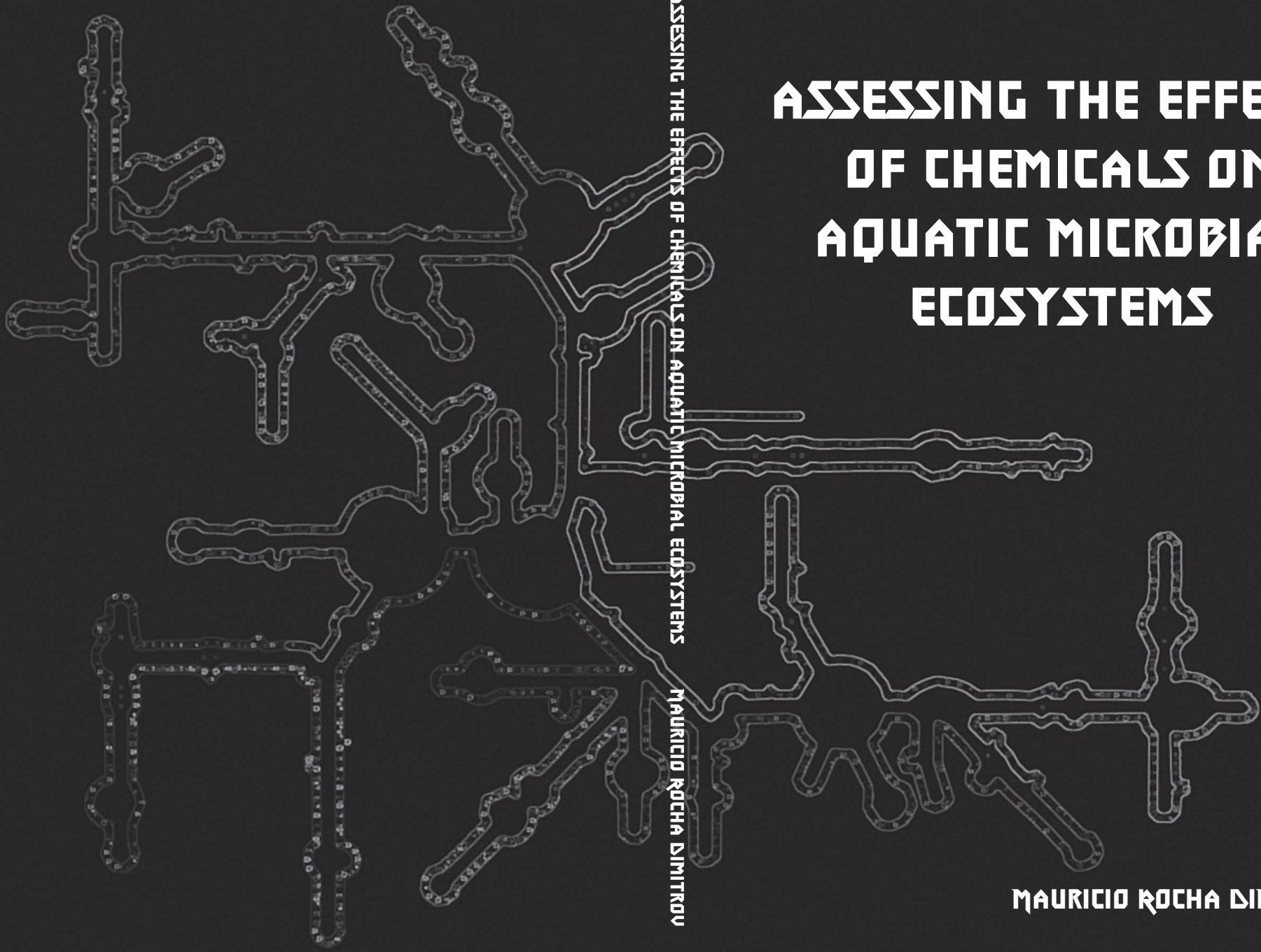

ASSESSING THE EFFECTS OF CHEMICALS ON AQUATIC MICROBIAL ECOSYSTEMS

MAURICIO ROCHA DIMITROV

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Mauricio Rocha Dimitrov

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Assessing the effects of chemicals on aquatic microbial ecosystems

Mauricio Rocha Dimitrov

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To my family.

"So the Maples formed a Union
and demanded equal rights
'The Oaks are just too greedy
We will make them give us light'
Now there's no more Oak oppression
For they passed a noble law
And the trees are all kept equal
By hatchet,
Axe,
And saw…"

- The Trees - Rush -
(1978)

