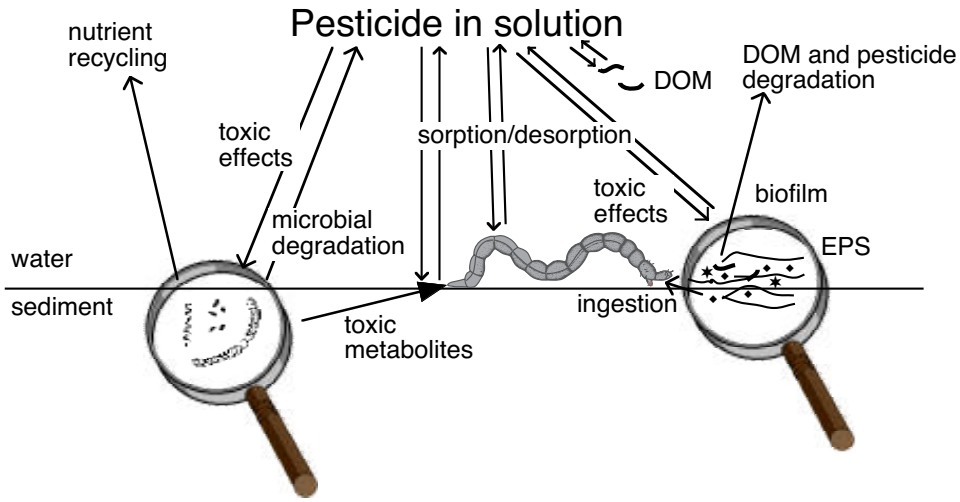

Fig. 2. An overview of the interactions between pesticides and sediment microorganisms that are considered in this thesis. Dissolved organic matter (DOM) can be found both in water and biofilms and extracellular polymeric substances (EPS) produced by microorganisms are the main component of biofilms. For further details, see the text.

Objectives

The overall aim of this doctoral thesis was to contribute to our understanding of the interactions between pesticides and microorganisms in freshwater sediment, both in the environment and in toxicity tests.

The major questions addressed were:

- Does exposure of functionally different pesticides at environmentally relevant (NC and MPC), and high concentrations (100xMPC), cause effects on heterotrophic microbial community function of a natural sediment? (paper I)
- Do commonly used pesticides, at environmentally relevant and high concentrations, affect the composition of sediment microbial communities? (paper II)
- What are the microbial characteristics of the peat-based artificial sediment and how does it relate to the microbial communities of natural sediments? (paper III)
- How do sediment microorganisms and/or their extracellular polymeric substances (EPS, i.e. biofilms) affect the bioavailability and toxicity of sediment-associated pesticides to deposit-feeding benthic invertebrates? (paper IV)

Materials and methods

In the following sections, I give a brief overview of the methods used in the different papers to enable a later discussion of the obtained results. For detailed descriptions of the methods, I refer to the original papers. The general approach for all studies was to use laboratory microcosms (glass beakers, 125 ml), and surface sediment and water from freshwater lakes.

Pesticide effects on sediment microbial communities (I and II)

In these toxicity tests we chose to work with ecotoxicologically relevant concentrations of pesticides instead of using a more traditional dose-response approach. This was done to include different types of pesticides and compare their effects on sediment microbes and because it felt more environmentally relevant. Pesticides were selected with regards to their target organisms, common use, and the availability of an estimated MPC (Crommentuijn *et al.*, 2000). Common for both studies were the phtalamide fungicide captan, the phenylurea herbicide isoproturon, and the insecticide pirimicarb (Table 1). In paper I, the pyrethroid insecticide deltamethrin was also included. In paper II, we included the widely used herbicide glyphosate. Glyphosate is often claimed to have no effects on non-target organisms and to be rapidly degraded after application (Baylis, 2000). However, the rather high concentrations detected in the environment (e.g. Sundin *et al.*, 2002) indicate that it might not be degraded as fast under field conditions at high latitudes as in laboratory experiments. Moreover, there are only a few studies on glyphosate toxicity to microorganisms (Busse *et al.*, 2001, Araujo *et al.*, 2003). Since there is no MPC reported for glyphosate by Crommentuijn *et al.* (2000), we used a concentration commonly observed in sediments (Sundin *et al.*, 2002) and denoted it MPC. Our nominal exposure concentrations were 0 (controls), NC, MPC and 100 times the MPC (100MPC) (paper I) or 0 (controls), low (MPC) and high (1000*MPC) (paper II) (Table 1).

Profundal sediment (12-16m) and water was collected from Lake Erken, a mesotrophic lake east of Uppsala that is relatively unaffected by agricultural activities. In the laboratory, the sediment and its microbial community were then exposed to the different pesticides and test concentrations in microcosms with overlying filtered (GF/C) lake water. Pesticide effects on the function of the microbial community were assessed by measuring end-points at different levels of microbial organization. For the whole microbial assemblage, community respiration (CO_2 production) was regularly measured over a 16-day period (Goedkoop *et al.*, 1997) and microbial biomass in the surface sediment was determined as ATP content (Tobin *et al.*, 1978) at the end of this exposure time. At the community level, the bacterial activity (^{3}H-leucine incorporation rate) of sediment after short-term pesticide exposure was determined according to van Duyl and Kop (1994), a modification for sediment samples of the method by Simon and Azam (1989). Also the effects on denitrifying bacteria were assessed by quantifying

denitrification rates after short-term exposures, using the ^{15}N-isotope pairing technique (Nielsen, 1992) with modifications by Svensson (1998).

Table 1. *Pesticides used in experiments, their active ingredients (IUPAC names), partition coefficients in octanol and water (log Kow), and the applied nominal exposure concentrations in sediment and water.*

Pesticide	Active ingredient	log K_{ow} [a)]	NC [b)] µg/kg dw, ng/l	MPC [b)] µg/kg dw, µg/l
Captan	(N-trichloromethyl-thiocyclohex-4-ene-1,2dicarboximide)	2.8	0.013 1.1	1.3 0.11
Deltamethrin	((S)-α-cyano-3-phenoxy-benzyl-(1R,3R)-3-(2,2-di-bromovinyl)-2,2-dimethyl-cyclo-propanecarboxylate)	4.6	0.013 0.003	1.3 0.0003
Glyphosate	N-(fosfonmetyl)glycin	-3.4		150
Isoproturon	(3-(4-isopropylphenyl)-1, 1-di-methylurea)	2.5	0.053 3.2	5.3 0.32
Pirimicarb	(2-dimethylamino-5,6-dimethylpyrimidine-4-yl dimethylcarbamate)	1.7	0.022 0.9	2.2 0.09

a) from (Tomlin, 1994)
b) from (Crommentuijn et al., 2000)
c) from (Sundin et al., 2002)

The long-term (31 d) effects of pesticide exposure to sediment microbial communities were studied in a new experiment. Effects of pesticide exposure on microbial community composition were determined by analyses of ergosterol (fungal biomass), phospholipid fatty acids (PLFA), and by genetic fingerprinting using terminal restriction fragment length polymorphism (T-RFLP) (paper II). PLFAs are present in all cell membranes, but since the amounts of certain fatty acids differ among microbial groups and some are biomarkers for specific groups, the PLFA-pattern provides a good overview of the major components of the microbial community (Frostegård *et al.*, 1991). Furthermore, the total PLFA content provides an estimate of the viable microbial biomass (Pinkart *et al.*, 2002). In T-RFLP analysis, DNA was extracted from the whole sediment and the bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR). Then the obtained PCR-product was digested with restriction enzymes that cut double-stranded DNA at specific base-pair sites, resulting in DNA fragments of different lengths depending on the bacterial type (Liu *et al.*, 1997). Principal component analysis (PCA) was used to compare the PLFA- and T-RFLP-patterns obtained in controls and treatments. In addition, bacterial activities (^{3}H-leucine

incorporation rate) were quantified during incubations and microbial biomass (∑PLFA) was determined at the termination of the 31-days experiment.

