



Effects of modern pesticides on the microbial community in a natural and in an artificial sediment - a microcosm study.

Master's thesis

by

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Abstract

In the present study effects of for functionally different pesticides (a herbicide; isoproturon, a fungicide; captan and two insecticides; deltamethrin and pirimicarb) on sediment microbiota were investigated in two laboratory microcosm studies. The objective of the study was to examine if the pesticides at negligible concentration (NC), maximal permissible concentration (MPC) and a hundred times the maximal permissible concentration (100xMPC) affected microbial respiration, ATP content and bacterial activity in an artificial and in a natural sediment. The NC and MPC were extracted from a recent RIVM report (National institute of public health and the environment, The Netherlands)

The results indicated that the tested concentrations had inconsistent effect on the tested biological parameters measured in this experiment. In the experiment with artificial sediment it was found that the isoproturon treatment had an overall effect on community respiration (Two-way ANOVA, $P = 0.0106$) and that captan had an overall significant effect. Deltamethrin and pirimicarb had no effect on community respiration. Pirimicarb was the only tested pesticide that had an general effect on sediment ATP concentration. The tested pesticides had no effect on bacterial activity. In the experiment with natural sediment no significant effect of the pesticides was found on community respiration. Of the tested pesticides only deltamethrin had an general effect on the ATP concentration.

Since no general effect on community respiration or ATP concentration were found no clear-cut conclusions regarding the environmental consequences using the chosen pesticides could be drawn. Accordingly, further studies are needed to evaluate effects at the species level, the community level and the ecosystem level.

Introduction

Pesticides are chemicals deliberately distributed all over the world to alter the species composition and dynamics of agricultural systems. The aim of pesticide application is to ensure a secure and economically beneficial outcome of the produced crops. Some of the environmental problems caused by the use of pesticides have been studied intensively, e.g. pesticides in groundwater (Jones 1990) and the fate of pesticides in soil (Arnolds and Briggs 1990), but a lot of questions concerning the fate of pesticides in soils and water systems still need to be answered (Roberts 1990). Pesticides may be applied directly in freshwater to control troublesome aquatic organisms like pests, parasites, vectors of diseases and aquatic weeds (Walker et al. 1996) or they may enter these freshwaters indirectly through run-off or leaching through tiledrains from land. Aquatic sediments both provide a habitat for many aquatic organisms and are a major repository for many of the more persistent chemicals. Contaminated sediments may be directly toxic to aquatic life or can be a source of contaminants that bioaccumulate in the food chain. Both benthic and non-benthic organisms are affected.

Generally, the hydrophobic character of many pesticides cause a strong association with particles. The availability of sediment-associated contaminants to aquatic organisms is dependent on numerous sediment and water characteristics, biological and chemical processes, toxicant characteristics and the organisms' exposure routes via contact and feeding (Bennett 1990, Burton, 1991). About 175 000 species associated with freshwater sediments have been described, with the main organism groups being bacteria, algae, fungi, protozoa, plants and invertebrates (Palmer et al. 1997).

The effects pesticides have on microbial communities (bacteria, fungi, unicellular microfauna), are not well studied. Nevertheless, in a microbial study Londry and Suflita (1997) have shown that organosulfur compounds have an inhibitory effect on microbial activity and that this could in turn influence the anaerobic biodegradation activities. It is known that microbial communities can respond quickly to changes in environmental conditions and therefore can be used as early warning indicators of ecosystem stress by using methods like Microtox® tests (Odum, 1985). According to Palmer et al. (1997) bacteria have an important function in the chemical and photosynthetic production and in the consumption and breakdown of organic matter and release of nutrients. Bacteria

are also involved in biofilm production that affects sediment stabilisation, sediment clogging and deposition of sulphur. Additionally, bacteria are important in processes like nitrification, denitrification, nitrogen fixation, oxidation and reduction of sulphur and iron, degradation of organic components and precipitation of heavy metals (Palmer et al. 1997). Since the microflora in sediments is diverse in its composition, the different functional groups determining the mode of action of the pesticides, i.e. their toxicity, might affect the microbiota in different ways. A fungicide presumably has a higher toxicity to microbiota than a herbicide, and it is therefore relevant to study how pesticides with different functional groups affect the microbial community (Walker et al. 1996)

Correspondingly, little is known about the effects that microbes have on the pesticide test concentrations although some studies have indicated that pesticides are concentrated and accumulated in bacterial cultures (Leschniowsky et al. 1993). On the other hand Streloke and Köpp (1995) suggested that bacterial degradation of test compounds may occur in standardised bioassays and long-term toxicity tests with sediment-dwelling organisms. Svensson and Leonardsson (1992) showed that relatively low concentrations of the fungicide fenpropimorph inhibited denitrifying bacteria in lake sediment and that this negative effect on denitrification is enhanced by prolonged exposure. Studies on terrestrial bacteria made by Martinez-Toledo et al. (1993 and 1997) showed that nitrifying bacteria in soil were negatively affected both by the insecticide lindan and the fungicide captan when used in accordance with the recommendations of the manufacture. In an other study on terrestrial bacteria, Shand et al. (1995) reported that pesticides were metabolised by different soil bacteria, indicating that these compounds could be utilised as a carbon source. Microbial degradation of organic pollutants also occurs in aquatic environments according to Larsson et al. (1988) and Larsson and Lemkemeiser (1989). If interactions occur between test compounds and sediment microbes in toxicity tests/bioassays the interpretations of test results may be seriously affected. One first step to clarify the role of bacteria-mediated processes is to answer the question: Are sediment microbes affected by pesticide concentrations commonly used in standardised toxicity tests.

In the present study effects of four functionally different pesticides (a herbicide, a fungicide and two insecticides) on sediment microbiota were investigated in two laboratory

microcosm studies. The objective of the study was to examine if the pesticides at 1) negligible concentration (NC), 2) maximal permissible concentration (MPC) and 3) a hundred times maximal permissible concentration (100xMPC) affected respiration, ATP content and bacterial activity in the microbial community in an artificial- and a natural sediment. Negligible concentrations and maximal permissible concentrations in sediment were extracted from a recent RIVM report (Crommentuijn et al. 1997) written on behalf of the Directorate-General for Environmental Protection, Directorate for Chemicals, External Safety and Radiation in the context of the project "Setting Integrated Environmental Quality Objectives", National Institute of Public health and the Environment, The Netherlands. The tested concentrations ranged from Negligible Concentrations, i.e. the highest concentration having no effect on sediment associated organisms to a 100 times the Maximal Permissible Concentrations; representing 100 times the highest value causing insignificant effects on sediment associated organisms. In this report MPCs and NCs for the environmental compartments (water, soil and sediment) were derived from ecotoxicological data using extrapolation methods. The extrapolation method used is a statistical extrapolation method and a modified EPA (US Environmental Protection Agency) method which uses assessment factors. These methods require ecotoxicological data, but in the case of sediment, no toxicity data for sediment dwelling organisms were available and NCs and MPCs were derived using the equilibrium partitioning method (Crommentuijn et al. 1997).