Contents

Chapter 01

General introduction and thesis outline

Nowadays, an increasing number of synthetic chemicals are used in a vast range of applications. World chemical sales were valued at 3,156 billion euros in 2013, with a 24 per cent increase in global sales compared to 2012 (CEFIC, 2014). When reflecting about our daily activities, it is not difficult to realize how much of our life style is dependent on synthetic chemicals. From the food we eat, medicines we take to the health care products we use, all have their fair share of synthetic chemicals. According to United Nations, the world human population reached a number of 7 billion people in 2013, with projections that surpass 10 billion people inhabiting our planet by 2050. Much of the increase in life expectance and quality of life seen in the last few decades can be also attributed to the development and use of synthetic chemicals. Human population growth will create an increased demand for food, energy, clean water, health care and a lot more. One of many critical obstacles is food production, which can only be overcome by the constant increase in technological developments in the fields of agriculture and aquaculture. Synthetic chemicals play a central role in the development of agriculture and aquaculture practices. For instance, the development and use of synthetic pesticides and fertilizers have revolutionized agriculture (Aktar et al., 2009; Bishopp and Lynch, 2015). The use of synthetic pesticides have greatly reduced losses due to pests and diseases, therefore, without these chemicals we would not be able to produce food at the rate we do today. Aquaculture has also been growing steadily in the last decades (FAO, 2012). To ensure and increase productivity, synthetic chemicals, such as, disinfectants, parasiticides and antibiotics, are constantly used to treat diseases or as a prophylactic measure. However, many chemicals used in agriculture, aquaculture or even in our daily activities are released into the environment, where they could become a threat to non-target organisms, ecosystems, as well as us, human beings (Kummerer, 2009; Steen et al., 2001).

Due to their use and application, synthetic chemicals may be found in terrestrial, aquatic and atmospheric environments. Aquatic environments receive direct and indirect inputs of chemicals such as pesticides, which have been detected not only in fresh and marine waters but also in the sediment associated to such ecosystems (Battaglin et al., 2011; Kreuger et al., 1999; Miles and Pfeuffer, 1997; Steen et al., 2001). Although synthetic chemicals like pesticides can be degraded by biotic or abiotic processes in the environment, many can persist for a long period. Despite being banned in the 1970s, the pesticide DDT (dichlorodiphenyltrichloroethane) and PCBs (polychlorinated biphenyls), which were widely used as insulating material in electric equipment, are still detected in sediments in many different places around the world (Zanaroli et al., 2015). Pesticides may have a direct or indirect effect on aquatic organisms, and may affect different trophic levels. For example, changes on the fungal community composition associated to leaf material, due to fungicide exposure, may alter feeding behaviour of leaf-shredding organisms (Bundschuh et al., 2011; Dimitrov et al., 2014), which

can impact important ecosystem functions, such as leaf breakdown (organic matter degradation). Moreover, pesticides have been found to alter microbial community composition in freshwater sediment (Widenfalk et al., 2008).

Microorganisms are an essential part of aquatic ecosystems, carrying out crucial ecosystem functions such as primary production, nutrient cycling and decomposition. Moreover, microbial communities have a large influence on abundance and diversity of higher organisms (i.e. benthic invertebrates) by controlling carbon dynamics and providing a food source. On the one hand, microorganisms may influence the degradation and bioavailability of synthetic chemicals. However, on the other hand, synthetic chemicals may affect microbial community composition and function in aquatic ecosystems. Studies assessing the toxicity of synthetic chemicals (i.e. pesticides) on microbial community composition and function are limited, especially in aquatic environments. Most studies have focused on the microbial degradation of pesticides, for instance, rather than on the effects on microbial communities (DeLorenzo et al., 2001). Therefore, little attention is given to the potential effects of chemicals on aquatic microbes, especially effects on heterotrophic microbes. Since microorganisms play a central role in many important ecosystem processes, understanding pollutant-induced effects on microbial communities in aquatic ecosystems is pivotal for the protection of such ecosystems.

Antibiotics form another group of chemicals that are frequently detected in aquatic ecosystems (Kummerer, 2009). Antibiotics are widely used in human and veterinary medicine, as well as in food animal production, and a vast range of antibiotic concentrations has been detected in terrestrial and aquatic ecosystems. Antibiotic levels detected in aquatic ecosystems vary from below the minimal inhibitory concentration for most bacteria to levels that exceed human therapeutic blood plasma concentrations (Kristiansson et al., 2011; Kummerer, 2009). Antibiotics can enter aquatic ecosystems directly, via discharge of effluents from wastewater treatment plants or aquaculture residues; and indirectly, by runoff and leaching of agriculture soils treated with manure from livestock facilities (Rico et al., 2014; Sarmah et al., 2006; Ternes et al., 2004; Zhu et al., 2013). Environmental pollution by antibiotics leading to harmful effects on microbial communities has been demonstrated by various studies (Ebert et al., 2011; Maul et al., 2006; Proia et al., 2013). As for pesticides, effects of antibiotics on aquatic microbial communities could lead to effects at higher trophic levels. Maul et al. (2006) observed an effect of the antibiotic ciprofloxacin on carbon source utilization of microbial communities associated to leaf litter. A change in functional endpoints could also indicate a shift in community composition, which could alter leaf breakdown by higher organisms. Therefore, antibiotic pollution may affect important ecosystem functions, especially functions that are mainly governed by bacteria, such as nitrogen

cycling, which could lead to alteration on water quality parameter and consequently effects on higher organisms. Antibiotics are also naturally produced by a vast range of microorganisms, however, their role in nature is not yet fully understood (Allen et al., 2010). Not surprisingly, resistance to antibiotics is a process that is common in natural environments and existed before the use of antibiotics by humans (Allen et al., 2010). Therefore, antibiotic resistance is not a process created by the extensive and uncontrolled use of antibiotics in human and veterinary medicine alone, but certainly enhanced by it. To this end, it is also interesting to note that expression of antibiotic resistance genes was observed in a range of natural microbial ecosystems not directly affected by anthropogenic exposure to antibiotics, including human and animal intestines, as well as marine and terrestrial environments (Versluis et al., 2015). Antibiotic resistance poses a real threat to human and animal health worldwide. Increased selective pressure from antibiotic pollution creates the ideal scenario for evolution and spread of resistance, which can happen by mutations and transfer of genetic elements among bacteria that carry resistance gene(s). Studies have reported high levels of antibiotic resistance in livestock and aquaculture production (Tamminen et al., 2011; Zhu et al., 2013), which could promote a direct transfer of antibiotic resistance bacteria from animals to humans. Therefore, studies assessing the risk of antibiotic pollution to microbial community composition, function and development of resistance are urgently required (Brandt et al., 2015).