Microbial characterization of artificial sediment (III)

In this study, results from a number of experiments with artificial sediment were compiled and compared with data reported for natural sediments. Additionally, the microbial community compositions of artificial sediment and a natural sediment were analyzed and compared. The first two experiments were performed using artificial sediment with 10% organic matter, prepared according to the OECD test guideline (OECD, 1994). The sediment consisted of mainly sand, kaolin clay, 10 % finely ground *Sphagnum* peat and $CaCO_3$. The sediment was transferred to microcosms, covered with M7 medium (Streloke and Köpp, 1995) and acclimated for 10 days at 20˚C under aeration. In the first experiment, community respiration was quantified 3 times a week for 28 days using methods described in Goedkoop *et al.* (1997). Bacterial abundance and mean cell volume were determined at day 0 and 21. In another experiment, bacterial activity ([^{3}H]-thymidine incorporation) and microbial biomass (ATP content) was quantified after 0, 8, 15, and 30 days of incubation according to Bell and Ahlgren (1987) and Tobin (1978), respectively.

The microbial community compositions were determined in control sediments from a toxicity test run with artificial sediment and control sediment from the experiment performed in paper II. The sediments had been incubated for 28 and 31 days, respectively, and the acclimation time for the artificial sediment was 16 days. Microbial (∑PLFA) and fungal (ergosterol concentration) biomass in these sediments were analyzed according to Frostegård *et al.* (1991) and Mille-Lindblom and Tranvik (2003), respectively. Differences in microbial community composition were determined from PLFA-patterns of the artificial and natural sediments using principal component analysis (PCA). The bacterial diversity of the artificial and natural sediment was determined by quantifying PCR-amplified 16S rRNA gene heterogeneity as described above and similarity of the obtained DNA fragment patterns in artificial and natural sediment was calculated. The fragment diversity, which is a proxy for bacterial genetic diversity, was computed as the Shannon-Wiener H' index.

Influence of biofilms on the bioavailability of chlorpyrifos (IV)

In a fourth study, the influence of sediment microorganisms and/or EPS on the bioavailability of the hydrophobic insecticide chlorpyrifos to benthic organisms was investigated. Chlorpyrifos is one of the world's most widely used active ingredient for pest control (Pesticide Action Network, 2004, http://217.154.68.186/pestnews/actives/chlorpyr.htm, accessed 26-Jan-2005) and is an acetylcholinesterase inhibitor that is moderately toxic to aquatic organisms (Ankley *et al*., 1994). Due to its high hydrophobicity, log K_{ow}= 5.25 (DeBruijn *et al*., 1989), it associates with organic matter in aquatic environments. We used uniformly labeled ^{14}C-chlorpyrifos to facilitate the quantification and the fate of

the pesticide in our experimental microcosms. Larvae of the midge *Chironomus riparius* Meigen were selected as test organisms due to both their ecological relevance and recommended use in toxicity tests (e.g. OECD, 1994). *Chironomus* larvae are deposit-feeders that construct tubes in the sediment, which exposes it to contaminants from various possible routes (Fig. 1).

Microcosms were established by transferring 5 g (dw) of sterilized silica gel (particle size 40-63 μm) to glass beakers and adding filter-sterilized lake water. The silica was added as an inert bottom substrate to reduce larval stress and the size fraction was chosen because *Chironomus riparius* are known to ingest particles in this size range (Davies *et al.* 1999). To these microcosms, low amounts of organic matter were added as a sediment microbial extract (microbial extract treatment), sediment (sediment treatment), sterile sediment (sterile sediment treatment), and dissolved humic acids (humic treatment). Also controls (no organic matter) were included. Sediment microbes were extracted according to Mermillod-Blondin *et al.* (2001) from a Lake Ekoln sediment, which was also used for the sediment treatments. Aliquots of the microbial extract and sediment were added to microcosms and biofilms were allowed to develop for 6 days in the dark, after which a fluffy layer could be observed (Fig. 3). The number of bacterial cells in microbial extract and sediment additions was determined by epifluorescence microscopy (Haglund *et al.*, submitted). Organic matter additions were quantified as total organic carbon (TOC), total nitrogen, and polysaccharides (EPS).

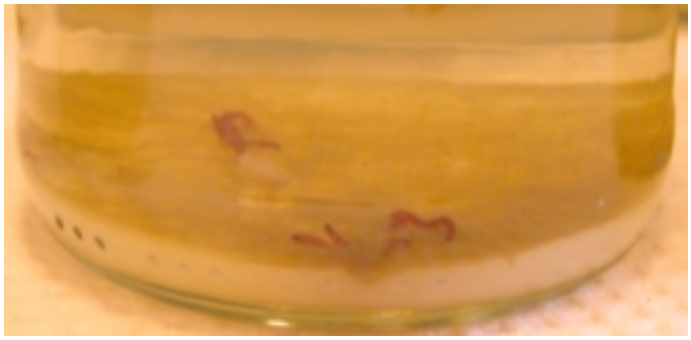

Fig. 3. Photo of *Chironomus* larvae in the biofilm during chlorpyrifos exposure. The brown fluffy layer is a biofilm developed from the microbial extract on top of the white silica gel.

Sterile-sediment treatments were prepared by adding autoclaved lake sediment to microcosms. Artificial humic water, prepared by adding humic acid sodium salt (Sigma-Aldrich) to sterile-filtered lake water, was added to the silica gel in the humic treatments. Controls contained only silica gel and sterile-filtered lake water (Fig. 4). ^{14}C-chlorpyrifos was added to all microcosms and the pesticide was allowed to sorb to organic matter and silica gel for 18h. Then 4th instar larvae were randomly allocated to microcosms. After 25 h of exposure, 3 of the larvae from each microcosm were taken for determination of chlorpyrifos uptake (liquid scintillation counting for ^{14}C). The remaining 5 larvae were allowed to depurate for

21h in clean microcosms containing sand and a food source (Tetraphyll®). Then the larval tissue concentrations of assimilated chlorpyrifos were quantified in these larvae. Additionally, to determine the partitioning and fate of the pesticide in the microcosms, chlorpyrifos concentrations in the overlying water, the organic matter/surface layer of silica gel, and the homogenized silica matrix were quantified.