Materials and methods

Experimental setup

Two experiments were performed, one with artificial sediment and the other with natural sediment. The experimental set-up and methods used in both experiments were based on the International Toxicity Ring-test (OECD, 1994), but with some minor alterations by Goedkoop and Johnson (1997). First, instead of 2.0 L test vessels, transparent glass vials were used with a volume of approximately 175 ml and a bottom area of 28 cm². Second, aeration was more intense meaning more air per time unit was supplied to the system, 3.0 L min⁻¹ instead of

1.2 L min⁻¹. The experimental set up consisted of one 160 ml Plexiglass tube, one 340 ml gas washing bottle and two 100 ml gas washing flasks connected to each other, followed by an air distribution unit and microcosms connected to 30-ml CO₂ traps (Figure 1). To measure the amount CO₂ produced by the bacteria and other microbes in the sediment in each microcosm, the ingoing air had to be CO₂ free. This was done by the soda lime pellets in the plexiglass tubes and 5M NaOH (*p.a.*) in the gas washing bottles. Thereafter, the air passed through the two E-flasks with deionized water to prevent possible effects on the pH in the microcosms due to NaOH-residue in the air. The pH of the deionized water in the gas-washing flasks was checked regularly to make sure it did not exceed 8. To prevent water vapour originating from the E-flasks reaching capillary tubes, the plastic tube leading from the last 100 ml-E-flask was equipped with a 15 cm long glasspipe. This allowed evaporated water to condense on the cold glass surfaces and thus prevented drift stops. CO₂-traps consisted of 30 ml glass vials filled with 5.0 ml of 0.2 M NaOH *p.a.* CO₂-traps were connected to the air-outlet of each microcosm to collect the CO₂ produced by the microbial community (Figure 1). Both experiments were performed in a climate room at 20.2 ± 0.3 °C with a 16:8 light:dark regime using a 22 W / 30°C Warmton, Warmwhite lamp as an indirect light source. Temperature was checked daily. Aquarium pumps were used for aeration during 15 min each hour, and the airflow was set at 3.0 L min⁻¹. Daily checks were made for the proper function of the aeration.

Artificial sediment

In the first experiment, artificial sediment with 10% organic matter was used. The sediment was prepared according to test guideline No. 207 (OECD, 1994) and it contained seasand (*p.a.*, Merck) with 60% of its particles less than 200 µm (larger particles were removed by sieving), kaolin clay (KEBO), finely ground peat (Original solmull®, Hasselfors Garden AB, <150 µm) and sodiumcarbonate (*p.a.*, Merck). All ingredients, except the peat, were dried in an oven at 105°C until its dry weight had stabilised. The relative distribution of the ingredients in the artificial sediment was 68.7% sand, 19.6% kaolin clay, 10.0% peat and 1.7% sodiumcarbonate of the total dry weight

Table 1: Active ingredients of test compounds and their test concentrations, negligible concentration (NC), maximum permissible concentration (MPC) and 100x MPC.

Pesticide	Active ingredient	NC (µg/l)	MPC (µg/l)	100x MPC (µg/l)
Captan	N-trichloromethylthio cyklohex-4-n-1,2-dicarboximid	1.1	110	11000
Deltamethrin	(S)-a-cyano-3-phenixibenzyl(1R,3R)- 3-(2,2-dibromovinyl)-2,2-dimethyl- cyclopropanecarboxylate	0.003	0.3	30
Pirimicarb	2-(Dimethylamino)-5.6-dimethyl- 4-pyrimidinylcarbamate	0.9	90	9000
Isoproturon	3-(4-isopropylphenyl)-1, 1-dimethylurea	3.2	320	32000

(DW) respectively. The dry ingredients were thoroughly mixed and M7 medium (Heimbach, 1995) was added. M7 medium is a synthetic water recommended to use by the OECD guideline. The sediment was then placed in the refrigerator (4°C) for a few days to let the dry sediment absorb the medium. Aliquots of artificial sediment (35.0 ± 0.10 g) were added to the glass vials (bottom area: 28 cm²) resulting in a sediment layer of about 1 cm. The microcosms were thereafter placed in the freezer over night to prevent resuspension and stratification of the sediment when the overlying M7 medium was added. The following day 150 ml well aerated, cold M7 medium was carefully added to each glass vial. The glass vials were then sealed with a polyethylene snap cap and placed in a climate room at 20°C and allowed to acclimate for 13 days.

Natural sediment

The natural sediments used in experiment two was collected in April in Lake Erken, a mesotrophic lake situated 60 km NNE of Stockholm (lake description by Pettersson, 1990). Surficial sediment was collected from a depth of approximately 12 m with an Ekman sampler. The overlying water was removed with a suction device and the top 2 cm of the sediment were carefully collected with a spoon. The surficial sediment layer was chosen since the highest bacterial and faunal activity is found in the top layer of the sediment. The sediment was aerated in a climate room at a temperature of 10°C for 5 days to slowly accumulate to laboratory conditions. The sediment was sieved (0.5 mm) to remove coarse

detritus and macrofauna and thereafter 35.0 ± 0.10 g of well stirred sediment was transferred to each of the glass vials. The bottom of each glass vial was covered with an approximately 1 cm thick sediment layer. 140 ml well aerated, GF/C filtered (glass microfibre filters, 4.5 cm) Lake Erken water was carefully added and the vials were sealed with a polyethylene snap cap. The microcosms were thereafter allowed to acclimate for eight days in a climate room at 20°C while being aerated (Karlsson, 1998).

Pesticides and pesticide addition

Four pesticides with different functions were chosen, a herbicide, (isoproturon), a fungicide, (captan) and two insecticides, (deltamethrin and pirimicarb). Isoproturon (3-(4-isopropylphenyl)-1, 1-dimethylurea) is a phenylurea herbicide that inhibits the photosynthetic electron transportation system in plants (Niesink et al. 1996). It is used to control annual grasses and broad leafed weeds in small grain cereals. Microbial degradation is considered to be the primary mechanism for its dissipation in soil and probably in sediment as well (Gaillardon and Sabar 1994). Captan (N-trichloromethylthio cyklohex-4-n-1,2-dicarboximide) is a fungicide used to control *Botrytis*, *Fusarium*, *Fusicoccum* and *Pythium*. It is a protectant fungicide applied to the plant surface before infection occurs and remain on the surface and kill any fungal spores or bacterial cells that come into contact with it. It has a fairly high log K_{ow} of 2.8 and it easily adsorbs to the sediment. Captan is no longer used in Sweden, but reaches Swedish lakes and streams by long-range transport (Karlsson, 1998). Deltamethrin

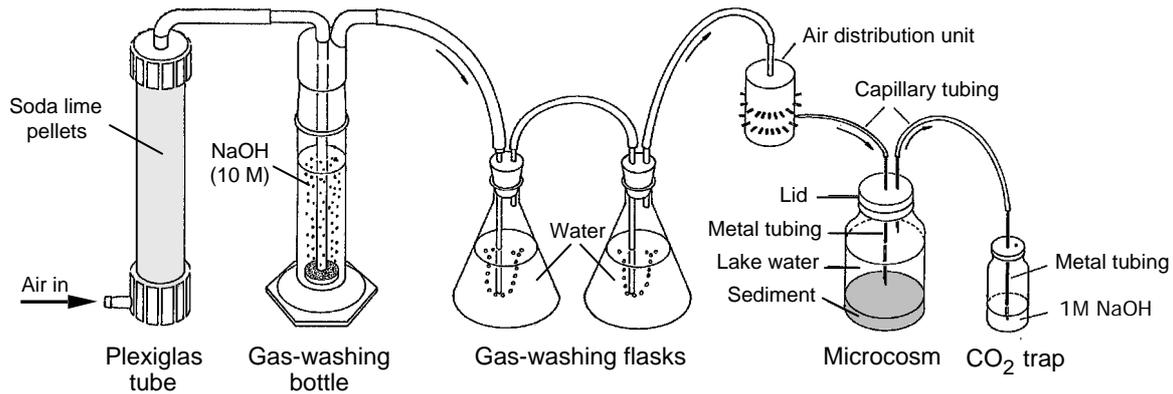


Figure 1. Experimental set up showing how the microcosms were connected to the equipment used to strip the ingoing air from CO₂, enabling measurements of community respiration.