Effects of chemical contaminants in aquatic ecosystems may be assessed by sediment toxicity and bioaccumulation tests (Diepens, 2013). Such tests are pivotal for the understanding of how chemicals may affect sediment biota, since chemical exposure in the sediment may be different from exposure in the aquatic phase. As previously stated, microorganisms may influence the degradation and bioavailability of chemicals in aquatic sediment, which consequently influence chemical exposure to higher organisms. Since natural sediments are highly complex and heterogeneous in time and space, sediment testing is often standardized by using artificial sediments rather than natural sediments. Studies have demonstrated that microbial communities are poorly developed in artificial sediments, however, even when poorly developed, microorganisms may directly or indirectly influence water and sediment quality (Goedkoop et al., 2005; Verrhiest et al., 2002). For example, invertebrate sediment toxicity tests should ideally be performed with single species, avoiding interactions with other organisms that could influence test outcome. However, it is difficult, if not impossible, to exclude microorganisms from such tests in a realistic test scenario. Moreover, tests conducted in the absence of microbial communities would not be realistic, leading to less ecologically relevant outputs. Therefore, studies evaluating the effects microorganisms might have on the fate of chemicals in the sediment are necessary, since microbes eventually may affect the outcome of sediment tests with higher organisms.

As previously stated, the understanding of pollutant-induced effects on microbial communities in aquatic ecosystems is of great importance for maintenance of a healthy ecosystem. Conventionally, toxicity effects of synthetic chemicals on microorganisms are often evaluated in laboratory tests with single species or by assessing microbial community level endpoints, such as the density of selected microbial populations (Kahru et al., 1996; Schafer et al., 2011). However, single species tests and community level endpoint analyses may fail to detect effects on microbial community composition, which could be associated with changes in important ecosystem functions (Widenfalk et al., 2008). In addition, characterization of microbial community composition in contaminated terrestrial and aquatic ecosystems has been traditionally limited to the marginal fraction of culturable microorganisms (Malik et al., 2008). Culture independent molecular techniques provide new prospects in the assessment of pollutant-induced changes in natural microbial communities (Adetutu et al., 2008; Malik et al., 2008). Although molecular biological techniques have not been routinely applied in the ecological risk assessment of synthetic chemicals, by targeting nucleic acids, which allow for a higher screening resolution then traditional techniques, molecular techniques have the potential to promote a deeper understanding of the effects synthetic chemicals may cause in aquatic microbial communities. For instance, the use of techniques such as high-throughput qPCR, metagenomics and metatrancriptomics allow researchers to acquire information with respect to a microbial community's functional capacity and actual activity, leading to a deeper comprehension of the interaction microorganisms might have with synthetic chemical. For example, Fang et al. (2014) used metagenomic analysis to assess the abundance and diversity of biodegradation genes as well as potential degradation pathways of persistent pesticides such as DDT in marine and freshwater ecosystems. By using metagenomics Fang et al. (2014) were able to identify nearly complete biodegradation pathways for two persistent pesticides (DDT and atrazine). Such finding may help to develop more efficient ways to remediate contaminated sites. Molecular technique may also be used for monitoring purposes of chemical pollution in the environment. A recent study used a high-throughput qPCR array to detected 149 unique antibiotic resistance genes associated to manure processing and land disposal in commercial swine farms in China (Zhu et al., 2013). Therefore, techniques such as next generation sequencing and high throughput quantitative PCR assays have the possibility to transform the study of how chemicals interact with and influence microbial communities.

The objectives of this thesis were to implement currently available molecular techniques for the assessment of potential effects of a variety of synthetic chemicals on aquatic microbial communities, and how in turn microbial communities might affect the fate of such chemicals in aquatic ecosystems.

Thesis outline

This thesis starts with an assessment of how much incomplete DNA extractions from an environmental matrix, such as soil, may affect microbial community characterization. In **Chapter 2**, six different soils are used to determine whether apparent composition of microbial communities, as well as microbial abundances, change due to successive DNA extractions performed on the same sample.

In **Chapter 3,** the effects of a widely used fungicide, tebuconazole, on non-target aquatic bacterial and fungal communities were assessed in a semi-field study. Moreover, indirect effects at a higher trophic level, resulting from the effects on the microbial communities, were studied when tebuconazole exposed leaf material was fed to leaf-shredding organisms. The fungicide concentration used in the semi-field study was chosen based on a threshold derived from a "non-microbial" species sensitivity distribution.

Chapter 4 describes the development of bacterial communities in artificial sediments during pre-equilibration and exposure phases of a whole-sediment test. Furthermore, the abundance of selected functional genes, which are involved in important ecosystem functions mediated by microbes, was determined during the whole-sediment test. Potential implications of bacterial community development during sediment toxicity tests are discussed and suggestions for test improvement are given.

