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Effects of pesticides on community composition and activity of sediment microbes – responses at various levels of microbial community organization

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Molecular techniques revealed pesticide-induced changes at lower levels of microbial community organization that were not detected by community-level end points.

Abstract

A freshwater sediment was exposed to the pesticides captan, glyphosate, isoproturon, and pirimicarb at environmentally relevant and high concentrations. Effects on sediment microorganisms were studied by measuring bacterial activity, fungal and total microbial biomass as community-level endpoints. At the sub-community level, microbial community structure was analysed (PLFA composition and bacterial 16S rRNA genotyping, T-RFLP). Community-level endpoints were not affected by pesticide exposure. At lower levels of microbial community organization, however, molecular methods revealed treatment-induced changes in community composition. Captan and glyphosate exposure caused significant shifts in bacterial community composition (as T-RFLP) at environmentally relevant concentrations. Furthermore, differences in microbial community composition among pesticide treatments were found, indicating that test compounds and exposure concentrations induced multidirectional shifts. Our study showed that community-level end points failed to detect these changes, underpinning the need for application of molecular techniques in aquatic ecotoxicology.

Keywords: Pesticides; Sediment; Microbial communities; PLFA; Genetic fingerprinting

1. Introduction

Sediment microbial communities provide important functions in aquatic ecosystems like the decomposition of organic material, the recycling of nutrients, and by constituting a major food source at the base of aquatic food webs. Additionally, microbes are able to detoxify or degrade sediment-associated organic pollutants and can thus remediate contaminated ecosystems. Pollutants may, however, also have adverse effects on many microorganisms in the sediments (DeLorenzo et al., 2001). Due to the complexity of detecting ameliorative effects of environmental contaminants on natural microbial assemblages, few studies have addressed this topic (Warren et al., 2003). Agricultural pesticides are a group of potentially toxic substances that are deliberately introduced in the environment over large spatial scales. Pesticides may unintentionally contaminate aquatic ecosystems through spray-drift, runoff, or soil erosion and have frequently been detected in both surface waters (Planas et al., 1997) and in sediments (Kreuger et al., 1999). Hence, the exposure of sediment microorganisms to a wide range of pesticides could affect important ecosystem functions. Despite the frequent occurrence of pesticides in

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the aquatic environment, little is known about their interactions with non-target sediment microorganisms (DeLorenzo et al., 2001).

Toxic effects of pesticides on microorganisms have traditionally been assessed in laboratory tests with individual bacterial strains (Bitton and Koopman, 1992) or by studying functional responses like overall microbial activity or respiration (Burton and Lanza, 1985). Such single species tests or community level end point analyses may, however, fail to detect more subtle, yet functionally important, changes in the composition of microbial communities in response to pesticide exposure. In other words, dramatic shifts in microbial community structure may occur upon toxicant exposure, but these changes may not be detected by crude functional end points due to inherent redundancy, i.e. tolerant species may compensate for loss of functions associated with more sensitive groups (Johnsen et al., 2001). Still, such changes may eliminate important ecosystem functions mediated by certain microbial populations and/or alter the sensitivity of certain functions to other environmental constraints (e.g. pH, temperature, redox conditions). Traditional culture-dependent techniques are of limited use for the analysis of microbial assemblages in soils or sediments, since more than 90% of indigenous microorganisms in nature escape cultivation (Ward et al., 1990). During the last decades, however, a number of culture-independent methods such as analysis of phospholipid fatty acids (PLFA) (Pennanen et al., 1996) and genetic fingerprinting (Polymenakou et al., 2005), have been applied to detect pollutant-induced changes in the composition of natural microbial communities. PLFAs are useful microbial biomarkers for some specific groups of microbes and also provide a good indirect estimate of viable microbial biomass (Pinkart et al., 2002). Genetic fingerprinting methods for analysis of mixed microbial communities often use ribosomal RNA (rRNA) sequence heterogeneity as a diversity proxy and one strategy to describe the rRNA heterogeneity is terminal restriction fragment length polymorphism (T-RFLP). This relatively novel technique is based on mixed-template PCR amplification of community rRNA genes and subsequent genotyping using restriction cleavage patterns (Liu et al., 1997). Applying these methods in ecotoxicology may provide new possibilities to quantify pesticide-induced changes in microbial communities.

In a previous study, we found that sediment bacterial activity (^{3}H-leucine incorporation rates) was inhibited in short-term exposures (hours) with four pesticides at environmentally relevant concentrations (Widenfalk et al., 2004). Somewhat surprisingly, no inhibition of bacterial activity was found at higher exposure concentrations for three of the four pesticides. We speculated that this was due to compensatory mechanisms, i.e. that some microbial groups were favored by high pesticide exposure and that their growth and increased activity were masking potential negative effects on other groups. Such shifts in microbial community composition have been described for soils after exposure to various pesticides (El Fantruossi et al., 1999) and for sediments after application of antifouling biocides (Petersen et al., 2004). In the present study we assessed whether sediment microbes were affected by exposure to the pesticides captan, glyphosate, isoproturon and pirimicarb at environmentally relevant and high pesticide concentrations, at both community and subcommunity (''species'') levels. Bacterial activity ([^{3}H] leucine incorporation), microbial biomass (ΣPLFA), and fungal biomass (ergosterol concentrations) were quantified as community-level end-points, while PLFA composition (microbial community structure) and T-RFLP (bacterial community composition) were analyzed to study effects on microbial community structure. We hypothesized that exposure to pesticides could affect microbial community function and could induce shifts in community structure.