[(S)-a-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate] is an insecticide that, like all the pyrethroides, affects biota by preventing the cellmembrane-associated sodium channels from functioning, thereby disturbing the transmission of nerve impulses. Deltamethrin is used on a broad range of crops, conforms strong adsorption to soil colloids and its main degradation route is through microbial decomposition (The British Crop Protection Council, 1998). Pirimicarb (2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate) is an insecticide that acts by inhibiting acetylcholinesterase and acts as a nerve poison in insects (Niesink et al. 1996). It is readily degraded by chemical and biochemical agents (The British Crop Protection Council, 1998).

On day 0, the four pesticides dissolved in ethanol (99 % *p.a.*) were added to the overlying water in three different proportions allowing the final concentrations representing negligible concentration, (NC), maximal permissible concentration, MPC, and hundred times MPC (100 MPC) (Crommentuijn et al., 1997). An overview of test concentrations is given in table 1. Additionally, controls without pesticide addition were run. The experiments comprised four treatments in four replicates each.

TetraPhyll addition

Fine particulate TetraPhyll® (dried algae, used as fish-food) to simulate natural sedimentation of organic material. This was done seven days prior to the addition of pesticides and on the same day (day 0) the pesticides were added. In each food addition 0.600 µg TetraPhyll® microcosm-1 was added. This addition is comparable to the International Toxicity Ring

test (Streloke and Köpp, 1995) where 150 µg ind-1d-1 is recommended and each microcosm contains four *Chironomus* larvae. In the first experiment with artificial sediment, 100 µl 6 mg TetraPhyll® ml⁻¹ was added to each microcosm. Microcosms not treated with pesticides received no TetraPhyll® but an equal volume of deionized water. In the experiment with natural sediment, 500 µl 1.2 mg TetraPhyll® ml⁻¹ was added.

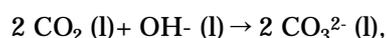
Termination of the experiment

On day 16 the experiments were terminated and an aliquot of the overlying water was taken from the controls for pH measurements. The rest of the water phase was removed using a pump and discarded. In the experiment with artificial sediment, the sediment of each microcosm was mixed thoroughly and aliquots for determination of bacterial activity (³H-thymidine incorporation) and ATP content were taken (see below). Visual observations of the sediment layer in the microcosms with artificial sediment indicated that the top millimetres seemed to be of different composition compared to the rest of the sediment. According to this observation samples from eight microcosms consisting only of the top millimetres were collected with a spoon and analysed for ATP-content. These measurements showed that ATP concentrations in surficial sediment were 4.8 times higher than that of the entire sediment. Consequently, only the top millimetres of sediment were used for ATP-content analysis in the experiment with natural sediment. A comparison between the activity in the mixed sediment and the top layer were made in the measurements of bacterial activity as well with similar results as in the analysis of ATP-content. The activity in

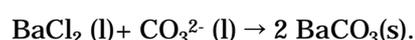
the top layer was found to be 2.9 times higher. No samples for the measurements of bacterial activity were collected in the experiment with natural sediment, since no effect on bacterial activity of pesticide exposure was found in the experiment with artificial sediment.

Respiration

Respiration (registered as CO₂ production) was measured on day -2, -1, 0, 1, 2, 4, 8, 12 and 16 in the experiment with artificial sediment and on day -2, -1, 0, 1, 2, 4, 8, 13 and 16 in the experiment with natural sediment. Prior to the decoupling of CO₂ traps, the microcosms were aerated continuously for 45 min to assure that CO₂ accumulated in the microcosms reached the traps. After removing the CO₂ traps, the small in- and outflow holes in the lid of each CO₂ trap were sealed immediately with tape. Respiration was determined by titrating the 0.2 M NaOH in the CO₂ traps with 0.097 M HCl (Torstensson, 1993). The titrations were made directly in the CO₂ traps and performed within one hour after removal of the CO₂ trap. One drop of phenolphthalein was used as an indicator and 2 ml of 1 M BaCl₂ (*p.a.*) was added right before titration to bind the produced CO₃²⁻ ions. In the CO₂ traps the following reaction occurs:



followed by



The remaining OH⁻ are then titrated with HCl (see above)

To correct for CO₂ originating from the water used as overlying medium the amount of CO₂ in the six blanks added to respective experiment (M7 medium and Lake Erken water) were determined using the same method as described above. The mean respiration of the blanks were subtracted from the treated microcosms and the difference in CO₂ production between the blanks and the treated microcosms were used as the amount CO₂ produced only by the sediment.

ATP-content

The ATP measuring method used in these experiments uses the firefly luciferases which catalyse a reaction between ATP, D-luciferin and O₂ to form AMP, inorganic pyrophosphate (PPi), oxyluciferin, CO₂ and light. The produced light can be quantified with a luminometer and

Table 2: Physical characteristics of the artificial sediment and the natural sediment. Values are means ± SD with the number of replicates (n) in parenthesis.

Sediment type	Loss on ignition (%dw)	Water content (%)
Artificial (OECD-guideline 207)	10.8 ± 0.005 n=4	45.3 ± 1.8 n=16
Natural (Lake Erken, 12 m)	19.5 ± 0.001 n=4	92.3 ± 0.5 n=16

the ATP concentration can be determined (Lundin 1999). For ATP measurements 1.0 g aliquot were transferred to 50 ml polypropylene centrifuge tubes with a spoon. ATP was extracted with 10.0 ml 10% TCA containing 4 mM EDTA. The samples were mixed for one minute and thereafter stored for two days in refrigerator (+4°C) enabling rupture of the cell of the bacteria membranes. Before the ATP measurement the samples were centrifuged and the supernatant was taken care of. Cold 0.8 ml of Tris-EDTA buffer (0.1 mM Tris(hydroxymethyl) aminomethane, 2 mM EDTA, pH 7.75, BioThema® Prod.nr.21-501) was added to 5 ml luminometer measuring tubes and allowed to reach room temperature. Thereafter the ATP content was measured with a luminometer (LKB-Wallace 1250) after adding 10 µl of extractant, 0.2 ml of reagent solution (lyophilised reagent containing D-luciferin and luciferase, BioThema® Prod.nr. 11-501) and an ATP standard (10⁻⁵ moles/L of ATP, BioThema® Prod.nr.45-051).

Bacterial activity

For bacterial activity measurements ([³H]-thymidine incorporation rate), 0.5 g of wet sediment from the microcosms exposed for the highest concentration of pesticide (100MPC) and 0.25 g overlying water was transferred to 10 ml Oak Ridge centrifuge tubes and analysed according to Bell and Ahlgren. The samples were processed by the Division of Limnology, Uppsala University. In this report, bacterial activity is given in DPM g⁻¹ ww⁻¹ h⁻¹.