Chapters 5 and 6 assess the ecological impact of the fluoroquinolone antibiotic enrofloxacin on the structure and function of tropical freshwater ecosystems, as well as on the development of antibiotic resistance. In **Chapter 5** the effects of enrofloxacin on the community structure of macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria are monitored. Two functional endpoints were assessed as well, i.e. organic matter decomposition and nitrogen cycling. **Chapter 6** describes the effect of enrofloxacin on the composition of sediment bacterial communities and relative abundance of multiple antibiotic resistance genes.

In **Chapter 7**, a general discussion of the main findings of this thesis is provided as well as future prospective for the use of molecular tools for the ecological risk assessment of chemicals.

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Chapter 2

Successive DNA extractions improve characterization of soil microbial communities

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Manuscript in preparation

Abstract

Currently, characterization of soil microbial communities relies heavily on the use of molecular approaches. Independently of the approach used, soil DNA extraction is a crucial step. Success of downstream procedures used for microbial characterization will depend on how well DNA extraction was performed. Often, studies describing and comparing soil microbial communities are based on a single DNA extraction, which may not lead to a representative recovery of DNA from all organisms present in the soil. To determine whether successive DNA extractions, performed on the same soil sample, would lead to different observations in terms of microbial abundance, community composition, we performed three successive extractions, with two widely used commercial kits, on six different soil samples. Successive extractions increased considerably DNA yield, as well as total bacterial and fungal abundances in most of the soil samples. 454-pyrosequencing analyses of the 16S and 18S rRNA genes revealed that microbial community composition (taxonomic groups) observed in the successive DNA extractions were similar. Successive DNA extractions revealed a few additional microbial groups, which were not observed with a single extraction. However, relative abundance of these additional groups was very low. Nevertheless, for some soil samples shifts in microbial community composition were observed, mainly due to shifts in relative abundance of a number of microbial groups.

Introduction

Microorganisms are key to various biogeochemical processes that drive life on Earth (Falkowski et al., 2008). Soil is one of the most diverse biomes found on Earth and a large reservoir of microbial diversity (Bardgett and van der Putten, 2014; Gans et al., 2005; Torsvik et al., 1990; Torsvik et al., 2002). Besides being essential drivers of biogeochemical processes, soil microorganisms also play an important role in processes such as plant nutrition (Mendes et al., 2013), disease suppression (Mendes et al., 2011), bioremediation (Maphosa et al., 2012), global warming mitigation (Bender et al., 2014), to just name a few. However, understanding the mechanisms behind all these processes is not an easy task, since the vast majority of microorganisms are still unculturable (Hawksworth and Rossman, 1997; Torsvik and Øvreås, 2002). The introduction of culture independent methodologies has revolutionized the way soil microbial communities are studied. Extracting and characterizing DNA has become trivial in most soil microbial ecology studies (Delmont et al., 2012; Navarrete et al., 2015; Pan et al., 2014; Tahir et al., 2015). Moreover, constant improvements and accessibility of high throughput sequencing technologies have allowed researchers to characterize soil microbial communities in an unprecedented way and at ecologically relevant scales and resolution of time, space and environmental conditions.

Due to its stability, DNA is often the nucleic acid of choice to be used to characterize microbial communities in soils. Once extracted, DNA can be used in a range of experiments that may provide insights with respect to the abundance, diversity and functional potential of soil microbial communities. Therefore, successful characterization of soil microbial communities is directly dependent on the quality of the DNA extracted from such soil sample. With the introduction of culture independent methodologies to study soil microbial communities, a variety of soil DNA extraction protocols have been developed (Berry et al., 2003; Burgmann et al., 2001; Liles et al., 2008; Robe et al., 2003; Zhou et al., 1996). However, DNA extraction from soil can be laborious and problematic (Braid et al., 2003; Dong et al., 2006; Frostegard et al., 1999; Robe et al., 2003). Often, as an alternative to simplify and standardize procedures, commercial DNA extraction kits are used. Comparison of different soil DNA extraction protocols, including commercial kits, has shown that DNA yield and purity varies greatly depending on the protocol and soil type (Burgmann et al., 2001; Inceoglu et al., 2010; Knauth et al., 2013). Therefore, every DNA extraction protocol has its own bias, and it will yield DNA that is representative of a portion of the microbial community present in the original soil sample (Delmont et al., 2011). Feinstein et al. (2009) analyzed DNA extraction efficiency of a commonly used commercial soil DNA extraction kit, and indicated that not all microbial DNA present in a soil sample is extracted with a single DNA extraction. When soil DNA, from successive DNA extractions performed on a single sample, was used to characterize microbial communities, substantial shifts

in the bacterial community were observed. Here, to investigate further how bias of incomplete soil DNA extraction may affect microbial characterization, we expanded on the reported findings by Feinstein et al. (2009). Two widely used commercial soil DNA extraction kits were used to extract DNA from a variety of soils. Successive DNA extractions were performed on six different soils collected throughout the Netherlands, and bacterial and fungal abundances, as well as community diversity and composition of each successive extraction, were assessed by using next-generation sequencing technology to sequence 16S and 18S ribosomal RNA (rRNA) gene amplicons.

Material and Methods

Soil samples

Soil samples as well as sampling procedure used in the present work have been described previously (Kuramae et al., 2012). Briefly, soil cores (8 cm diameter X 30 cm deep) were sampled in six contrasting fields located in different regions throughout the Netherlands (Figure S1). Soil samples were chosen to represent five of the most important land management practices in the Netherlands (conventional and organic arable field, pasture, pine and deciduous forest). Moreover, soil samples were chosen and separated according to sand and clay content (Table S1). After sampling, soil samples were sieved and stored at -80 ºC until further processing.