Fig. 4. Photo showing microcosms of the different treatments in the chlorpyrifos bioavailability study. The white layer in the bottom is the silica gel. Treatments from the left are; microbial extract, sediment, control, sterile sediment, and humic acids.

The bioavailability of chlorpyrifos was estimated by calculating bioconcentration factors (BCF) and bioaccumulation factors (BAF). These factors were defined as $BCF=C_{larvae}/C_{water}$ and $BAF=C_{larvae}/C_{sediment}$, where C_{larvae} is the chlorpyrifos concentration in larvae, C_{water} is the concentration in overlying water, and $C_{sediment}$ is the concentration in the surface layer of the silica.

Results and discussion

Effects on community function (paper I)

In the first study, our results showed that exposure to pesticides at environmentally relevant concentrations could affect the non-target microbial communities of natural sediments. After 8 h of exposure at maximum permissible concentrations (MPCs), bacterial activities were decreased by 20-24% compared with controls by all pesticides tested (Fig. 5). Inhibitions of bacterial activity were even found for the two pesticides deltamethrin and isoproturon at negligible concentrations (NCs). However, the bacterial activity did not differ from controls at the highest exposure concentration (100MPC) of deltamethrin and pirimicarb. Instead, in these high exposures activities were higher than at MPC. For the whole microbial community, a lower microbial biomass (ATP-content) was found in MPC-

treatments with deltamethrin than in controls, whereas NC-treatments with isoproturon had lower microbial biomass than at MPC- and 100MPC-treatments. Conversely, no pesticide effects were detected for the community respiration, and denitrification rate, at the lowest level of microbial organization.

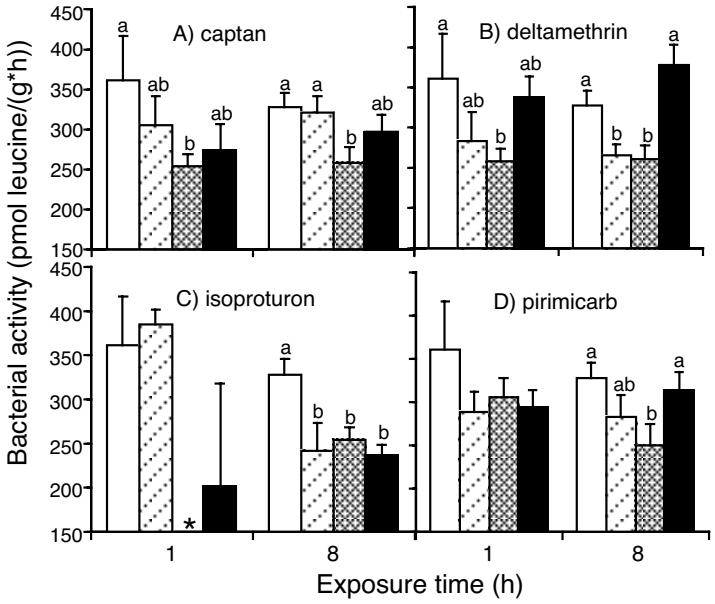

Fig. 5. Mean bacterial activity (^{3}H-leucine incorporation, as pmol/g·h) in sediment treated with captan A), deltamethrin B), isoproturon C), and pirimicarb D) after 1 and 8 h of exposure, respectively, at exposure concentrations of NC (striped), MPC (checked), and 100MPC (black) and in controls (white). Letter codes denote significant differences between means (Bonferroni/Dunn $p < 0.0083$). Error bars denote ±1 SD. *) Data omitted.

In our study, isoproturon had the strongest inhibiting effect on bacterial activity with decreased activities observed at all three exposure concentrations. This was interpreted as a toxic effect by isoproturon, since this compound is known to harm a wide variety of non-target organisms (Tomlin, 1994, Tixier *et al.*, 2002). Thus, it may seem strange that the observed effects on bacterial activity did not show a consistent, inverse relationship with increasing concentrations of the other pesticides included in the study. However, studies with other pesticides have also shown stimulated bacterial production (leucine incorporation) at the highest exposure concentrations (Petersen *et al.*, 2004, Downing *et al.*, 2004), probably due to increased activity of tolerant microorganisms. Therefore, high exposures with captan, deltamethrin, and pirimicarb could have induced a similar response in our study. The scientific literature proposes a number of processes that can cause favorable growth conditions for tolerant microbes. Firstly, toxic effects on sensitive microorganisms may result in decreased competition for nutrients and an increased release of labile organic matter from decaying microorganisms. Hence,

those groups of microorganisms that are not negatively affected by the pesticide exposure could take the competitive advantage and increase their growth. Furthermore, some types of microbes may experience a competitive advantage by their capability of using pesticides as an energy source (El Fantruossi *et al.*, 1999). Consequently, pesticide exposure may induce shifts in the dominance of certain groups of microbes in microbial communities and I conjectured that the high bacterial activities observed at 100 MPC in our study most likely were due to pesticide-induced changes in the microbial community composition.

In this experiment, we found no net effect of pesticide exposure on denitrification and microbial community respiration. One interpretation of these results is that the pesticides do not affect these processes. Despite this lack of an observed effect on the response variables, the pesticides might still have affected the microbes. Denitrification is a general process that can be performed by a large number of bacterial species (Petersen *et al.*, 2004). Hence, a substitution of the dominant denitrifying bacterial types upon pesticide exposure could result in a net-zero effect on denitrification. Likewise, one can argue that respiration is a quite unspecific endpoint to detect effects from pesticide exposure on microbial communities, since a decrease in respiration in some microbial groups could have been masked by an increased respiration by more tolerant groups. Alternatively, overall microbial respiration may not have responded to pesticide exposure due to a flaw in the experimental set-up. In this experiment, the pesticides were spiked to the overlying water according to the OECD guidelines for toxicity tests (OECD, 1994), resulting in the highest pesticide exposure at the very surficial layer of the sediment where sorption was highest. Therefore, any effects on respiration in the surface layer may have been obscured by an absence of effects deeper in the sediment. This conjecture was further supported by the observed effects on microbial biomass (ATP) in the top 2-mm of the sediment in treatments with deltamethrin and isoproturon. From these results I concluded that overall respiration and denitrification are not very sensitive parameters for assessing the toxicity of pesticides to aquatic microorganisms.

Effects on community composition (paper II)

Based on the results of paper I, I chose to expand the experimental endpoints to also include changes in sediment microbial community composition. In this study, we applied a longer exposure time (one month) to allow the changes to develop. Our results showed that environmentally relevant pesticide concentrations (see above) may affect non-target sediment microorganisms in various ways. Subtle, albeit significant, shifts in the bacterial community composition of this natural sediment, were detected as changes in T-RFLP patterns after captan and glyphosate exposure (Fig. 6). Also the PLFA analyses indicated that pesticide exposure caused shifts in the sediment microbial composition. Generally, treatments with low and high pesticide concentrations resulted in shifts of the microbial community in different direction from the controls, again suggesting a lack of a unidirectional concentration-dependent response to pesticide exposure.