2. Materials and methods

2.1. Test sediment

Profundal sediment (16 m depth) and surface water were collected in February 2003 from Lake Erken, a mesotrophic lake that is situated 60 km North-East of Stockholm, Sweden (59° 51′N, 18°35′E) and is relatively unaffected by agricultural activities. Sediment was collected from the ice with an Ekman grab. The overlying water was removed with a hose and the top 2 cm of the sediment was collected with a spatula. The sediment was brought back to the laboratory and kept dark at 4 °C under aerated water, i.e. mimicking winter conditions in the profundal zone of Lake Erken. After one week, the sediment was sieved through a 0.5-mm mesh and stored under aerated lake water for 3 d at 10 °C and subsequently for 4 d at 15 °C to gradually acclimate to experimental conditions. During storage, ground fish food (Tetraphyll®) was added twice as organic matter to avoid nutrient depletion.

The water content (105 °C, over night) of the experimental sediment was 89.7 ± 0.1% ($n = 4$) and the organic matter content (loss on ignition, 550 °C over night, Nabertherm oven Mod N54E) was 22.0 ± 0.1% (of dw, $n = 4$). The carbon content was 9.1% and nitrogen content 1.2% (of dry weight), as determined with an elemental analyzer (Carlo Erba NA1500).

2.2. Pesticides and exposure concentrations

To investigate whether the toxicity to non-target microorganisms is dependent on the specific toxicity to target organisms, four functionally different pesticides; captan, glyphosate, isoproturon, and pirimicarb (Ehrenstorfer Reference Substances, determined purity ≥98%) were chosen as test compounds. The fungicide captan belongs to the class phtalimides for which the exact mechanism(s) of action is unknown, but there are possibly several mechanisms (Ecobichon, 1991). Glyphosate is a widely used systemic, broad-spectrum herbicide (active ingredient in e.g. Roundup®), which most importantly inhibits the production of certain amino acids by acting on at least one enzyme system in plants (Franz et al., 1997). Isoproturon is a phenylurea herbicide that inhibits the photosynthetic electron transport system in plants (Roberts, 1998), but it is also directly or indirectly toxic to a wide variety of organisms (Tomlin, 1994). Pirimicarb is an insecticide that disables the nervous system by the inhibition of acetylcholinesterase (Ecobichon, 1991).

Sediments were exposed to either ''low'' or ''high'' concentration of each pesticide. The ''low'' exposure concentrations of captan, isoproturon, and pirimicarb were the environmentally relevant Maximum Permissible Concentrations (MPC), i.e. the highest permissible concentration of a single substance before ecotoxicological effects can be expected (Crommentuijn et al., 2000). We used previously reported MPCs for sediments, which are based on equilibrium partitioning due to the lack of ecotoxicologically relevant data. Since no MPC-value is available for glyphosate, we chose a low concentration that was similar to detected concentrations of glyphosate in stream sediments in agricultural areas of Sweden (Sundin et al., 2002); 150 μg/kg DW. Our ''high'' pesticide concentrations were 1000 times higher than the ''low'' concentrations. All nominal pesticide exposure concentrations are given in Table 1. Stock solutions of the pesticides were prepared in DMSO (dimethylsulfoxide, *p.a.* Merck, Darmstadt, Germany), which is non-toxic to bacteria (Kahru et al., 1996).

Table 1
Pesticides used in the experiment, their chemical names (from International Union of Pure and Applied Chemistry), partition coefficients in octanol and water (log K_{ow}), and the applied nominal exposure concentrations (Maximum permissible concentration, MPC) in sediment

Pesticide	Active ingredient	Log K_{ow}[a]	MPC µg/kg dw[b]	1000*MPC µg/kg dw
Captan	(N-trichloromethyl-thiocyclohex-4-ene-1, 2dicarboximide)	2.8	1.3	1300
Glyphosate	N-(phosphono-methyl) glycine	−3	150[c]	150 000
Isoproturon	(3-(4-isopropylphenyl)-1, 1-dimethylurea)	2.5	5.3	5300
Pirimicarb	(2-dimethylamino-5, 6-dimethylpyrimidine-4-yl dimethylcarbamate)	1.7	2.2	2200

[a] From Tomlin (1994).
[b] From Crommentuijn et al. (2000).
[c] From Sundin et al. (2002), detected environmental concentration.

2.3. Microcosms and experimental setup

Replicate microcosms ($n = 4$) were created by transferring 25.0 g of well-mixed wet sediment to glass jars (175 ml, bottom area 28 cm^2), resulting in a sediment layer of approximately 1 cm. The experimental sediment was spiked with 25 µl of pesticide stock solution to achieve the proper nominal low and high exposure concentrations. An equal volume of pure DMSO was added to the controls. After pesticide/DMSO addition the sediments were thoroughly mixed and then covered with 125 ml of GF/C-filtered (glass microfibre filters, Whatman International, Maidstone, England) Lake Erken water. Microcosms were covered with lids, placed in a dark climate room at 15 ± 0.5 °C and were aerated with approximately one air bubble per second using Pasteur pipettes. Organic matter (300 µg Tetraphyll® per microcosm) was added at the start of the experiment and then once a week for the remainder of the experiment (in total 1.5 mg, corresponding to 0.3% of the total organic matter pool of the sediment). This was done to mimic natural additions of particulate organic matter to the sediment and avoid starvation of the microbial community. For measurements of bacterial activity (see below), microcore sediment samples were taken after 1, 3, 7, 14, and 31 days using 10-ml sterile syringes (without the needle) and transferred to 10 ml Oak-Ridge tubes (Nalgene, Rochester, NY, USA) and weighed (250 ± 25 mg WW).