DNA extraction and quantification

Soil DNA was extracted from each soil sample using two different commercial kits, which are widely used for such purpose (Kuramae et al., 2012; Martin-Laurent et al., 2001; Mendes et al., 2014; Sutton et al., 2013). Three replicates were extracted from each soil sample. The PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) and the FastDNA Spin kit for soil (MP Biomedicals, Solon, OH, USA) were used according manufacturer's instructions, with few modifications. The bead-beating step of the PowerSoil DNA isolation kit (PS) was done at 5.5 m s^{-1} for 10 min, using a Retsch MM301 mixer mill (Retsch GmbH, Haan, Germany). Samples extracted with the FastDNA Spin kit for soil (FS) were processed using a FastPrep24 instrument (MP Biomedicals). For both kits, an initial DNA extraction was followed by a successive extraction, which was then followed by another extraction after samples had been stored overnight at -20 ºC (Figure S2). Therefore, three DNA extractions were performed on three replicates of the six soil samples, resulting in a total of nine DNA extracts per soil and extraction kit. In total, fifty four DNA extractions were performed per DNA extraction kit. After the bead-beating step of the first extraction (E1), tubes containing beads and soil were kept on ice until extraction had been finished. To start the second extraction (E2), while using PS, solution from new PowerBead tubes, without beads, was added to PowerBead tubes used in the first extraction. After that, DNA extraction proceeded exactly in the same way as for E1. After the bead-beating step of E2, tubes containing beads and soil were store overnight at -20 ºC. The third and final extraction (E3) was performed as described for E1 and E2. The procedure described for PS was also used for FS; when initiating a new DNA extraction sodium phosphate buffer and MT buffer were added to the lysis matrix tubes containing beads and soil. Volumes of added buffers were always in line with manufacturer's instructions. Extraction proceeded normally afterwards. Supernatant recovery, throughout the whole DNA extraction procedure, was done carefully in order to obtain a complete recovery and minimize carryover of DNA from one extraction to another. When higher volumes of

supernatant were recovered, compared to manufacturer's instructions, adjusted volumes of solutions were used in order to maintain the proper concentration of reagents. Total DNA quantity and quality were measured using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), as well as visualized on 1% (w/v) agarose gel under UV light after staining with ethidium bromide. Tris-acetate-EDTA (TAE) buffer was used for gel preparation and electrophoresis.

Bacterial and fungal abundances

Quantitative real time PCR (qPCR) was used to determine total bacterial and fungal abundances in each soil sample by targeting the 16S and 18S rRNA genes, respectively. qPCR reactions were performed in a 384-well plate (Bio-Rad, Hercules, CA, USA) using a CFX384 Real-Time PCR Detection system (Bio-Rad). All samples (108 soil samples) were analyzed in triplicate, and reactions were carried out in a total volume of 10 μL. qPCR reactions were prepared using 5 μL of iQ SYBR Green super mix (Bio-Rad), 0.4 μL of forward and reverse primers (10 μM), 0.1 μL of BSA (20 mg/mL), 0.1 μL of VisiBlueTM qPCR mix colorant (TATAA Biocenter, Gothenburg, Sweden) and 4 μL of DNA (2.5 ng/μL). Primer combinations and cycle conditions are described in Table 1. At the end of each qPCR run, a melting curve analysis was performed from 60 to 99 °C with an increase of 0.5 °C every 10 s. Purity of the qPCR products was checked by the observation of a single peak on the melting curve, while correct size of the amplicons was confirmed on a 1% (w/v) agarose gel. For each qPCR reaction, a standard curve comprising serial 10-fold dilutions of the target gene was created. Standards were obtained by amplifying the target genes from the following sources: *Escherichia coli* (16S rRNA gene) and *Aspergillus niger* (18S rRNA gene).

Fungal community analysis by T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) was used to determine whether the fungal community of a soil sample was different among the three successive DNA extractions performed. Internal transcribed spacer (ITS) regions were used to target fugal community by amplifying ITS1 region, 5.8S rRNA gene and ITS2 region using specific primers (Table 1). A single replicate from each soil sample was chosen to determine the fungal community by T-RFLP. Therefore, only eighteen samples per DNA extraction kit were analyzed. Three PCR reactions were performed for each sample. PCR was performed with final volume reaction of 25 μL contained 2.5 μL of 10X PCR reaction buffer with 20 mM of $MgCl_2$ (Roche Applied Sciences, Indianapolis, IN, USA), 200 μM of dNTPs, 1 μM of each primer, 1.25 U of Fast Start DNA polymerase (Roche Applied Sciences) and 5 μL of template DNA (5 ng/μL). Cycle conditions are given in Table 1. The forward primer was labelled with the fluorescent dye 6-FAM, while the reverse primer was labelled with NED (Applied Biosystems, Foster City, CA, USA).

Successful amplification was confirmed by running PCR products on a 1.5% (w/v) agarose gel. PCR products were digested with 10 units of *Hha*I (Thermo Scientific) at 37 °C for 3 h. Enzyme inactivation was performed by incubation at 80 °C for 20 min. After inactivation, digested PCR products were purified using ethanol precipitation. Appropriate dilutions, based on test runs of terminal restriction fragments (TRFs), were analyzed with an ABI 3130 sequencer using GeneScan™ - 500 LIZ (Applied Biosystems) as a size standard.