Sediment characterisation

For determination of the water content the sediment samples were dried at -45 °C for four days using an Edwards 4K Freeze dryer

Modulyo“ (table 2). Loss on ignition was determined after combustion in a Nabertherm“ oven (Mod N54E) at 550°C over night. The natural sediment had an organic content of 19.5% and the artificial sediment consisted of 10.8% organic matter table 2)

Statistical analyses

All data were ¹⁰log-transformed prior to statistical analyses. Respiration data were analysed using two-way ANOVAs and Scheffé's post-hoc test was used for pairwise comparisons. Data on ATP-content and bacterial activity were analysed using one-way ANOVA. The significance level was set at 0.05.

Results

Respiration

Results are presented in figure 2. As seen from this figure all concentrations of isoproturon had an inhibitory effect on respiration in microcosms with artificial sediment. An effect was also observed in the captan MPC treatment compared to the control in the experiment with artificial sediment. None of the other functionally different pesticides affected respiration at any experimental concentration in artificial sediment. Respiration in the experiment with natural sediment was not affected by any of the tested pesticides. Community respiration ranged from 24.4 ± 1.4 to $140.6 \pm 16.4 \mu\text{g g}^{-1} \text{d}^{-1}$ (mean \pm SD, used throughout) in the microcosms with artificial sediment, and from 26.2 ± 6.0 to $100.0 \pm 8.3 \mu\text{g g}^{-1} \text{d}^{-1}$ in the microcosms with natural sediment. A significant overall time-effect (Two-way ANOVA, $P < 0.05$) was found for all four pesticides in both in the artificial- and the natural sediment.

In the experiment with artificial sediment, the herbicide isoproturon had an overall effect on respiration (table 3). Scheffé's post-hoc tests showed significant differences between all tested experimental concentrations and controls ($P = 0.0275$). Respiration in microcosms treated with isoproturon treatments in artificial sediment ranged from 51 ± 5.5 to $141 \pm 16.4 \mu\text{g g}^{-1} \text{d}^{-1}$ with the highest value ($141 \pm 16.4 \mu\text{g g}^{-1} \text{d}^{-1}$) occurring on day 2 (figure 2A). Microcosms with artificial sediments treated with the other pesticides exhibited their highest respiration on day 8. The fungicide captan had an overall inhibitory effect on respiration as well (table 3). The maximal permissible concentration (MPC) had, according to Scheffé's

post-hoc test, a significant inhibitory effect on respiration ($P = 0.0191$). Community microbial respiration in the captan treatments varied over time from 26 ± 4.0 to $107 \pm 8.2 \mu\text{g d}^{-1} \text{g}^{-1}$. Respiration reached its highest value 8 days after the addition of the pesticide ($107 \pm 8.2 \mu\text{g g}^{-1} \text{d}^{-1}$) and declined thereafter to $67 \pm 6.4 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 12 (figure 2B). No effect on respiration was detected for the insecticide deltamethrin at any tested concentration (table 3). Community respiration in microcosms treated with deltamethrin in artificial sediment ranged from 24 ± 1.4 to $120 \pm 10.7 \mu\text{g g}^{-1} \text{d}^{-1}$ and reached a maximum value on day 8 ($120 \pm 10.7 \mu\text{g g}^{-1} \text{d}^{-1}$) and thereafter, like microcosms treated with captan, respiration declined to $71 \pm 4.3 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 12 (figure 2C). The treatments with the insecticide pirimicarb did not have any effect on microbial respiration in the artificial sediment (table 3). Variation in respiration over time in microcosms treated with pirimicarb ranged from 22 ± 3.1 to $115 \pm 4.7 \mu\text{g g}^{-1} \text{d}^{-1}$. Respiration in pirimicarb treatments were also highest on day 8 ($115 \pm 4.7 \mu\text{g g}^{-1} \text{d}^{-1}$) and declined to $78 \pm 7.9 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 12 (figure 2D).

In the microcosm-study with natural sediment no significant effect on respiration was found in either treatment at any tested concentration (table 4). Respiration in the treatment with captan varied from 30 ± 5.8 to $100 \pm 8.3 \mu\text{g g}^{-1} \text{d}^{-1}$. Respiration in the captan microcosms treated with natural sediment peaked 8 days after the addition of the pesticide ($100 \pm 8.3 \mu\text{g g}^{-1} \text{d}^{-1}$) and declined to $50 \pm 6.3 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 13 (figure 3B). No observed effect on respiration was detected in the microcosms treated with deltamethrin and natural sediment either (table 4), with temporal variation in respiration ranging from 34 ± 5.2 to $72 \pm 4.1 \mu\text{g g}^{-1} \text{d}^{-1}$. Also in the deltamethrin treatments the highest values were again obtained on day 8 ($62 \pm 12.1 \mu\text{g g}^{-1} \text{d}^{-1}$) and respiration then declined to $35 \pm 21.0 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 13 (figure 3C). The microcosms treated with isoproturon did not significantly affect the respiration in natural sediment at any of the tested concentrations (table 4). Respiration varied over time from 27.8 ± 2.6 to $74.7 \pm 23.0 \mu\text{g d}^{-1} \text{g}^{-1}$. Also in the microcosms treated with isoproturon, respiration was highest on day 8 ($74 \pm 23.0 \mu\text{g g}^{-1} \text{d}^{-1}$) and declined to $43 \pm 7.6 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 13 (figure 3A). Pirimicarb did not affect the community respiration in any of the added concentrations (table 4). Microbial respiration in pirimicarb treatments with natural sediment varied between 26 ± 6.0 and $95 \pm 12.8 \mu\text{g g}^{-1} \text{d}^{-1}$. Also in pirimicarb treatments respiration obtained its highest value day 8 ($95 \pm$