Oxygen concentrations (YSI oxygen meter, model 51B equipped with a 5739 electrode, YSI Scientific, Yellow Springs, OH, USA) and pH (Metrohm 691 pH-meter, Metrohm Ltd, Herisau, Switzerland) in the overlying water of each microcosm was measured on days 14 and 31. Incubations were terminated after 31 days by removing the overlying water and thoroughly mixing the remaining sediment. The pH of these sediments was analyzed as described above. Subsamples of 6 g were transferred to 50-ml Falcon tubes and stored at −20 °C for ergosterol analysis. The remaining sediment was frozen directly in the microcosms at −20 °C. Prior to analysis, the microcosms were thawed in a fridge, the sediment thoroughly mixed again, and subsamples of 1.0 g were transferred to 50 ml Kimax tubes for PLFA analysis, and subsamples of 0.5 g were transferred to 1.5 ml sterile Eppendorf tubes (Plastibrand, Wertheim, Germany) and stored at −70 °C for T-RFLP-analysis.

2.4. Bacterial activity

Bacterial activity was measured as ^{3}H-leucine incorporation rates according to Simon and Azam (1989), with modification for sediment samples according to van Duyl and Kop (1994). Briefly, 0.25 nmol leucine (L-[4,5 ^{3}H]-leucine, 161 Ci/mmol, Amersham Pharmacia Biotech) diluted with non-radioactive leucine to 24 Ci/mmol, was added to the tubes containing sediment samples. The tracer was gently mixed into the sediment and incubated in darkness at 15 °C for 1 h. Leucine incorporation was terminated by adding 1 ml of 80% ethanol. A blank, where bacterial activity was stopped with 80% ethanol before ^{3}H-leucine addition, was also included in each measurement to determine the adsorption of the isotope to sediment particles. After a number of rinsing steps with 80% ethanol and 5% ice-cold TCA, radioactivity was measured by liquid scintillation counting (LKB-Wallac, 1217 Rackbeta). Scintillation counts were corrected for quenching using internal standard ratios.

2.5. PLFA-analysis

Duplicate samples from controls and each pesticide treatment incubated for 31 days were randomly selected for PLFA analysis. Lipids were extracted from 1.0 g of wet sediment using the one-phase Bligh/Dyer method (Bligh and Dyer, 1959) with some modifications (Frostegård et al., 1991). One blank sample (without sediment) was also included in the extraction. Extracted lipids were separated on silicic acid columns into neutral, glyco- and polar lipids (King et al., 1977), the latter including the phospholipids. The methyl ester non-adecanoic acid (19:0) was added as an internal standard immediately before the phospholipids were converted to fatty acid methyl esters (FAME) by mild alkaline methanolysis (Dowling et al., 1986). The FAME preparations were dried at room temperature under a stream of N_2 and stored at −20 °C until gas chromatographic analyses.

The FAMEs were quantified and identified by GC-FID and GC-MS as described in Steger et al. (2003). Since the peaks of PLFAs 18:2 and 18:3 often were small and incompletely separated, their areas were summed and treated as one. Quantification was carried out by correlating the peak areas for fatty acids to that of the internal standard 19:0. For identification and quantification of monounsaturated fatty acids, including cis and trans conformations, DMDS derivatized samples were analyzed with GC-MS as previously described (Nichols et al., 1986).

PLFAs are named by standard nomenclature: the total number of carbon atoms, followed by a colon and the number of double bonds. The position of the first double bond is indicated by "ω" followed by the number of carbon atoms counted from the aliphatic end. Methyl branching at the iso and anteiso positions and at the 10th carbon atom from the carboxyl end is designated by the prefixes "i", "a", and "10Me", respectively. The prefix "cy" denotes cyclopropane fatty acids.

2.6. T-RFLP analysis of 16S rRNA genes

The FastDNA-Spin Kit for soil (Q-Biogene, Carlsbad, CA, USA) was used to extract total sediment DNA from the controls, the high pesticide treatments and four of the low pesticide treatments (captan 1 and 4, glyphosate 2 and 4). Additionally, Tetraphyll® was directly extracted to evaluate the contribution of this organic matter addition to the 16S ribosomal RNA profiles in the samples. Sediment DNA from the remaining low pesticide treatments were extracted using Ultra Clean Soil DNA kit (Mo Bio laboratories, Solana Beach, CA, USA). Prior to DNA extraction, sediment aliquots of 0.5 g were centrifuged (30 s, 10 000 rpm) and the supernatant was discarded. The sediment was resuspended in the lysis solution provided in the respective kits and transferred to individual Lysing Matrix/Bead solution tubes, also provided in the kits. DNA extraction was then carried out according to the manufacturers, except that a Mini-Beadbeater (Biospec products) set to 3000 rpm for 30 s was used for the mechanical extraction step. Recovered DNA extracts were stored at −70 °C and analysis of extracted DNA by 1% agarose gel electrophoresis, ethidium bromide stain and UV-transillumination showed that the concentration of genomic DNA in the extracts exceeded 50 ng/µl with an average fragment size that exceeded 20 kb in length. DNA extracts were diluted 10 times to obtain DNA concentrations suitable for polymerase chain reaction (PCR).

16S rRNA genes were amplified by PCR using the bacteria-specific primer 27f labeled with hexachlorofluorescein and the universal primer 519r (Eiler and Bertilsson, 2004). Triplicate 30-µl reactions were prepared for each individual sample to minimize bias due to random events during the first cycles of the PCR. Each reaction tube contained 3-µl DNA template, PCR buffer (10 mM Tris–HCl, pH = 9, 50 mM KCl, 0.1% Triton X-100 and 2 mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 100 nM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA). Amplification was carried out in a Stratagene Robocycler with an initial 3 min

denaturation at 94 °C followed by 28 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C followed by a single 7 min extension at 72 °C. Replicate PCR tubes were pooled and treated with Mung Bean Nuclease (New England Biolabs) to remove single stranded products and thus avoid formation of pseudo terminal restriction fragments (TRFs) (Egert and Friedrich, 2003). Nucleases were inactivated and removed by purification of the PCR products using Qiaquick PCR purification kit (Qiagen, Germany).