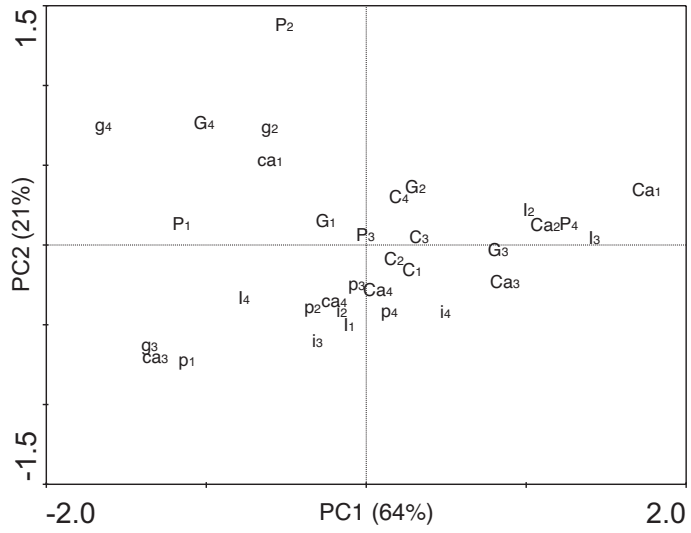

Fig 6. Principal component analysis (PCA) with percentages of individual terminal restriction fragments, showing the sediment microbial T-RFLP patterns in controls (C) and in treatments with low (lower case letters) and high (capital letters) concentrations of the pesticides captan (ca and Ca, respectively), glyphosate (g, G), isoproturon (i, I), and pirimicarb (p, P).

Our results suggested that different groups of microorganisms were stimulated depending on which pesticide that was applied. For example, some bacterial groups were relatively more common after exposure to low concentrations of glyphosate, as was supported by changes in bacterial composition in this treatment (Fig. 6). This could have been due to an ability of these groups to use the glyphosate molecules as a carbon source (Busse *et al.*, 2001). PLFA-analysis indicated that also fungi and actinomycetes were favored by exposure to low concentrations of glyphosate. In contrast, low concentrations of the fungicide captan probably enhanced the growth of some bacterial groups due to a combination of their ability to use the pesticide as a carbon source, and a decreased fungal competition. This conjecture was supported by changes in bacterial composition combined with PLFA-data showing that treatments with captan were less associated with fatty acids typical for fungi and actinomycetes than controls. Treatments with high concentrations of captan, on the other hand, resulted in a bacterial composition that was significantly different from that in treatments with low concentrations of captan, indicating that other groups were affected by the high exposure.

Our PLFA-data further showed that many branched saturated fatty acids (i14:0, i15:0, a15:0, and i16:0) that are indicative of gram-positive bacteria (Pinkart *et al.*, 2002) were relatively more abundant in treatments with high concentrations of glyphosate, isoproturon, and pirimicarb. Gram-positive bacteria are considered to be more stress-tolerant, possibly due to their relatively thick cell wall and ability to form endospores that are highly resistant to stress, including toxic chemicals

(Stainer *et al.*, 1977). In line with this, a predominance of PLFAs indicating gram-positive bacteria has been found in heavily polluted sediments (Rajendran *et al.*, 1994) and soils (Pennanen *et al.*, 1996). It was therefore concluded that the shifts in community compositions in our treatments with high pesticide concentrations (Fig. 6) could be caused by proliferation of tolerant microbial groups or persistence in the form of endospores, possibly gram-positive bacteria.

The community-level endpoints bacterial activity and total microbial biomass were not affected by pesticide exposure in this study. The lack of pesticide effects on bacterial activity may seem contradictory to the results obtained in paper I. However, this discrepancy could be due to a short-term effect of the pesticides and the longer exposure times applied in the second study. The bacterial activity could have been affected directly after pesticide exposure, but this was not detected at the first measuring occasions after 1 and 3 days. Additionally, the bacterial activity measurements showed quite large standard deviations, making it more difficult to detect differences among treatments. Short-lived effects of sulfonylurea herbicides (e.g isoproturon) have been reported by Blair and Martin (1988) and Sheehan et al. (1986) found an initial stimulation of bacterial activity by high levels of atrazine followed by a return to control levels. Furthermore, inactive or diapausing cells (e.g. endospores) were included in the analysis of community composition, but did not contribute to the microbial activity. This could also partly explain that effects were found only at the sub-community level.

Possible responses of microbial communities to pesticide exposure

There are a number of different ways in which heterotrophic microbial communities may respond to pesticide exposure (Fig. 7). The undisturbed community is represented by community A, which may then develop to any of the communities B-E upon pesticide exposure. First of all, the pesticide may have no or minor effects on the microbial community, resulting in a stable community with virtually the same activity and composition as in A. This appeared to be true for long-term exposure of pirimicarb at MPC in paper II. In scenario B some types of microorganisms are stimulated by pesticide exposure, for example by an ability to use the pesticide as an energy source. Other types of microbes may not be directly inhibited by the pesticide, but may experience a competitive disadvantage against microorganism that have the ability to degrade the pesticide. The sum effect on microbial activity could be zero. Such a net-zero response may have been the case in the exposure of low concentrations of glyphosate in Paper II. In scenario C, some groups of microorganisms are inhibited (abundance and/or activity) by pesticide exposure, whereas some other groups are indifferent, resulting in decreased total microbial activity. An example of this could be the inhibition of bacterial activities by all pesticide exposures at MPC in paper I. As illustrated in scenario D, an alternative response is that some groups of microorganisms are inhibited or killed, while others are stimulated due to decreased competition, increased nutrient leakage from dead microbial cells, and/or an ability to use the pesticide as a carbon source. This could induce a rapid initial short-term increase