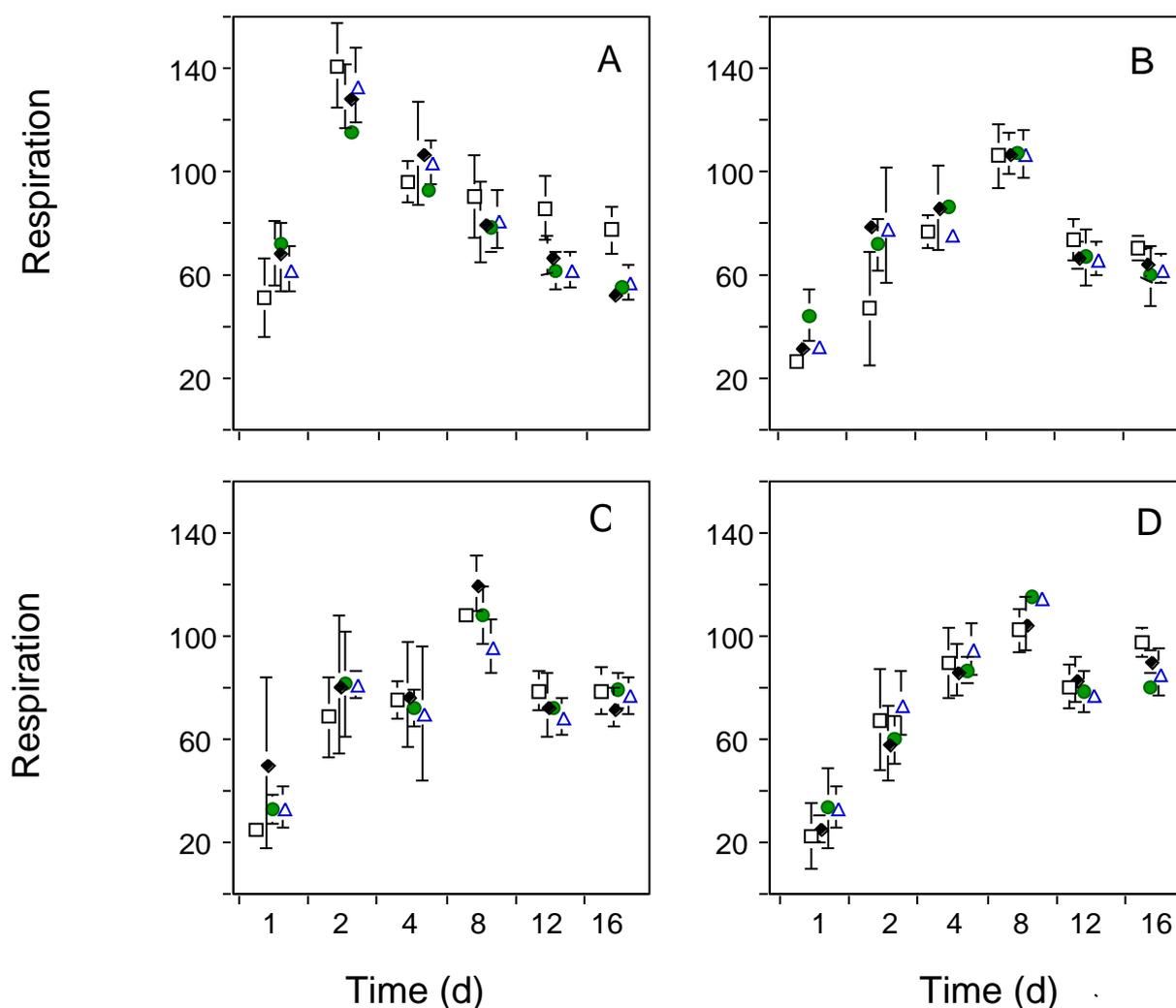


Figure 2: Respiration ($\mu\text{g CO}_2 \text{g}^{-1} \text{d}^{-1}$) in artificial sediment exposed to isoprotruron (A) ($n=61$), captan (B) ($n=68$), deltamethrin (C) ($n=67$) or pirimicarb (D) ($n=66$) for controls (squares), negligible concentrations (diamonds), maximum permissible concentrations (circles) and 100x maximum permissible concentrations (triangles). Error bars give $\pm 1\text{SD}$.

12.8 $\mu\text{g g}^{-1} \text{d}^{-1}$) and then declined to $26 \pm 6.0 \mu\text{g g}^{-1} \text{d}^{-1}$ on day 13 (figure 3D). In the microcosm study with natural sediment there was much less temporal variation compared to the microcosm study with artificial sediment.

In general, the variation in respiration among replicates was remarkably large and partly caused by malfunction of the aeration of the microcosms. Prior to the statistical analysis a number of respiration values were excluded based on following criteria: (1) if aeration disturbances (i.e. the microcosms were not sufficiently aerated) were noticed in the daily checks and the measurement was the lowest among its replicates (2), if respiration was unreasonably low, i.e. lower than $10 \mu\text{g g}^{-1} \text{d}^{-1}$, resulting in a minor difference between the mean respiration calculated from the blanks and the respiration from the microcosm, and (3) if a disturbance of

the aeration was detected and the measurement was 10% lower than the other replicates. Consequently, in the experiment with artificial sediment 4 out of 96 values (4%) were excluded in the captan treatment, 5 values (5%) were excluded in the deltamethrin treatment, 11 values (11%) were excluded in the isoprotruron treatment and in the pirimicarb treatment, 6 values (6%) were excluded. In the experiment with natural sediment 10 out of 96 values (10%) were excluded in the treatments with captan, 12 values (13%) were excluded in the treatments with deltamethrin, 12 values (13%) were excluded in the treatments with isoprotruron and in the treatment with pirimicarb, 8 out of 96 values (8%) were excluded.

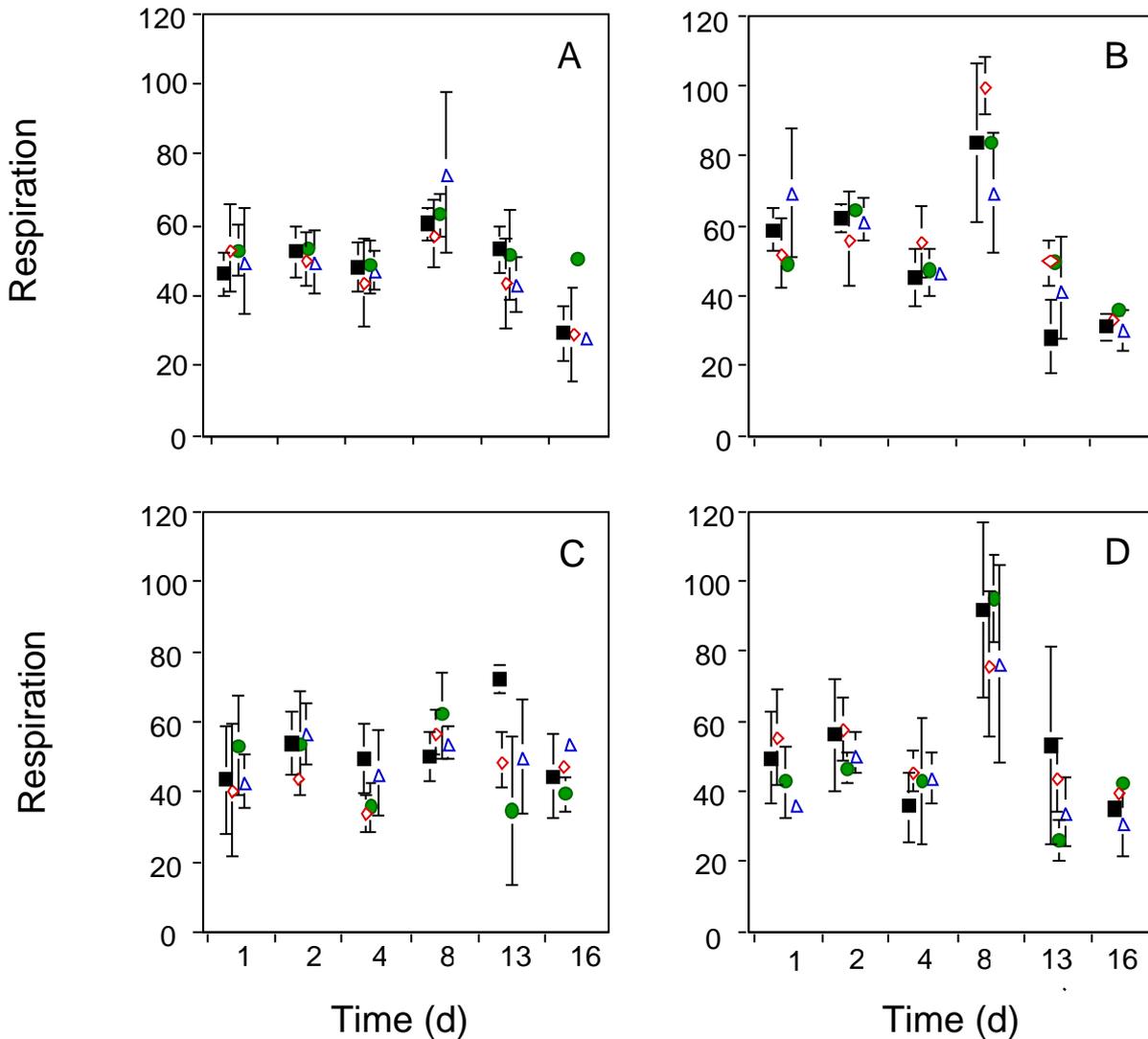


Figure 3: Respiration ($\mu\text{g CO}_2 \text{g}^{-1} \text{d}^{-1}$) in natural sediment exposed to isotroturon (A) ($n = 60$), captan (B) ($n = 62$), deltamethrin (C) ($n = 60$) or pirimicarb (D) ($n = 64$) for controls (squares), negligible concentrations (diamonds), maximum permissible concentrations (circles) and 100x maximum permissible concentrations (triangles). Error bars give $\pm 1\text{SD}$.