The final concentration of nuclease-treated PCR product was determined by comparison to a low DNA mass ladder (Invitrogen, Carlsbad, CA) on a 1% agarose gel with ethidium bromide staining and UV-transillumination. Approximately 60 ng of each PCR product were digested separately with the restriction enzymes HaeIII, HhaI, and RsaI (Invitrogen, Carlsbad, CA) for 16–18 h at 37 °C. Fragment size was determined on an ABI 3700, 96-capillary sequencer after addition of 500 bp ROX-labelled size markers (Applied Biosystems). Terminal restriction fragments were sized and quantified using the GeneScanView 1.1.4 Software (C.R.I.B.I., University of Padova). Fragments with size differences less than 0.5 base pairs were considered identical and fragments with area percentages less than 0.5%, as well as fragment sizes below 60 bp and above 500 bp, were excluded from further analysis.

Digestions with HhaI and RsaI produced a low or highly variable number of terminal restriction fragments (11–54 and 15–110 TRFs, respectively) making comparisons between the samples difficult. Digestions with the restriction enzyme HaeIII, produced a higher and more uniform number of TRFs in most samples, and therefore only data generated with HaeIII were further evaluated. Samples used in the analysis contained between 33 and 73 (average 63) terminal restriction fragments (TRFs). The performance of the two DNA-extraction kits was equal regarding the quantity of DNA recovered from the samples (data not shown). This is not surprising since both kits are based on a combination of mechanical and chemical cell lysis. The similar performance of the two extraction procedures are further supported by the high similarity (89%) of T-RFLP patterns from separate microcosms of the same treatment where different DNA extractions kits were used (e.g. culture g3 and g4, Sørensen similarity index).

2.7. Ergosterol analysis

Ergosterol concentrations, a measure of fungal biomass, were quantified for controls and high pesticide treatments. Ergosterol was extracted from 0.40 g of lyophilized sediment and analyzed using HPLC (Mille-Lindblom and Tranvik, 2003). Briefly, sediment samples were mixed with 10% KOH in methanol and 1 ml cyclohexane, sonicated for 15 min and refluxed at 70 °C for 60 min. After cooling, 1 ml Milli-Q water and 2 ml cyclohexane was added and samples were vortexed for 30 s. After centrifugation (5 min, 1000 × *g*) the cyclohexane supernatant was transferred to new tubes. The pellet was then washed with 2 ml of cyclohexane and the centrifugation step was repeated. The ergosterol-containing cyclohexane was then dried at 40 °C under a gentle flow of air. The dry samples were reconstituted in methanol, incubated for 15 min at 40 °C, and filtered (0.45 µm) directly into autosampler vials. Samples were analyzed using HPLC (Gilson) equipped with a Phenomex column (Sherisorb 5 ODS (2) 250 × 4.60 mm) and a UV detector (282 nm). The mobile phase was methanol (1 ml min^{-1}). An ergosterol standard (Fluka) was used to identify the ergosterol peak in samples.

2.8. Statistical analysis

Bacterial activity and ergosterol concentration data were log_{10}-transformed prior to statistical analysis. Effects of pesticide exposure and time of incubation were analyzed by Analysis of variance (ANOVA), and Bonnferroni/Dunn tests for pairwise comparisons using StatView 5.0 for MacIntosh (SAS Institute, Cary, NC, USA) with alpha (α) set to 0.05. Fatty acid concentrations were converted to relative mole percentages for each sample and then arcsine-transformed prior to principal component analysis (PCA) using Canoco for Windows 4.5 (ter Braak and Smilauer, 2002). To avoid strong influence of cy17:0, that was detected at unrealistically high concentrations in two samples, and 22:0, a PLFA most common in eucaryotic cells and occurring irregularly in our samples, these FAs were excluded from the PCA. For T-RFLP-data, the relative areas of terminal restriction fragments were analyzed by PCA in the same way as the PLFA data. However, the low proportions yielded made arcsine-transformation unsuitable (Zar, 1974), and consequently ^{10}log-transformed data were used. Differences among treatments and controls were tested using Monte Carlo permutation tests, while pairwise comparisons of sample scores were done using Tukey–Kramer HSD tests. Moreover, to evaluate the similarity between different samples based on the T-RFLP-analysis we used the Sørensen similarity index, $S = 2J/(N_A + N_B)$, where J is the number of common bands (TRFs) and N_A and N_B is the total number of bands in sample A and B, respectively.

3. 3 Results

Bacterial activity (leucine incorporation) ranged from 100 ± 8.3 pmol/(g*h) to 208 ± 19 pmol/(g*h) across all treatments and was not affected by pesticide exposure (two-way ANOVA, $p \geq 0.29$) (data not shown). Incubation time, however, had an overall effect on bacterial activity (two-way ANOVA, $p < 0.0001$), caused mainly by a markedly elevated activity on day 14 ($p < 0.0001$). Pair-wise comparisons showed that bacterial activity in all treatments was higher on day 14 than on the other days, except for day 31 in the low treatment concentration of pirimicarb. Additionally, bacterial activity on day 3 was lower than on day 31 in the low concentration of isoproturon and the high concentration of glyphosate ($p = 0.0003$ and 0.0025, respectively). Similarly, in treatments with high concentration of captan, bacterial activity was lower on day 3 than on both day 1 and 31 ($p = 0.0040$ and 0.0004, respectively).