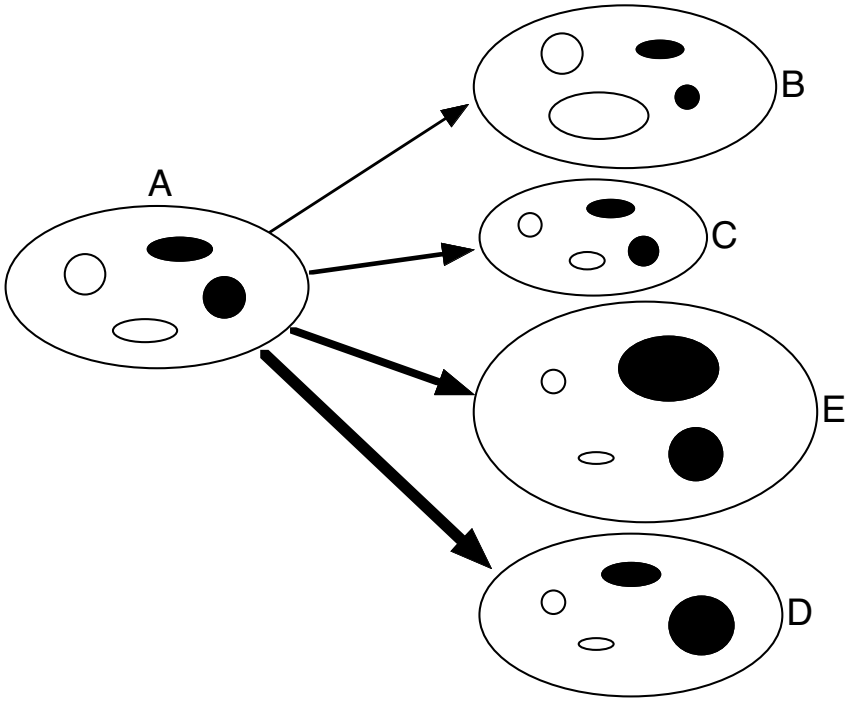

Fig. 7. Examples of possible responses of a microbial community to pesticide exposure. Black symbols represent tolerant and white symbols sensitive groups of microbes. The rods represent microbial groups that could have a fast proliferation and the circles groups that have a slower response. The arrows symbolize the shift (or pesticide exposure) and the size of the ovals represents the activity level of the microbial community. For further details, see text

in bacterial activity as was observed in 100MPC-exposures of deltamethrin and pirimicarb in paper I. Lastly, in response E, some types of microbes are inhibited while other types are stimulated. In this scenario, there are changes in microbial community composition, but these changes are not accompanied by a change in the total microbial activity. The exposure to high concentrations of captan in Paper II, that probably affected fungi negatively, but tolerant bacteria could increase their numbers, can be seen as an example of this. However, for all these changes, there is always a possibility that the microbial community recovers when the pesticide exposure has ceased and return to state A.

For many of the general biogeochemical processes performed by the sediment microbes, for example degradation of labile organic matter and denitrification, there is probably a quite large functional redundancy in the sediment microbial communities (van Beelen and Doelman, 1997). Even though some micro-organisms are inhibited or disappear upon pesticide exposure, (Engelen *et al.*, 1998, El Fantruossi *et al.*, 1999), there may always be another group of micro-organisms around that can perform the same function. Additionally, there are indications that the communities can recover from effects caused by short-term

exposures at low pesticide concentrations (Larsen *et al.*, 1986). However, repeated exposures may lead to more severe toxic effects or result in the development of a more tolerant community by elimination of the sensitive microbes, as has been observed for periphyton communities (Nyström *et al.*, 2000). Moreover, pesticides rarely occur one at the time in aquatic systems, but rather as a mix of several pollutants, that may act in concert to give antagonistic, additive, and/or synergistic effects in organisms (e.g. DeLorenzo and Serrano, 2003, Belden and Lydy, 2000).

Specialized processes may show less redundancy than the general processes. For example, only a few types of specialized bacteria are able to convert ammonia to nitrate (Petersen *et al.*, 2004), making the nitrification process quite vulnerable. Also the degradation of refractory organic matter (e.g. lignin and cellulose) may require a chain of degradation steps, some of which are performed by highly specialized microorganisms. For example, fungi constitute an important component in many degradation pathways (Kominkova *et al.*, 2000), and negative effects on fungi could possibly break the chain of reactions needed to degrade certain substances. Thus, the effects that changes in community composition will have on sediment microbial function will ultimately depend on the ability of tolerant species to maintain important biogeochemical processes. The long-term ecological consequences of the development of a tolerant community could be a limited ability to respond to new perturbations, a decreased nutrient regeneration rate, and reduced capacity to degrade refractory sources of organic matter. Pesticide effects on these ecosystem functions need to be further addressed.

Microbial characterization of artificial sediment (paper III)

Our comparisons between the artificial and natural sediment consistently showed that the artificial sediment has a poorly developed microbial community. Microbial biomass (as ∑PLFA) was on average only 10% and fungal biomass 57%, of the values found for the natural sediment (Table 2). Bacterial production in the artificial sediment, 0.062-0.113 μg C g^{-1} h^{-1} was in the same range as that of a natural sediment (Goedkoop and Törnblom, 1996), but orders of magnitude lower than that reported for several other lakes (Tuominen, 1995, Bell and Ahlgren, 1987). In this study, PLFA analysis indicated that different groups of microorganisms were dominating the two sediments, with a higher share of aerobic bacteria in the natural sediment (30%) than in the artificial (16%). Our PLFA data also showed that fungi were relatively more abundant in artificial sediment, 5.4%, compared to 3.0% in the natural sediment. Furthermore, genetic fingerprinting (T-RFLP) indicated a lower bacterial diversity (fragment diversity) in the artificial sediment than in natural sediment (Table 2). Likewise, the T-RFLP pattern similarity was 0.869 among samples of natural sediment and 0.834 between samples of artificial sediment, whereas the similarity between natural and artificial sediment was much lower, 0.395. Combining all these data convincingly show that the artificial sediment is a poor model for natural sediments with respect to the microbial community.

Table 2. *Microbial characteristics (means ± standard deviations) of artificial and a natural sediment (Lake Erken)*

Variable	Unit	Artificial	Natural
Microbial biomass	nmol PLFA g^{-1} WW	9.83±3.6	97.6±15.6
	nmol PLFA g^{-1} DW	14.0±2.4	911±96.5
Fungal biomass	µg C g^{-1} WW	38.6±4.4	67.9±9.1
	µg C g^{-1} DW	57.6±6.57	659±88.4
Bacterial richness	# T-RF	31±0.7	58±5.9
Bacterial diversity (Shannon-Wiener)	-	4.16±0.03	4.55±0.09

A low bacterial production and biomass in the artificial sediment could be expected, considering that the organic fraction consists of peat which is a highly humic and biologically refractory pool of organic matter. Also the very low nitrogen and phosphorus content of the artificial sediment and the high demands of bacteria for these nutrients (Vrede *et al.*, 2002), makes it unlikely that the artificial sediment can sustain high bacterial growth rates and abundances. Neither is it surprising that the microbial composition and diversity of the artificial sediment differ from that of a natural sediment, since the community of the artificial sediment is restricted to establish from microorganisms associated with the peat and to microbial input during sediment preparation.