ATP- content

The artificial sediment used had generally a low ATP concentration, showing an overall range from 48 to 142 ng g^{-1} . In the experiment with artificial sediment, Pirimicarb had an overall inhibitory effect on sediment ATP concentrations ($P = 0.035$). However, pairwise comparisons revealed no difference between exposure concentrations (Scheffé's test, $P = 0.087$). The results from the ATP measurements varied from 62 ± 14 to $83 \pm 9 \text{ ng g}^{-1}$ in the microcosms treated with pirimicarb (figure 4A). None of the other pesticides tested affect sediment ATP-levels (figure 4A). ATP concentration in artificial sediment varied from 82 ± 19 to $123 \pm 35 \text{ ng g}^{-1}$ in the treatments with captan, from 49 ± 4 to $72 \pm 21 \text{ ng g}^{-1}$ in the microcosms

treated with deltamethrin and from 88 ± 14 to $143 \pm 63 \text{ ng g}^{-1}$ in the microcosms treated with isotroturon.

Compared to artificial sediment, natural sediment generally had a much higher ATP content ranging from 402 to 1058 ng g^{-1} . An overall significant effect on ATP content was observed in the treatment with deltamethrin ($P = 0.005$). Scheffé's post-hoc tests showed that there was no significant difference between controls and the lowest (NC) and the highest tested concentration (100xMPC), respectively (Scheffé's test, $P = 0.566$ and $P = 0.214$). Yet, deltamethrin affected ATP content at MPC ($P = 0.006$). The ATP concentration in the microcosms treated with deltamethrin varied from 581 ± 189 to $1009 \pm 352 \text{ ng g}^{-1}$ (figure 4B). For microcosms treated with isotroturon no effect

Table 3: Two-way ANOVA table for log-transformed community-respiration in the microcosm study with artificial sediment.

	Captan			Isoproturon			Deltamethrin			Pirimicarb		
	df	F	P	df	F	P	df	F	P	df	F	P
Conc.	3	3.065	0.034	3	4.067	0.011	3	1.152	0.335	3	1.020	0.390
Time	5	72.868	<0.001	5	63.534	<0.001	5	49.357	<0.001	5	69.875	<0.001
Interaction	15	2.958	0.001	15	1.034	0.003	15	1.034	0.433	15	0.824	0.648

Table 4: Two-way ANOVA table for log-transformed community-respiration in the microcosm study with natural sediment.

	Captan			Isoproturon			Deltamethrin			Pirimicarb		
	df	F	P	df	F	P	df	F	P	df	F	P
Conc.	3	1.686	0.179	3	2.418	0.075	3	1.926	0.135	3	2.368	0.079
Time	5	32.660	<0.001	5	14.308	<0.001	5	2.755	0.026	5	17.834	<0.001
Interaction	15	2.010	0.0012	15	1.105	0.372	15	1.813	0.054	15	1.374	0.188

was observed at any of the experimental concentrations (One-way ANOVA, $P = 0.128$). ATP concentrations in the isoproturon treatments in the experiment with natural sediment varied from 402 ± 157 to 920 ± 266 ng g⁻¹ (figure 4B). Correspondingly, pirimicarb had no significant effect on the ATP content (One-way ANOVA, $P = 0.352$). ATP concentrations in natural sediment varied from 402 ± 133 to 1058 ± 157 ng g⁻¹ (figure 4B). The fungicide captan had no significant effect on the ATP content in the natural sediment (One-way ANOVA, $P = 0.604$) and the ATP concentration varied from 669 ± 175 to 801 ± 163 ng g⁻¹.

Unfortunately, also in the ATP dataset some extreme outliers had to be omitted prior the statistical analysis to reduce the risk of misinterpretation of the data. The criterium for omission was that if a value was at least two times higher or lower than the mean it was excluded. In the experiment with artificial sediment 2 out of 16 values (13%) was excluded in the treatments with captan and pirimicarb, 1 value (6%) was excluded in the treatment with deltamethrin and 3 values (19%) were excluded in the treatment with isoproturon. In the experiment with natural sediment 1 value out of 16 was (6%) excluded in the treatments with captan and deltamethrin, 3 values (19%) were excluded in the treatment with isoproturon and 2 values (13%) were excluded in the treatment with pirimicarb.

Bacterial activity

The ³H-Thymidin incorporation rate was measured both on the whole mixed artificial sediment and on the top millimetres of the artificial sediment. The top millimetres of the sediment generally had a more than 3-fold higher activity (ranging from 153,891 to 313,841 DMP g⁻¹ h⁻¹) than the whole mixed sediment (ranging from 55,704–76,320 DMP g⁻¹ h⁻¹). However, no effect of any test concentration on bacterial activity was observed (One-way ANOVA, $P > 0.05$) (figure 5).

Discussion

The data in the present study showed that the tested substances had no consistent concentration-dependent effects on respiration or ATP concentration in neither the artificial sediment nor the natural. In some instances significant differences were found between controls and MPC, for example in the microcosms treated with captan in the experiment with artificial sediment, but no difference was found between controls and the highest concentrations. Consequently, assumptions could either be made that the negligible- and maximal permissible concentrations don't have the effect they are referred to have or that a possible effect was not detected by the tested parameters.

Macroinvertebrates, sediment microbes and toxic compounds interact in a highly complex

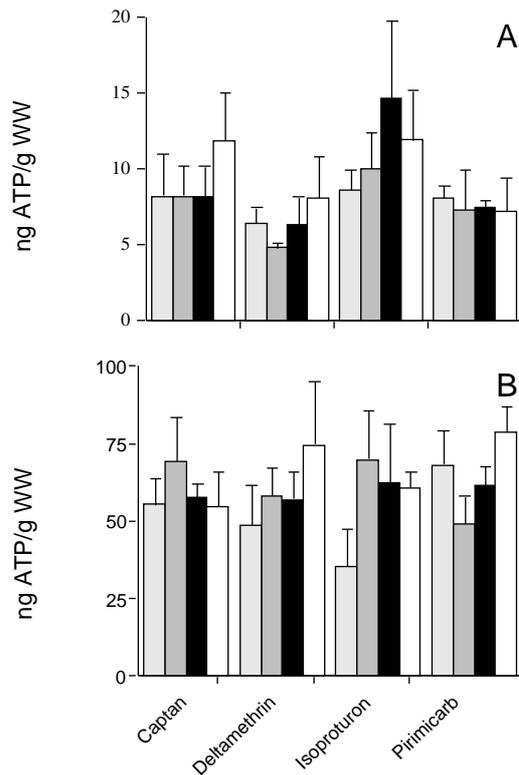


Figure 4. ATP content in artificial (A) and natural sediments (B) for controls (white bars) and for negligible concentration (striped bars), maximal permissible concentration (grey bars), and 100x maximal permissible concentration (black bars). The number of observations in the experiment with artificial sediment were 10 for each pesticide. In the experiment with natural sediment the number of observations was for captan and deltamethrin 11, for isoproturon 9 and for pirimicarb 10. Error bars give \pm 1SD.