Total microbial biomass ($\sum$PLFA) ranged from 771 ± 9.25 nmol/g dw (glyphosate, high concentration) to 992 ± 169 nmol/g dw (glyphosate, low concentration) and was not affected by pesticide exposure (Fig. 1). Also for fungal biomass (ergosterol concentrations), analyzed in controls

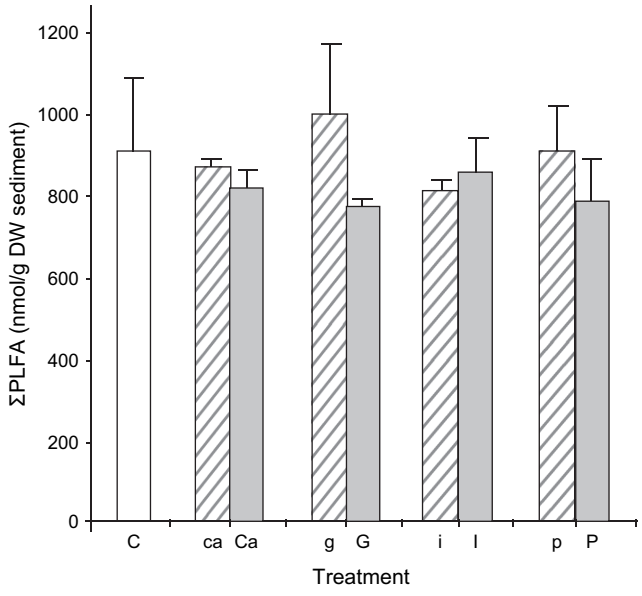

Fig. 1. Mean microbial biomass (total PLFA concentration) in sediment of controls (C) and in treatments with low and high concentrations of the pesticides captan (ca and Ca, respectively), glyphosate (g, G), isoproturon (i, I), and pirimicarb (p, P). Error bars denote ±1 standard deviation.

and treatments with high pesticide concentrations, no differences among treatments were found (One-way ANOVA, $p \geq 0.19$). Ergosterol concentrations ranged from 7.4 ± 0.95 (isoproturon) to 9.1 ± 0.91 μg/g dw (pirimicarb) across all treatments.

Bacterial community composition, however, showed significant pesticide-induced changes for captan (permutation tests, $p = 0.001$ for the first and all PC-axes) and glyphosate (permutation tests, $p = 0.035$ and $p = 0.039$ for the first and all PC-axes, respectively). The first axes explained between 45 and 65% of the variation in T-RFLP, while the second axes explained between 14 and 37% (Fig. 2). Controls were generally close together in the PCA plot while treatments spread along the first and second axes. For example, treatments with low concentrations of captan and glyphosate were placed to the right in the plots, not overlapping with controls along the first PC-axis. However, pairwise comparisons showed that only treatments with low concentration of glyphosate were significantly different from controls. For captan, the high concentration treatments were situated more to the left, but showed some overlap with controls along the first PC-axis and were thus only significantly different from treatments with low concentration. For exposures with pirimicarb, Monte Carlo permutation test showed no differences among treatments and controls, but pairwise comparisons revealed that high concentrations differed from low concentrations along the second PC-axis. Sørensen similarity index showed that two of the controls, C1 and C4, were similar to 90%, while L3 and H1 in the captan treatment that were most distant in the PCA-plots, showed 68% similarity.

Certain TRFs were present in controls, but absent in all replicates of some treatments. For example, a 166 base pairs (bp) TRF was absent in exposures with high concentrations of glyphosate, and a 316 bp TRF in treatments with low concentrations of captan and in those with high concentrations of glyphosate and isoproturon. Similarly, a TRF of 294 bp was absent in exposures with pirimicarb, and a 235 bp fragment was missing in exposures with high concentrations of isoproturon. Three other fragments (211, 250, and 309 bp) were missing in three or more of the control samples, but appeared in several of the treatments. However, all these TRFs only occurred at low percentages, 0.5–1%, which is close to the detection limit. Tetraphyll® samples were dominated by a single TRF (85%) that was absent from all sediment samples, indicating that Tetraphyll® additions did not interfere with our T-RFLP analyses.

A total of 26 PLFAs were identified in sediment samples and among these 16:0, 16:1ω7 and 18:1ω7 were dominating (Table 2). PCA with PLFA data showed that the first PC-axis explained 47% of the variation and largely represented