The observed low microbial biomass, activity, and diversity in the artificial sediment may have implications for the fate of test compounds in toxicity tests. A low overall microbial activity will result in a slow decomposition of organic matter and likewise slow degradation of organic contaminants. If the relatively low activity of the microbial community in an artificial sediment accomplishes a slower degradation of the test compound, the exposure concentrations may remain higher than in similar conditions in natural systems, and the standardized test may overestimate the toxicity. In addition, a low diversity of the bacterial community may affect the capability to degrade sediment contaminants or test compounds. A microbial consortium with members harboring complementary functions is often a prerequisite for the degradation of organic pollutants. For example, (Senior *et al.*, 1976) demonstrated that seven different microorganisms were required to degrade the herbicide dalapon. Thus, in an artificial sediment where certain species in the degradation chain are missing, the microbial community may be unable to decompose the test compound (Schwarzenbach *et al.*, 1993), whereas this would occur in a natural sediment. Microbial modification of test compounds may also generate metabolites that are more toxic than their parent compounds (Tixier *et al.*, 2002), depending on microbial biomass, activity, and diversity. In artificial sediments, the microbial communities may not produce metabolites or produce alternative (less toxic) metabolites compared with indigenous microorganisms in natural sediments. In this case the standardized toxicity test may underestimate

pollutant toxicity in nature. These microbial processes may need to be considered when evaluating toxicity test results.

Influence of microorganisms on pesticide bioavailability (paper IV)

In this study, living microbes and biofilms were found to markedly increase the bioavailability of chlorpyrifos to larvae of *Chironomus riparius*. Larval uptake of chlorpyrifos (label associated with both tissue and gut tracts) was 1.5 times higher in the sediment treatment and 2 times higher in the microbial extract treatment, respectively, than in controls (Fig. 8). Conversely, larval uptake in treatments with sterile sediment and humic acids did not differ from controls. After depuration, the same pattern in tissue concentrations prevailed, with three times more chlorpyrifos assimilated in larvae in the microbial extract treatment, and more than twice as much in sediment treatment, respectively, compared to controls (Fig. 8). Additionally, the accumulated larval concentrations of chlorpyrifos were higher in the microbial extract treatment than in the humic and sterile sediment treatments. Most likely, the observed increase in uptake and assimilation of chlorpyrifos in *Chironomus* larvae in treatments with biofilms was due to a higher uptake both from food ingestion (particle-associated) and through the skin from overlying and pore water (dissolved and colloid-associated). However, with this experimental design, it was difficult to distinguish which uptake route was quantitatively most important.

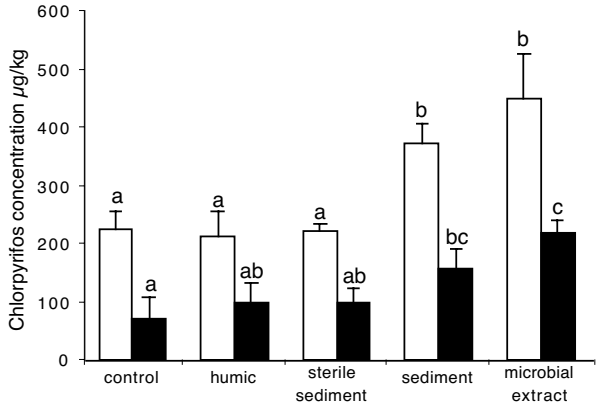

Fig. 8. Larval concentrations of chlorpyrifos (means ±1sd ng/g ww) before (open bars) and after (filled bars) depuration in treatments and controls. Letter codes denote significant differences in pairwise comparisons (Bonferroni/Dunn), i.e. treatments that share letters are not significantly different.

Supporting uptake through food ingestion is that most deposit feeders digest the microbial epigrowth of particles, rather than using non-living organic matter

(Taghon *et al.*, 1978). *Chironomus* larvae are also known to ingest large numbers of bacteria (Johnson *et al.*, 1989), implying a coingestion of EPS associated to the bacterial cells. The EPS concentrations were 10-30 times higher in the sediment and microbial extract treatments than in controls. It is thus likely to assume that the higher uptake and assimilation of chlorpyrifos in *Chironomus* in the treatments with additions of sediment and microbial extract was due to a high feeding rate on biofilms with associated chlorpyrifos. Conversely, the low uptake of chlorpyrifos by larvae in the sterile sediment treatment may be due to a low feeding rate of the larvae on this non-living, but apparently organic-rich sediment. Thus, in accordance with other studies (Gunnarsson *et al.*, 1999, Wolfaardt *et al.*, 1994) I conjectured that an increased bioavailability of organic contaminants could occur due to ingestion of a labile food source, in this case biofilms.

Uptake of dissolved/colloidal chlorpyrifos through larval skin likely contributed to the total larval uptake as stated above. Overlying water concentrations were higher in the humic, sediment, and microbial extract treatments than in controls and sterile sediment treatments. The mechanism behind these higher water concentrations were likely higher concentrations of dissolved organic carbon (DOC) in the form of humic and dissolved/colloidal EPS, respectively, to which chlorpyrifos could associate. We further found that the bioconcentration factors (BCF) in larvae before depuration were high, on average ranging from 321 to 666. However, despite similar overlying water concentrations of chlorpyrifos, the BCF value of the microbial extract treatment was 2-times higher than that of the humic treatment, showing that the bioconcentration factors did not show a straight correlation with chlorpyrifos concentrations in overlying water. This observation may be due to ingestion being the major uptake route, making water concentrations less good predictors for chlorpyrifos uptake. On the other hand, the different types of DOC and their binding characteristics could have influenced the bioavailability of chlorpyrifos. Dissolved/colloidal EPS, with a high dissociation rate of loosely bound pesticide to larval skin may have increased the chlorpyrifos bioavailability. Conversely, humic acids could have reduced the bioavailability due to a strong binding of chlorpyrifos, resulting in DOC-contaminant complexes too large for entering larval membranes. Some support for the conjecture that DOC quality may influence bioavailability comes from Kukkonen and Oikari (1991) who reported decreasing BCF values with increasing amounts of hydrophobic acids within humic acids (resulting in stronger binding). Ankley *et al.* (1994) found closer agreement between predicted and measured concentrations of chlorpyrifos in pore water upon adjustment for potential DOC binding. They also concluded that the organic carbon partitioning model fairly well predicted the toxicity of chlorpyrifos to *Chironomus tentans*. Our findings suggest that besides the total concentration of DOC the quality and its binding characteristics may need to be considered when estimating the risk for bioaccumulation of dissolved organic contaminants.

As expected for this hydrophobic contaminant, high chlorpyrifos concentrations were found in the silica surface layer or the organic layer on top of this. The results indicated that the organic matter and biofilms had an efficient sorption of

chlorpyrifos. Also the bioaccumulation factor value (BAF) was 3 times higher in the microbial extract treatment than in the control. The observed tissue concentrations caused toxic effects in larvae, such as impaired behavior (spastic motility) or mortality, in all treatments. The percentage of larvae with normal behavior was highest in controls (60%) and lowest in the microbial extract treatment (0%). Accordingly, mortality was highest in treatments with microbial extract (76%) and lowest in controls (20%). These mortality rates demonstrate that even if the same amount of pesticide was added to all microcosms, the response of the test organism varied substantially depending on the partitioning of the compound and concentrations in the microhabitat of *Chironomus* (Fig. 3). These results further stress that the quality of organic matter in sediment toxicity tests may influence the obtained toxicity results.