Prokaryotic and fungal community analyses by 454-pyrosequencing

Prokaryotic and fungal community composition, as well as diversity were investigated by 454-pyrosequencing (454 Life Sciences, Roche). The prokaryotic community (bacterial and archaeal communities) was targeted by amplification of V3 and V4 regions of the 16S rRNA gene, while the fungal community was assessed by amplification of V7 and V8 regions of the 18S rRNA gene. Amplicons were generated by PCR reactions of 50 µL (total volume) containing 5 µL of 10X PCR reaction buffer with 20 mM of $MgCl_2$ (Roche Applied Sciences), 200 µM of dNTPs, 1 µM of each primer, 1.25 U of Fast Start DNA polymerase (Roche Applied Sciences) and 5 µL of template DNA (5 ng/µL). PCR reactions were performed in duplicate, which were mixed prior to purification. As for T-RFLP, only a single replicate of each sample was used (same replicate as used for T-RFLP). Hence, only eighteen samples per DNA extraction kit were analysed. Primer sequences and cycle conditions are shown in Table 1. Confirmation of amplification was performed by electrophoresis of PCR products on a 1.5% (w/v) agarose gel. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific), and quantified using a Qubit 2.0 Fluorometer (Life Technologies). Purified and quantified PCR products were then mixed in equimolar amounts at a final concentration of 500 ng/µL. The equimolar mixture was purified by electrophoresis of pooled amplicons in a 1.5% (w/v) agarose gel and excision of the band. DNA was recovered from agarose using the GeneJET Gel Extraction Kit (Thermo Scientific). Gel purified amplicons were sequenced using an FLX genome sequencer in combination with titanium chemistry (Macrogen Inc., Seoul, South Korea).

Table 1. Adaptors and primers used for targeting prokaryotic and fungal community.

Primers	Sequence 5'– 3'	Target	Application	Cycle conditions	References
ITS1F	TCCGTAGGTGAACCTGCGG	Fungi	T-RFLP	95 °C – 5 min; 35 cycles of 95 °C – 30 sec, 55 °C – 40 sec, 72 °C – 90 sec	White et al. (1990)
ITS4R	TCCTCCGCTTATTGATATGC	Fungi	T-RFLP		White et al. (1990)
BACT1369F	CGGTGAATACGTTCYCGG	Bacteria	qPCR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec.	Suzuki et al. (2000)
PROK1492R	GGWTACCTTGTTACGACTT	Bacteria	qPCR		Suzuki et al. (2000)
FF390	CGWTAACGAACGAGACCT	Fungi	qPCR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 52 °C – 45 sec, 72 °C – 60 sec	Modified from Vainio and Hantula (2000)
FFR1	AICCATTCAATCGGTAIT	Fungi	qPCR		Vainio and Hantula (2000)
515F	GTGCCAGCMGCCGCGGTAA	Archaea and Bacteria	Sequencing	95 °C – 2min; 25 cycles of 95 °C – 30 sec, 53 °C – 45 sec, 72 °C 60 sec.	Bates et al. (2011)
806R	GGACTACVSGGGTATCTAAT	Archaea and Bacteria	Sequencing		Bates et al. (2011)
FF390	CGWTAACGAACGAGACCT	Fungi	Sequencing	95 °C – 2min; 25 cycles of 95 °C – 30 sec, 52 °C – 45 sec, 72 °C – 60 sec	Modified from Vainio and Hantula (2000)
FR1	AICCATTCAATCGGTAIT	Fungi	Sequencing		Vainio and Hantula (2000)

Data analysis

Differences in DNA yield, bacterial and fungal abundances, represented as percentage of recovery after first, second and third extraction, were analyzed by two-way ANOVA using software IBM SPSS Statistics for Macintosh, version 23 (IBM Corp., Armonk, NY, USA). Similarly, a two-way ANOVA was used to analyze differences in total DNA yield as well as bacterial and fungal abundances observed after all three successive extractions. T-RFLP analyses were performed according to Mendes et al. (2012). Briefly, profiles were analyzed using PeakScanner v1.0 software (Applied Biosystems), and TRFs of less than 50 bp and bigger than 800 bp were excluded. The relative abundance of a single TRF was calculated as percent fluorescence intensity relative to total fluorescence intensity of the peaks (Culman et al., 2008). An average of three replicates was calculated for each individual sample. Principal Component Analysis (PCA) was used in order to assess differences in fungal community composition between successive DNA extractions of a single sample, within a single DNA extraction kit. Multivariate statistical analysis was performed using software Canoco 4.5 (Biometris, Wageningen, the Netherlands).