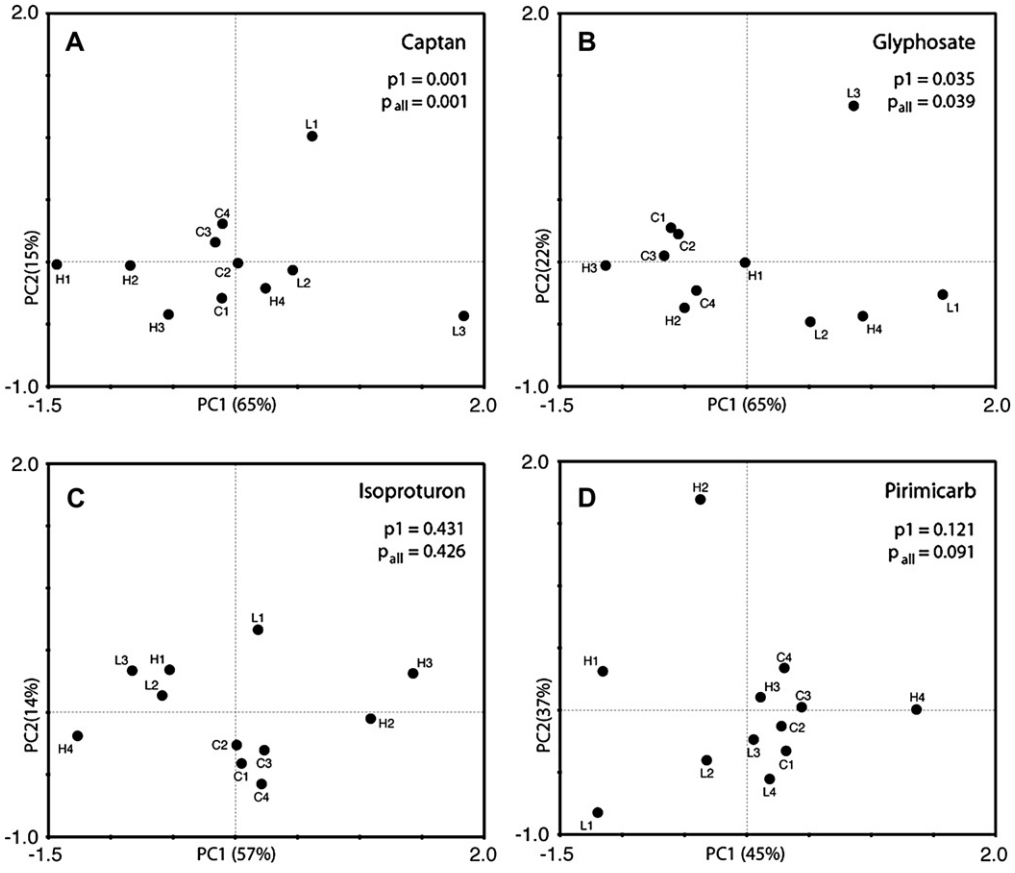

Fig. 2. Principal component analysis (PCA) with bacterial terminal restriction fragments for captan (A), glyphosate (B), isoproturon (C), and pirimicarb (D). "H" refers to high and "L" to low exposure concentrations, while "C" refers to controls. Numbers refer to the different replicates.

Table 2
Mean mol percentages (±1 standard deviation) of phospholipid fatty acids (PLFAs) and sum PLFA (as nmol/g dw) in sediment in controls and after exposure to pesticides for 31 days

PLFA	Control	Captan		Glyphosate		Isoproturon		Pirimicarb	
		Low	High	Low	High	Low	High	Low	High
i14:0	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.1 ± 0.4	1.2 ± 0.1	1.1 ± 0.0	1.5 ± 0.1	1.1 ± 0.5	1.3 ± 0.0
14:O	4.0 ± 0.3	4.5 ± 0.7	4.7 ± 0.2	4.1 ± 1.2	4.4 ± 0.2	4.2 ± 0.1	4.8 ± 0.1	4.1 ± 1.3	4.8 ± 0.0
i15:0	4.0 ± 0.0	4.1 ± 0.5	4.2 ± 0.1	4.0 ± 0.5	4.3 ± 0.0	4.1 ± 0.2	4.6 ± 0.0	4.2 ± 0.8	4.5 ± 0.2
a15:0	6.2 ± 0.0	6.6 ± 0.5	6.6 ± 0.1	6.2 ± 0.9	6.8 ± 0.0	6.6 ± 0.1	7.4 ± 0.3	6.3 ± 1.1	7.0 ± 0.2
15:O	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	1.1 ± 0.1
i16:0	1.3 ± 0.1	1.3 ± 0.0	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.0	1.3 ± 0.0	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
16:1ω9	1.6 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.5 ± 0.1
16:1ω7	19.6 ± 0.8	21.2 ± 1.8	20.9 ± 2.3	21.0 ± 1.5	19.7 ± 1.1	18.6 ± 1.6	19.5 ± 0.2	20.1 ± 2.1	19.5 ± 1.6
16:1ω6	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.0	2.1 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.0	2.2 ± 0.0	2.1 ± 0.1
16:1ω5	3.0 ± 0.1	3.4 ± 0.3	3.3 ± 0.1	3.0 ± 0.5	2.9 ± 0.2	3.3 ± 0.3	3.5 ± 0.1	3.2 ± 0.1	3.0 ± 0.4
16:O	19.4 ± 1.2	18.6 ± 0.7	19.3 ± 2.6	19.0 ± 0.2	19.8 ± 0.5	20.6 ± 0.7	19.3 ± 0.1	18.8 ± 1.1	19.7 ± 0.2
10Me16:0	4.6 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.2 ± 0.3	4.2 ± 0.1	4.3 ± 0.2	4.3 ± 0.5	4.3 ± 0.2	4.3 ± 0.2
i17:0	1.4 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.2	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.3	1.2 ± 0.0
a17:0	2.4 ± 0.0	2.2 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.7 ± 0.2	2.3 ± 0.2	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
cy17:0	1.9 ± 0.8	1.4 ± 0.2	1.3 ± 0.0	1.5 ± 0.3	1.4 ± 0.1	2.5 ± 1.5	1.3 ± 0.0	1.3 ± 0.3	1.3 ± 0.1
17:O	1.1 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
10Me17:O	0.8 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.4 ± 0.0	0.8 ± 0.4	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.1
18:2 + 18:3	3.0 ± 0.6	2.3 ± 0.8	2.8 ± 0.7	3.2 ± 0.4	2.2 ± 0.6	3.0 ± 0.2	2.4 ± 0.4	2.7 ± 0.2	2.8 ± 0.3
18:1ω9	4.6 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	4.5 ± 0.3	4.5 ± 0.1	4.6 ± 0.2	4.3 ± 0.3	4.6 ± 0.5	4.3 ± 0.0
18:1ω7	10.6 ± 0.4	10.8 ± 1.3	9.9 ± 0.8	10.9 ± 1.7	10.0 ± 0.4	9.6 ± 0.9	9.4 ± 0.2	11.2 ± 1.9	9.9 ± 0.7
18:1ω5	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
18:0	2.6 ± 0.2	2.6 ± 0.6	2.4 ± 0.3	2.6 ± 0.3	3.0 ± 0.2	3.2 ± 0.0	2.8 ± 0.0	2.8 ± 0.6	3.0 ± 0.6
10Me18:0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.0
cy19:0	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0
20:1	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.8 ± 0.1	0.4 ± 0.3	0.5 ± 0.4	0.5 ± 0.2	0.4 ± 0.0
22:0	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.0	1.6 ± 0.0	1.7 ± 0.1	0.9 ± 1.2	1.7 ± 0.0	1.6 ± 0.2	1.7 ± 0.2
Sum PLFA nmol/g dw	909 ± 169	860 ± 6	959 ± 252	992 ± 169	771 ± 9	805 ± 18	863 ± 95	904 ± 103	783 ± 96