Implications for pesticide bioavailability in standardized toxicity tests

By combining the results from papers III and IV, it becomes evident that the low microbial biomass found in the peat-based artificial sediment may also affect the bioavailability of the test compound to the test organism in standardized toxicity tests. Considering the low microbial biomass and the low nutrient content (i.e. high C/N-ratios) in the artificial sediment it is likely to assume that the EPS production and biofilm development will be poor in this system. Since EPS and microorganisms also sorb hydrophobic contaminants and act like a vector for uptake of these pollutants, their relative role for contaminant uptake in artificial sediment will be less than in natural sediments. Conversely, the refractory organic matter, peat, in artificial sediments, is known to form tight associations with hydrophobic contaminants and to reduce their bioavailability (Freidig *et al.*, 1998, Kukkonen and Oikari, 1991). Another complication with standardized toxicity tests is that *Chironomus riparius* rather feeds on added food particles than the artificial sediment (Åkerblom and Goedkoop, 2003), further decreasing larval exposure to the sediment-bound test contaminants. Hence there may be a high risk that the bioaccumulation and subsequent toxicity of sediment-associated contaminants in a common test organism like midge larvae (*Chironomus* sp), will be underestimated in standardized toxicity tests compared to identical exposure concentrations in natural sediments. Therefore, in accordance with for example Harkey *et al.* (1994) our results stress the importance to use whole sediment toxicity tests, with a living microbial community, for providing better predictions of toxic effects and bioaccumulation. To improve the microbial component of artificial sediments we suggest that a microbial extract from a natural sediment could be added in the sediment preparation procedure. Also the use of another organic substrate would make the artificial sediment more closely represent natural sediment and give more realistic toxicity estimates.

Main conclusions and future perspectives

- Natural microbial communities in sediments can be affected by pesticide exposure at environmentally relevant concentrations, i.e. both estimated safe concentrations and those detected in aquatic sediments.

- Different groups of microorganisms may be affected by different pesticides and exposure concentrations, making straightforward interpretations of toxic effects complicated. It is therefore highly important to use an endpoint that is sensitive for the effects of interest.

- The long-term effects caused by pesticides and repeated pesticide exposures on sediment microbial function need to be further addressed, as well as possible additive, synergistic, or antagonistic effects of mixtures of contaminants.

- With the growing data base of T-RFLP data it should in the future be possible to distinguish which bacterial "species" that are affected (negatively or positively) by pesticide exposure. Their presence or absence in natural sediments could then potentially be used as microbial indicators of contaminant exposure.

- The microbial biomass, activity, and diversity of the peat-based artificial sediment commonly used in toxicity tests is several-fold lower than in a natural sediment. This "lack" of microbes and microbial activity could affect the behavior of the test substance in standardized toxicity tests and ultimately toxicity tests results.

- Sediment microorganisms and biofilms increase the bioavailability of the organic hydrophobic pesticide chlorpyrifos to larvae of the midge *Chironomus riparius*. This indicates that the quality of organic carbon and the interaction of the test organism with this sediment organic pool in toxicity tests need to be considered.

- A recommendation for the improvement of artificial sediment used in toxicity testing could be the enrichment with a microbial extract originating from a natural sediment. A better development of a microbial component should provide more realistic estimates of toxicity.

- Microbes in biofilms are known to degrade pesticides and as biofilms also are vectors for pesticide uptake these two processes are counteracting each other. The relative importance of either of these processes likely depends on the contaminant and biofilm properties. This is an issue that needs further consideration in aquatic ecotoxicology.

Svensk sammanfattning

Mikroorganismer i sediment är betydelsefulla för ekosystemets funktion genom att de bryter ned organiskt material och på så sätt frigör näringsämnen som andra organismer kan använda. Dessutom utgör de en viktig födokälla i basen av den akvatiska näringsväven. Bekämpningsmedel från bland annat jordbruk kan spridas till sjöar och vattendrag där de ofta binds till sediment på grund av deras kemiska egenskaper. Trots detta har väldigt få studier undersökt hur bekämpningsmedel och sedimentlevande mikroorganismer påverkar varandra. Tänkbara interaktioner är allt från upptag av bekämpningsmedel i mikroorganismer till toxiska effekter och/eller mikrobiell nedbrytning av pesticidmolekylerna. I denna avhandling presenteras laboratoriestudier som utförts för att öka kunskaperna om interaktionerna mellan bekämpningsmedel och mikroorganismer i sötvattensediment.

Vid kortvariga exponeringar visade det sig att alla fyra bekämpningsmedel som testades minskade den mikrobiella aktiviteten med 20-24% vid koncentrationer som beräknats vara säkra för miljön (MPC; Maximum Permissible Concentrations). Vid exponering för en högre koncentration (100*MPC) däremot, var det bara ett av bekämpningsmedlen som orsakade en minskad mikrobiell aktivitet. Denna avsaknad av effekt på aktiviteten tolkades som att den höga koncentrationen av bekämpningsmedel var så giftig för vissa känsliga grupper av mikroorganismer att de hämmades i sin tillväxt eller dog. Andra toleranta grupper av mikroorganismer kunde då utnyttja den minskade konkurrensen eller läckage av näringsämnen från döda celler och åtminstone kortvarigt öka sin aktivitet.

Efter längre exponeringar (en månad) av sediment för fyra olika bekämpningsmedel visade molekylära metoder att små förändringar skett i sammansättningen av mikroorganismer. Det konstaterades att de låga och höga koncentrationerna generellt gav förskjutningar i olika riktningar i förhållande till kontrollerna. Det skulle kunna bero på att vissa grupper av mikroorganismer gynnas av låga pesticidexponeringar, exempelvis om de har en förmåga att bryta ned pesticidmolekylerna och på så sätt kan utnyttja dem som en kolkälla. Vid de höga koncentrationerna kan dessa grupper däremot påverkas negativt och endast toleranta grupper av mikroorganismer kan överleva, möjligen som sporer som är mindre känsliga för påverkan. En förskjutning av mikrobsamhällena mot allt fler toleranta mikroorganismer skulle kunna påverka förmågan till nedbrytning av organiskt material.

En tredje studie visade att ett artificiellt sediment (bestående av sand, torv och lerpartiklar) som ofta används i standardiserade toxicitetstester har flera gånger lägre mikrobiell aktivitet, biomassa och diversitet, samt även en annan sammansättning av mikrober, än naturligt sediment. Detta kan påverka hur testsubstansen (exempelvis bekämpningsmedel) bryts ned och dess giftighet, vilket man bör beakta när man utvärderar resultat från toxicitetstester. Vi föreslår att man tillsätter ett extrakt av mikroorganismer från ett naturligt sediment till det artificiella sedimentet för att få det mer likt naturliga exponeringsförhållanden.