way through various processes as illustrated in figure 6. Invertebrate activity like bioturbation and excretion has a positive effect on the sediment microbes (van de Bund et al. 1994) and since the microbes functions as food for the invertebrates they in turn stimulate the invertebrates. Toxic compounds may have negative effects on the activity of both macroinvertebrates and sediment microbes. On the other hand, activity of the invertebrates has a positive effect on the microbial degradation of the toxic compounds. In the present study, no macroinvertebrates were present since the sediment was either artificial or sieved (0.5mm). The microbial processes in the artificial sediment are not the same as those in natural sediment and therefore the sediments composition might have affected the measured parameters. Goedkoop and Törnblom (1996) calculated the bacterial production at 20°C and the bacterial abundance in sediment from mesotrophic Lake

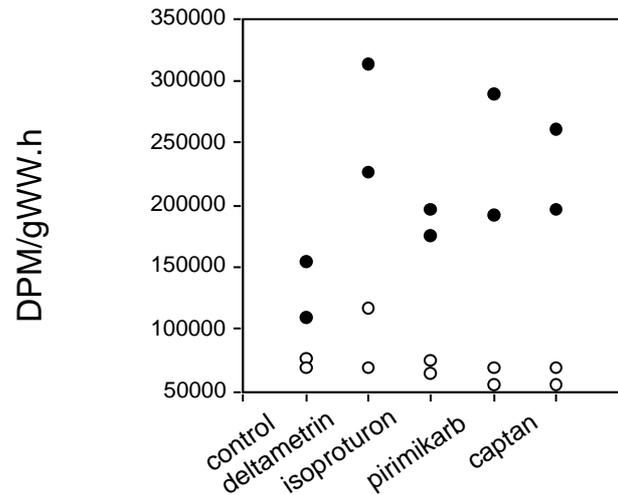


Figure 5. ³H-Thymidin incorporation rate (bacterial activity) for the top mm (black dots) and whole mixed samples of artificial sediment after exposure with pirimicarb, captan, deltamethrin or isoproturon at 100 x MPC. n=4.

Erken in July 1991 and found that these parameters were 12 and 60 times higher, respectively compared with an artificial sediment. In the present experiment, both respiration and ATP content in the artificial sediment were similar to those measured by Karlsson (1998). The respiration rate in the natural sediment was similar to that in the artificial, but the ATP contents were at least 5 times higher. The amount of organic carbon in sediment affects the partitioning of non ionic substances between particulate and interstitial water phase, and organic carbon has a substantial effect on the toxicity to benthic organisms of these compounds (Lamberson et al. 1992). In sediments with a high amount of organic matter it is more likely that toxicants like pesticides have a strong association to the particles in the sediment, i.e. their bioavailability is low. This may explain why an effect on respiration was observed in both the microcosms treated with captan and the microcosms treated with deltamethrin in the experiment with artificial sediment but not in the experiment with natural sediment. The organic content in the natural sediment was markedly higher than that of the artificial sediment (table 2).

The three test concentrations of each pesticide were chosen based on a study conducted by Crommentuijn et al. (1997). In this report Maximal Permissible Concentrations (MPCs) and Negligible Concentrations (NCs) for 70 pesticides were determined in water, soil and sediment. The concentrations for the environmental compartments were first based on eco-

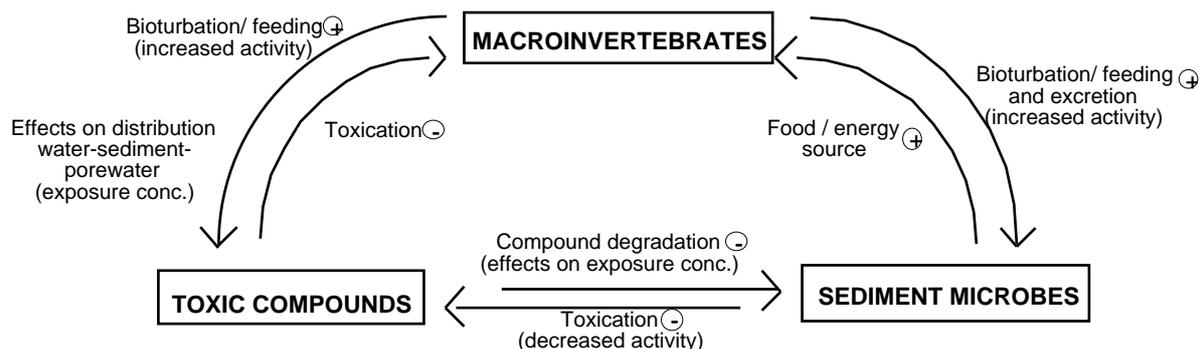


Figure 6. Overview of the complex interactions, through various processes, involving macroinvertebrates, sediment microbes and toxic compounds.

toxicological data using extrapolation methods and were then harmonised using the equilibrium partition method (see below). However, for sediment dwelling organisms no toxicity data were available and all MPCs and NCs were derived entirely by using the equilibrium partitioning method. MPCs and NCs derived by equilibrium partitioning are regarded as less reliable than MPCs and NCs based on ecotoxicological data (Crommentuijn et al. 1997). The MPC for sediment species using equilibrium partitioning was calculated using the formula:

$$MPC_{(sed/soilEP)} = MPC_{(water)} * K_{p(ssoil/ssed)}$$

In which:

$MPC_{(sed/soilEP)}$ = Maximum Permissible Concentration for terrestrial or sediment species using the equilibrium partition theory.

$MPC_{(water)}$ = Maximum Permissible Concentration for aquatic species .

$K_{p(ssoil/ssed)}$ = partition coefficient for standard soil or sediment in 1/kg.

(Crommentuijn et al. 1997)

Using this method, three assumptions are made. Firstly, the bioaccumulation and toxicity are closely related to the pore water concentrations. Secondly, the sensitivities of benthic organisms are comparable with the sensitivities of organisms living in the water. Thirdly, that equilibrium exists between the chemical sorbed to the particulate sediment organic carbon and the pore water and that the pore water and these concentrations are related by a partition coefficient, K_{oc} (= organic carbon normalised partition coefficient in 1/kg). In other words, this approach assumes that the expo-

sure in a water-biota system is comparable to the exposure in a sediment-water-biota system and that the toxicity of chemicals is determined by the concentrations in pore water (figure 7). This being the case has been shown for instance by Di Toro et al. (1991) in an experiment where biological effects (mortality and growth rate suppression) of a group of different substances on sediment organisms occurred at comparable concentrations if these were expressed as the concentrations in the pore water.