a gradient between saturated and mono-unsaturated PLFAs (Fig. 3). The second PC-axis explained 23% of the variation in the data and primarily represented a gradient in the occurrence of fungi- (18:2, 18:3) and/or actinomycete-specific (10Me-PLFA) PLFAs. The controls were very similar along the first PC-axis, but showed more variation along the second PC-axis. Treatments with high pesticide concentrations, with the exception of captan, were correlated with branched, saturated fatty acids, e.g. i14:0, i15:0, a15:0, and i16:0, typical for gram-positive bacteria. Treatments with the fungicide captan, on the other hand, did not show a correlation with the fungi-indicating PLFAs 18:2 and 18:3, except for one sample. For treatments with low concentrations of glyphosate, captan, and pirimicarb, there were conspicuous differences between duplicates along the first PC-axis.

pH in the overlying water ranged 6.7–6.8 among all treatments after 14 days of incubation. After 31 days, pH in the overlying water differed significantly among the treatments (One-way ANOVA, $p = 0.004$) and ranged between 4.8 ± 0.11 in treatments with low concentrations of glyphosate to 6.5 ± 0.46 in high concentrations of captan. Sediment pH, however, ranged from 6.1 to 6.6 by the end of exposure, and did not differ among treatments (one-way-ANOVA, $p = 0.147$). Dissolved oxygen concentrations ranged from 7.7 to 10.4 mg/L across all treatments, indicating good oxygenation in our experimental units.

4. Discussion

The study of contaminant effects on microbial communities is precarious, especially so in sediments, but also an intriguing challenge to environmental science. Our study contributes to the scanty information in this field by showing subtle, albeit significant effects of pesticide contamination on the microbial community composition in a natural lake-sediment, detected as changes in T-RFLP patterns after exposures with captan, glyphosate, and pirimicarb (Fig. 2). In all PCAs, controls showed a high degree of similarity, while treatments with low and high pesticide concentrations generally induced shifts in microbial community structure in opposite directions compared to controls. Also our PLFA analyses indicated shifts in the sediment microbial community structure upon pesticide exposure (Fig. 3). A lack of a unidirectional, dose-response relationship is not an unusual observation in microbial toxicology tests (Petersen et al., 2004; Widenfalk et al., 2004) and requires a new way of thinking in terms of toxic responses.

Our study further showed that community-level end points, like bacterial activity, fungal- and total microbial biomass were not affected by pesticide exposure. This is in line with reported differences in sensitivity among microbial response variables in reviews by van Beelen and Doelman (1997) and Johnsen et al. (2001). These papers both concluded that the

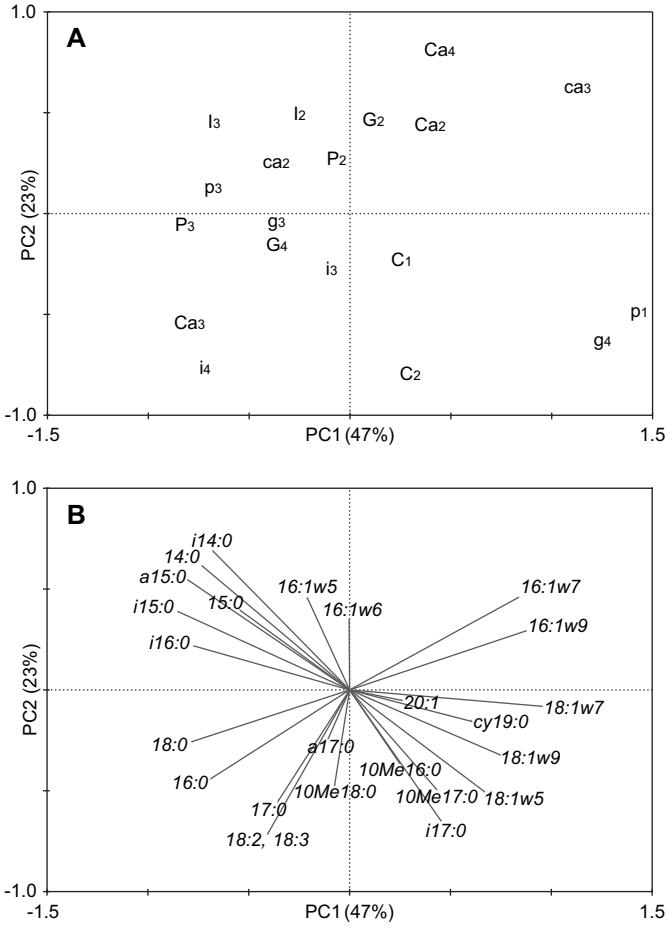

Fig. 3. Principal component analysis (PCA) of phospholipid fatty acids (PLFA) in sediment samples after exposure to pesticides for 31 days. (A) Score plot showing relationships among the different treatments with low and high concentrations of the pesticides captan (ca and Ca, respectively), glyphosate (g, G), isoproturon (i, I), and pirimicarb (p, P). (B) Loading plot for individual PLFAs.