Sequencing analyses were performed using a Snakemake workflow (Koster and Rahmann, 2012), which follows a standard operating procedure for 454 data in mothur version 1.33.2 (Schloss et al., 2009). Flowgrams were demultiplexed allowing two mismatches on the barcodes and three mismatches on the forward primer, flowgrams were trimmed to a size of 635 flows. Flowgrams were corrected using the shhh.flows command, which is a mothur implementation of the original PyroNoise algorithm (Quince et al., 2011). Afterwards, results of the different sff files were combined for further analysis. Merged sequences were aligned and classified with SINA (Pruesse et al., 2012) against

the SILVA 115 database (Quast et al., 2013). After alignment, some reads did not align to the same region as most of the reads. Therefore, reads were kept if containing at least 90% of its sequence aligned to a region common to all reads. To reduce sequence errors, sequences that were within two mismatches of each other were merged. Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Operational taxonomic Units (OTUs) were formed at maximum distance of 0.03 using the dist.seqs command and average neighbor clustering. For each OTU a consensus taxonomy was determined using the classify.otu command. Representative sequences for each OTU were re-aligned to the SILVA reference alignment, and a neighbor joining tree was created using the clearcut program (Sheneman et al., 2006). Taxonomic classification and OTU clustering data were combined into the BIOM format (McDonald et al., 2012) for further downstream analyses with the Phyloseq (McMurdie and Holmes, 2013) package for R (R-Core Team, 2014). Due to a low quality sequencing output, E2 performed with FS on soil 23 was removed from the fungal dataset after sequence quality control. Numbers of reads were not rarefied among samples (normalized to the lowest number of reads) before clustering analysis, since recent work has shown that rarefying is unnecessary (McMurdie and Holmes, 2014). Instead, OTU raw abundances were transformed to relative abundances prior analysis, which has been shown to be an alternative to rarefying (McMurdie and Holmes, 2014). Prior to clustering analysis, sample-wise singletons and doubletons were discarded, where an OTU would be kept only if observed in at least one sample and contained at least three reads.

Diversity analyses were performed using OTU tables that had been rarefied to the lowest number of reads and included singletons and doubletons. The lowest number of reads for the 16S rRNA dataset was 3180, whereas for the 18S rRNA dataset it was 3440. An additional filtering step was done only for the 18S rRNA gene dataset, where only reads belonging to the kingdom Fungi were kept for downstream analyses. For all statistical tests performed, statistical significance was accepted at $p < 0.05$.

Results

DNA yield

Overall, DNA extraction with both commercial kits yielded a substantial amount of DNA, regardless of soil type. Independently of soil type, efficiency was higher when using FS than PS. FS yielded around four times more DNA from clay soils and around three times more DNA from sandy soils than PS (average values of all replicates) (Figure 1, Table S2). For almost all soil samples, DNA extractions performed with both kits yielded the highest DNA concentration in E1, except for clay soil 18 (PS extraction) and sandy soil 23 (extraction performed with both kits), which showed the highest DNA yield after E2 (Table S2). DNA extraction of soil 12 (clay) was similar for both kits, where the highest amount of DNA was extracted in E1 (around 60% of total DNA obtained), with E2 and E3 showing lower DNA concentration compared to previous extractions (Figure 1A, 1C and Table S2A). Less than half of the total DNA obtained from soil 18 (clay) was extracted in E1 for both kits, with E2 and E3 still yielding substantial amounts of DNA (Figure 1A, 1C and Table S2A). Soil 19 (clay) showed distinct DNA extraction patterns when comparing both kits (Figure 1A and 1C). Using PS, around 95% of total DNA extracted was recovered in E1 (Figure 1A). However, when using FS, only 40% of the total DNA extracted was recovered in E1 (Figure 1C). Among clay soils, regardless of the kit used, soil 19 yielded the highest total DNA concentration followed by soil 12 and soil 18. The same trend was also observed for sandy soils, where soil 2, pasture soil as soil 19, yielded the highest total DNA concentration, followed by soil 23 and soil 4 (Figure 1B, 1D and Table S2B). DNA extractions of soil 2 showed a similar pattern regardless the kit used, with 55-60% of DNA obtained in E1 (Figure 1B, 1D and Table S2B). The same was observed for soil 4, with approximately 55% of the total DNA recovered being extracted in E1 (Figure 1B, 1D and Table S2B). Comparing all three successive extractions, soil 23 showed the highest DNA concentration in E2, for both kits (Figure 1B, 1D and Table S2B). Approximately 50% of the total DNA extracted was obtained in E2, with E1 yielding more DNA than E3. Different soil management influenced the total amount of DNA obtained, regardless of DNA extraction methodology. Pasture soil yielded a considerably higher amount of total DNA compared to other managements, with pine forest soil showing the least amount of DNA. DNA extraction kit had a significant effect on the total DNA yield, with FS extracting significantly more DNA than PS ($p < 0.05$), however, soil type did not significantly influence DNA yield ($p > 0.05$). Neither soil type nor DNA extraction kit had a significant effect on DNA yield in E1, E2 and E3 (recovery percentage) ($p > 0.05$).