Sedimentlevande organismer som äter av biofilmer (mikroorganismer med omgivande ”slem”) har föreslagits vara en viktig upptagsväg för föroreningar i akvatiska näringsvävar. I denna studie visades att koncentrationerna av ett fettlösligt insektsmedel i fjädermygglarver (*Chironomus riparius*) ökade i behandlingar som innehöll utvecklade biofilmer. Behandlingar med humussyror och ett sterilt sediment ökade däremot inte upptaget. Den bakomliggande orsaken tros vara att de extracellulära polymera substanserna (EPS) som produceras av mikrober i biofilmer har en effektiv, men ändå svag bindning, av många organiska föroreningar. När bentiska organismer äter biofilmer med associerade föroreningar frigörs dessa därför lätt under nedbrytningen i magen och kan tas upp i vävnad, vilket leder till en ökad biotillgänglighet. Andra organiska material, ex humusämnen, har däremot en stark bindning av organiska föroreningar, vilket kan leda till en minskad biotillgänglighet. Därför kan kvaliteten och bindningskapaciteten hos det organiska materialet i sediment avgöra upptaget av sedimentbundna föroreningar.

Sammantaget visar dessa studier att mikroorganismer i sediment kan påverkas av pesticidexponering, men även själva kan påverka biotillgängligheten av pesticider till andra organismer. Därför bör man ta hänsyn till mikrobiella processer vid riskbedömningar av pesticider.

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Acknowledgements

The time as a PhD-student can feel like a long journey, especially if it is interrupted by the births of two children. Now when I have finally reached the goal, I would like to thank everyone that in one way or another contributed to my continued sailing and to the thesis you are now holding in your hand. Thanks are written in Swedish to those who understand this language and in English to those who don't.

Först och främst vill jag tacka min huvudhandledare **Willem Goedkoop** för att du gav mig möjligheten att jobba med detta intressanta och spännande forskningsområde. Du har dessutom varit en exemplarisk handledare; handlett med lagom frihet, full av idéer, alltid till hands för en kort fråga eller längre fundering och manus som lämnats för granskning har kommit tillbaka så snabbt att jag knappt hunnit pusta ut. Med åren har du också blivit en kär vän och långt ifrån alla samtal har handlat om forskningen. Så tack för att du har fått mig att växa, och inte bara som forskare.

Tack också till **Henrik Kylin**, min biträdande handledare, och **Peter Sundin**, som fanns med i handledargruppen i början. Ni har varit till stor hjälp när jag har haft frågor som rör organisk miljökemi.

Stefan Bertilsson, som var min handledare under ex-jobbet, tackas för att du med din entusiasm fick mig att inse att lab-arbete och forskning är riktigt kul. Dessutom fick jag förmånen att samarbeta med dig igen under avhandlingsarbetet, vilket tillförde väldigt mycket. Ett varmt tack riktar jag även till mina övriga medförfattare, **Jonas Svensson**, **Ingvar Sundh**, **Kristin Steger** och **Ann-Louise Haglund**, för era olika bidrag till uppsatserna. Inte minst tackar jag **Anna Lundqvist** som kom in som en frisk fläkt i slutet av mitt projekt och jag är tacksam både för energitillskottet, bra samarbete och manusgranskningar.

Stort tack till alla **institutionens doktorander**, både de som fanns med från början och de som tillkommit under åren, för Göteborgsresa, roliga spex, fnittriga doktorandfika, höghöjdsbana och allt annat kul. Ett särskilt tack riktar jag till **Nina Åkerblom** och **Staffan Åkerblom**, mina trogna rumskompisar under större delen av doktorandtiden (både på institutionen och på konferensresor) för samtal, diskussioner, stöd och skratt.

Richard Johnson och **Joakim Dahl** tackas för att ni läste och kommenterade manus innan halvtidsuppföljningen.

Tack alla andra på **Institutionen för miljöanalys**! Alla på **OMK**- och **geokemi**-lab, ni är riktiga klippor när man vill ha hjälp och analyser gjorda! **Micke** har hjälpt till med både figurer, pdf-filer och provtagning och **Herman** har sett till att datorn funkat. De som inte har varit direkt inblandade i min avhandling har skapat en trevlig atmosfär att arbeta i, inte minst vår prefekt **Ewa Bringmark**. Ett speciellt tack vill jag rikta till **Béa**. Vi fann varandra trots en viss åldersskillnad

och hon var delvis min länk mellan Naturvårdsverket och Institutionen för miljöanalys. Jag saknar dig!

Josefin Päiviö och **Maria Persson**, examensarbetare inom projektet, var trevligt sällskap i labbet och på tågresor och uträttade en del försök och labarbete medan jag var hemma med barn och förtjänar därför ett tack för den insatsen.

Thanks are also due to **Dr Alan Decho**, who introduced me to the fascinating world of biofilms and bioavailability studies and who took care of me and my family during our stay in South Carolina, USA. I would also like to acknowledge **Adriana Bejarano**, for being such a nice friend and lab partner and **Tomohiro Kawaguchi** for taking care of me in the lab and teaching me new useful techniques.

Institutionen för ekologi och evolution, **Limnologi**, vid Uppsala universitet är ett trevligt ställe med bred kompetens. Tack för assistans med mina ergosterol-, DNA, C/N- och bakterieabundans-analyser och tack alla för att jag fick trängas med er på lab och i lunchrum.

Ifall det skull vara någon annan som känner sig delaktig i denna avhandling, men som jag har glömt att tacka, så ber jag om ursäkt och gör det nu- TACK!

Livet är så mycket mer än bara jobb. Varmt tack till **släkt och alla mina vänner**, gamla som nya, ingen nämnd och ingen glömd, för att ni finns och gör tiden utanför jobbet meningsfull. Ett särskilt tack dock till "**biologiska familjen**" för att ni gjorde tiden på grundutbildningen i biologi så kul att de flesta av oss aldrig ville sluta studera. Dessutom förtjänar mina svärföräldrar **Magge** och **Lewi** ett extra tack för upp- och barnpassning under åren.

Jag växte upp i världens bästa familj som jag har mycket att tacka för att jag är den jag är! Tack **mamma** och **pappa** för att ni alltid tror på mig, ställer upp och stöttar mig, vilka beslut jag än fattar. Ett särskilt tack för all hjälp med barnpassning och annat under slutfasen av doktorandtiden. Tack **Magnus** och **Håkan** för att ni alltid varit de bästa bröder man kan tänka sig, både som små och vuxna.

Sist, men inte minst, vill jag tacka min egen underbara familj! Min älskade man **Olof** för att du alltid stöttar och tror på mig och för att du har stått ut med mig under slutfasen av avhandlingens tillkomst. De senaste 8 åren har vi fixat 3 barn, 1 husbygge och 2 avhandlingar, vad blir nästa projekt tro? Mina fantastiska barn**, Agnes, Siri och Edvin,** vill jag tacka för att ni ger mig perspektiv på tillvaron och även om mygglarver, sediment, bakterier, bekämpningsmedel och datorn har tagit mycket av min tid på sistone, så är det ni som är viktigast i mitt liv.

TACK!