overall community metabolism is not a suitable response variable for detecting toxic effects of pesticides. The observed lack of community-level effects may still seem surprising considering that our high exposure concentrations were quite high and that the same low concentrations of three of the pesticides tested here caused an inhibition of bacterial activity in an earlier study (Widenfalk et al., 2004). Likely, this discrepancy among results can be attributed to differences in the administration of the pesticides and the time frame over which bacterial activity was measured in these studies (see also Blair and Martin, 1988). In our earlier study, we ran short-term exposures and measured bacterial activity after a few hours, while in the present study bacterial activity was quantified after one or more days of exposure. A comparison of these results suggests that bacterial activity shows an almost instantaneous response to pesticide exposure, but also that recovery, due to physiological adaptations or community shifts (towards more tolerant types) of bacteria and/or decreased bioavailability when pesticides are sorbed by sediment particles or degraded, can be very rapid.

Evidence of bacterial community shifts comes from the observed changes in T-RFLP-composition in treatments with captan and glyphosate, suggesting that certain groups of bacteria were stimulated at low exposure concentrations. Furthermore, PLFA-data for treatments with high concentrations of glyphosate, isoproturon, and pirimicarb revealed a close association with branched saturated fatty acids (i14:0, i15:0, a15:0, and i16:0) indicative of gram-positive bacteria (Pinkart et al., 2002) (Fig. 3). Gram-positive bacteria are considered to be stress-tolerant, possibly due to their relatively thick cell wall and ability to form endospores (Stainer et al., 1977). 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). Probably, the changes in microbial community composition would have been more pronounced if the pesticide additions had been repeated. Such an exposure scenario is also environmentally more realistic, since pesticide application in the field results in repeated inputs of the pesticide to the aquatic environment.

Our PLFA-results (Table 2 and Fig. 3) further suggest that low exposure concentrations of the fungicide captan likely stimulated some bacterial groups, either through their ability to use the pesticide as a carbon source or by negatively affecting fungi and thereby releasing bacteria from fungal competition. In line with this, PCA of PLFA-data showed that in all but one case, treatments with the fungicide captan were less associated with fatty acids indicating fungi and actinomycetes than were controls (Fig. 3). This can be interpreted as a decrease in fungal populations and a concomitant increase in total bacterial biomass upon captan exposure. Similar effects have been reported after application of captan on soil by Martinez-Toledo et al. (1998). Remarkably, the fungicide captan did not affect fungal biomass (ergosterol concentration). This lack of response for fungal biomass may be due to either a shift in fungal species or, more likely, to a slow degradation of ergosterol associated with dead and decaying fungal cells (Mille-Lindblom et al., 2004). Also glyphosate is known to stimulate microbial activity since it is an easily available carbon substrate (Araujo et al., 2003). Our finding of a higher mean percentage of fungal PLFAs (18:2 and 18:3) (Table 2) and a significant change in bacterial community structure (Fig. 2) in treatments with low concentrations of glyphosate lends some support to this.

Pesticide-induced changes in microbial community composition were also detected by the presence/absence of TRFs in our T-RFLP-analysis. Exposure to each of the four pesticides either caused the disappearance of one or more TRFs, or the appearance of certain ‘‘new’’ fragments that were missing in controls. Our PCR-based methodology does not allow us to make inferences about the natural abundance of TRFs (i.e. operational taxonomic units) that appear or disappear in the respective treatments (Liu et al., 1997). Nonetheless, these TRFs could represent bacteria that perform critical ecological functions and may potentially serve as microbial indicators of pesticide exposure. This, however, would require positive identification of the populations with rRNA cloning and sequencing paralleled by quantitative population detection

methods for these putative bioindicators. Both molecular methods identified treatment-induced changes in microbial community composition. An advantage of PLFA-analysis is that it is quantitative and provides insight in effects on major microbial groups. T-RFLP-analysis on the other hand, using bacterial primers, provides a high resolution of the bacterial component of the microbial community. Both methods, however, could potentially be influenced by the presence of inactive microbial cells such as algae, cyanobacteria or detrital material.

We observed changes in the composition of sediment microbial communities after exposures to both environmentally relevant and high concentrations of pesticides. Any shifts in community structure will, however, only have consequences on ecosystem function if the tolerant microorganisms cannot compensate for biogeochemical functions normally carried out by inhibited or eliminated microbial groups. The large functional redundancy in sediment microbial communities may likely constitute an inherent buffer against the loss of important ecological functions due to environmental constraints. Our results also indicate a certain degree of stochasticity in how microbial communities are affected by and recover from pesticide exposure. A stochastic component in the response of communities to disturbance is in line with ecological theory. Our study also showed that community-level end points, like bacterial activity and total microbial biomass ($\sum$PLFA), failed to detect changes that were found at lower levels of microbial organization, underpinning the need for the application of high-resolution molecular techniques in aquatic ecotoxicology. Future studies should address the effects of repeated exposures of low pesticide concentrations on the structure and function of microbial communities and the implications of this on ecosystem-level processes.

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