The assumptions underlying the equilibrium partitioning method are discussed and questioned and studies have pointed out errors in toxicity modelling that may be introduced by the use of this method. Alexander (1995) and Belfroid et al. (1996) have shown that the bioavailability or toxicity of chemicals in soil decreases with an increasing contact time of the substance in the soil, an incident called aging. Today, there are too few data to apply a factor for this aging phenomenon in the risk assessment of chemicals, and consequently this may overestimate the risks. Besides aging, a variation in bioavailability between classes of chemicals may also lead to an over- or under-estimation of the risks. Furthermore, due to kinetical constraints, equilibrium between the different compartments illustrated in figure 7 will probably not be reached for compounds readily degradable in the environment such as many pesticides (Belfroide et al. 1996). According to Van Brummelen et al. (1996) the habitat and physiology of an organism are other important parameters determining if organisms and pore water reaches equilibrium or how close they come from reaching it. Thus, in reality there may not always be an equilibrium situation among the different compartments in the ecosystem. To base NC and MPC on the equilibrium partitioning method

Table 5. Overview showing the role of freshwater sediment organisms in key ecological processes. (Modified from Palmer et al. 1997)

Biotic Group	Functional role
Bacteria	Chemical or photosynthetic production (autotrophy), consumption of organic matter (heterotrophy), biofilm production (effects sediments stabilization, sediment clogging, deposition of sulphur), breakdown of organic matter and release of nutrients, nitrification, denitrification, dinitrogen fixation, oxidation and reduction of sulfur and iron compounds, degradation of organic components, precipitation of heavy metals.
Algae	Photosynthetic production of new plant material, biofilm development, sediment formation.
Fungi	Decomposition of organic matter, biofilm production, mediative activities.
Protozoa	Regulation of decomposition (bacterial grazing), og fungal and bacterial population densities
Plants	Photosynthetic production of new plant material and oxygenation of sediment, baffle water flow, sediment trapping, and sediment stabilization.
<i>Invertebrates</i>	
Aschelminthes	Regulation of decomposition via microbial grazing, bioturbation.
Annelida	Regulation of decomposition and autotrophy through grazing, bioturbation, sediment formation, repacking of sediment.
Mollusca	Bioturbation, sediment formation, repacking of sediment.
Acari	Regulation of decomposition
Crustacea	Bioturbation, regulation of decomposition through grazing, physical breakup of detrius (shredding), repacking of sediments.
Insecta	Bioturbation, regulation of decomposition through grazing, physical breakup of detrius (shredding), repacking of sediments.

may thus cause an overestimation of the environmental risk in handling pesticides. Instead of having to base NC and MPC on this method the concentrations of each pesticide were based on toxicity data for water. When deriving toxicity data for water, chronic NOECs must be available for less than 4 species from different taxonomic groups. Assessment factors to be applied to toxicity data are also a requirement. The magnitude of this factor depends on the number and kind of these data. The toxicity data for water was lower than the toxicity data for sediment as organisms living in the water are more sensitive to toxic substances (Walker et al. 1996) and this may explain why the pesticides used in this experiment had no general effect on the microbial community.

As shown in table 5, sediment is a complex medium that is a habitat for many biotic groups with varying functional roles.

The chosen endpoints in this experiment, (respiration, ATP concentration and bacterial activity) are unspecific, measuring the whole benthic community and thus only measure community-level effects. Maybe an effect could have been noticed if instead of measuring community-level effects, effects on species-level were measured. (table 3). Goedkoop et al. (1997) calculated that the respiration in meiofauna in Lake Erken sediment represented only a few percent of the total community respiration.

Consequently, a negative effect on the meiofauna in these experiments would likely not have been detected. Examples of more specific tests that could be used are Microtox, and toxicity tests using *Daphnia* sp. and *Chironomus*.

Respiration in the experiments fluctuated considerably over time. According to Karlsson (1998), a possible source of these fluctuations could be a succession of the microbial community. The observed sharp decline in respiration after day eight could be the result of a community crash due to competition for essential nutrient (Sander and Kalff, 1993). Due to the Tetraphyll® addition an increased respiration was expected the days following the addition. This occurred in the first experiment with artificial sediment (figure 2), but not in the experiment with natural sediment (figure 3). The temporal variation in respiration rate in the experiment with natural sediment was much less. The reason for this has probably to do with the natural sediments different composition with a higher and more diverse proportion of organic material compared to the proportion of organic matter in the artificial sediment.

The large variation in respiration among the replicates may partly be introduced by Tetraphyll® additions. Tetraphyll® was added to increase the activity in the sediment prior to addition of the pesticide and on the same day the pesticide was added to the microcosms. In

the feeding procedure, one microcosm, by mistake, may have received two food additions, leading to a higher activity in that microcosm compared to its replicates. Tetraphyll[®] was in the experiment with artificial sediment added in a small volume of water (100 ml), and insufficient stirring may have caused a relatively large variation in the amount of Tetraphyll[®] added. In the experiment with natural sediment the suspension was stirred throughout the addition and a larger volume (500 ml) of the Tetraphyll[®]-suspension was added as an attempt to minimise the variation observed in the first experiment. However, the variation between the replicates in experiment two did not decrease, indicating that another source caused the variation.

In performing the microcosm experiments, the sediment-water system has to be aerated to supply oxygen to the microbiota in the sediment. Since all the CO₂ from the ingoing air was removed the amount CO₂ produced by the microbiota in the sediment could be measured and used to quantify respiration. The aeration of the microcosms was checked on a daily basis and sometimes the aeration was malfunctioning due to condensed water stopping up the capillary tubes between the microcosm and the CO₂ trap. As a consequence, not all CO₂ produced was transferred to the CO₂ trap and this probably was a source of variation among the replicates. In calculating the amount CO₂ produced, a comparison was made between the amount CO₂ produced in the glass-vials only containing M7 or Lake Erken water with the amount CO₂ produced in the experimental microcosms containing sediment and water. This was done to quantify the amount CO₂ originating from the overlying water. It was thereafter assumed that the difference in respiration between the glass-vials and the microcosms was equal to the amount CO₂ produced by the sediment. This is an indirect measurement of respiration and introduces uncertainty and may be another explanation to the large variation among the replicates.

The variation among the replicates in the ATP content was large as well. A pilot study showed that the concentration was highest in the top millimetres. The fact that the whole sediment was used in the experiment with artificial sediment can explain some of the variation in these measurements i.e. insufficient mixing of the artificial sediment may have caused variation in ATP content among the replicates. In the experiment with artificial sediment and deltamethrin a significant reduction was found for the ATP-concentration com-

paring the control with the MPC (One-way ANOVA, $P = 0.006$). Since a reduction was not observed in the 100xMPC treatment the reduction in the MPC treatment might be the result of the large variation between the measurements.

Since no or inconsistent effects on community respiration or ATP concentration were found, no clear-cut conclusions regarding the environmental consequences using the chosen pesticides could be drawn. Further studies are needed to evaluate effects at the species level, the community level and the ecosystem level. It is also necessary to produce more data to improve the modelling of MPC. In statistical analysis there is always a risk of a type 1 error i.e. a false significant result. The inconsistent significant results for example in the artificial sediment treated with captan at MPC indicate that such an error has occurred. In the analysis of the results from the ATP measurement one possible type 1 error was detected in the microcosms treated with deltamethrin at MPC in the experiment with natural sediment. As stated above, if parameters related to species level had been measured instead of community level effects the results may have said more about the specific effects the pesticides might have on the complex matrix of sediment.

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