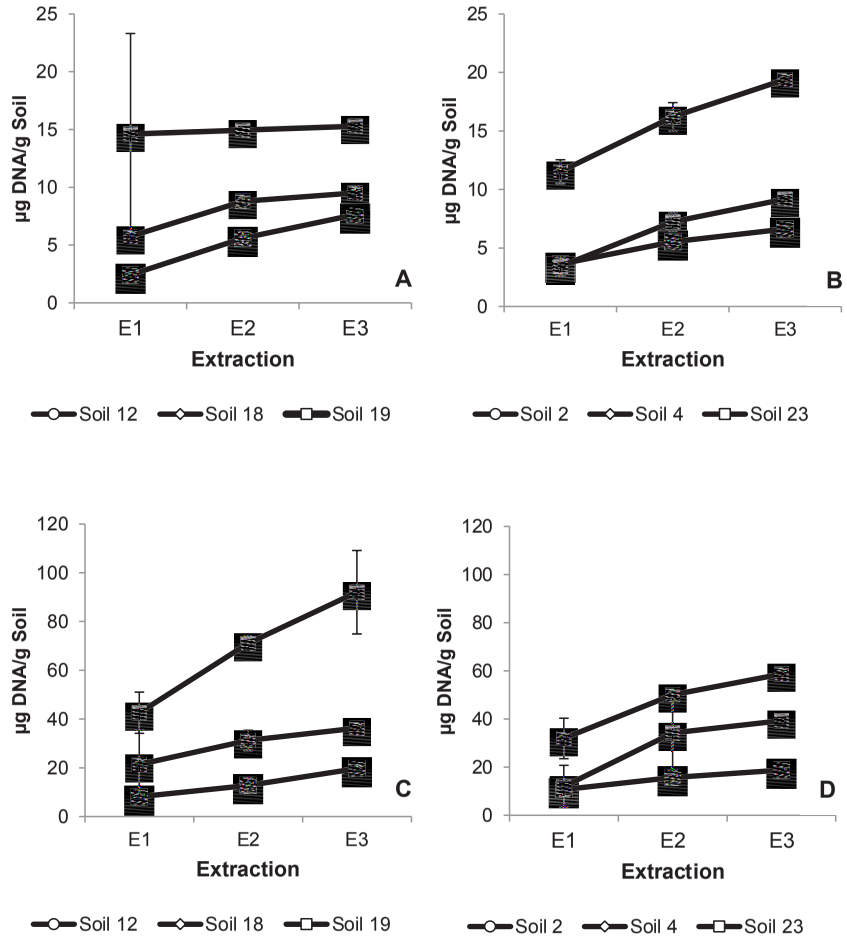

Figure 1. Cumulative DNA yields in successive extractions. Average and error bars (SD) of all three biological replicates are presented for each DNA extraction. (A) clay soils extracted with PS; (B) sandy soils extracted with PS; (C) clay soils extracted with FS; (D) sandy soils extracted with FS.

Bacterial and fungal abundances in successive DNA extractions

Overall, qPCR results of both targeted genes (16S and 18S rRNA) indicated similar patterns as observed in DNA quantification of all soil samples (Figures 1, 2 and 3). Bacterial and fungal abundances in soil 12 (clay) decreased with successive extractions, for both DNA kits, where no substantial increase in cumulative abundance could be seen after E2 (Figures 2A, 2C, 3A and 3C). Interestingly, soil 18 (clay) presented almost constant bacterial and fungal abundances in all three DNA extractions, which resulted in a linear cumulative abundance increase with successive extractions for both kits (Figures 2A, 2C, 3A and 3C). Differently from the other two clay soils, soil 19 (clay) showed

contrasting results when comparing DNA extraction kits. When using PS, bacterial and fungal abundances in E2 and E3 were very low compared to E1, indicating that almost all bacterial and fungal DNA available had been already extracted in E1 (Figures 2A and 3A). When using PS, around 97% and 99% of total bacterial and fungal DNA was recovered in E1, respectively. However, when using FS, bacterial and fungal abundances as measured in E2 and E3 were considerably high compared to E1 (Figures 2C and 3C), indicating that substantial amounts of bacterial and fungal DNA were still present in the soil sample after E1. As observed for total DNA yield, bacterial and fungal abundances in clay soils were higher in samples extracted with FS compared to PS. The same was not observed for sandy soils, where bacterial and fungal abundances were always higher in soil samples that had DNA extracted with PS. Irrespective of the kit used, bacterial and fungal abundances in soil 2 (sandy) samples were highest in E1 (Figures 2B, 2D, 3B and 3D). However, when using PS, around only half of total bacterial and fungal DNA was recovered in E1, whereas, when using FS, these values were around 80%. A very distinct pattern was observed for bacterial and fungal abundances in soil 4 (sandy), when comparing DNA extraction kits. Samples extracted with PS had a recovery of around 45% of total bacterial and fungal DNA in E1, whereas samples extracted with PS showed a recovery of more than 80% of total bacteria and fungi abundance in E3 (Figures 2B, 2D, 3B and 3D). After all three successive extractions, cumulative bacterial and fungal abundances were five times higher in samples extracted with PS compared to those extracted with FS, even with DNA yield being higher in samples extracted with FS. The total number of bacterial 16S rRNA gene copies in soil 23 (sandy) was similar for both kits (Figure 2B and 2D). The same was observed for total fungal abundance. Total bacterial and fungal abundances were slightly higher in samples extracted with PS, however, samples extracted with either kit presented higher fungal abundance in E2 and E3 compared to E1 (Figure 3B and 3D), which was not noticed for bacterial abundance. As observed for soil 2 and 4, a higher total DNA yield in samples of soil 23, which had been extracted with FS, did not result in higher bacterial and fungal abundances. Such contrasting result was particular of sandy soils, since clay soils with higher DNA yield presented higher bacterial and fungal abundances. Different DNA extractions kits did not affect significantly bacterial nor fungal abundance in clay and sandy soils ($p > 0.05$). However, bacterial abundance was significantly affected by soil type ($p < 0.05$).

The use of different commercial kits for DNA extraction did neither affect bacterial nor fungal abundance (percentage of recovery) observed after first, second and third extractions ($p > 0.05$). Soil type, however, had a significant effect on the total bacterial abundance ($p < 0.05$) and fungal abundance ($p < 0.05$) only after the first and third extraction.

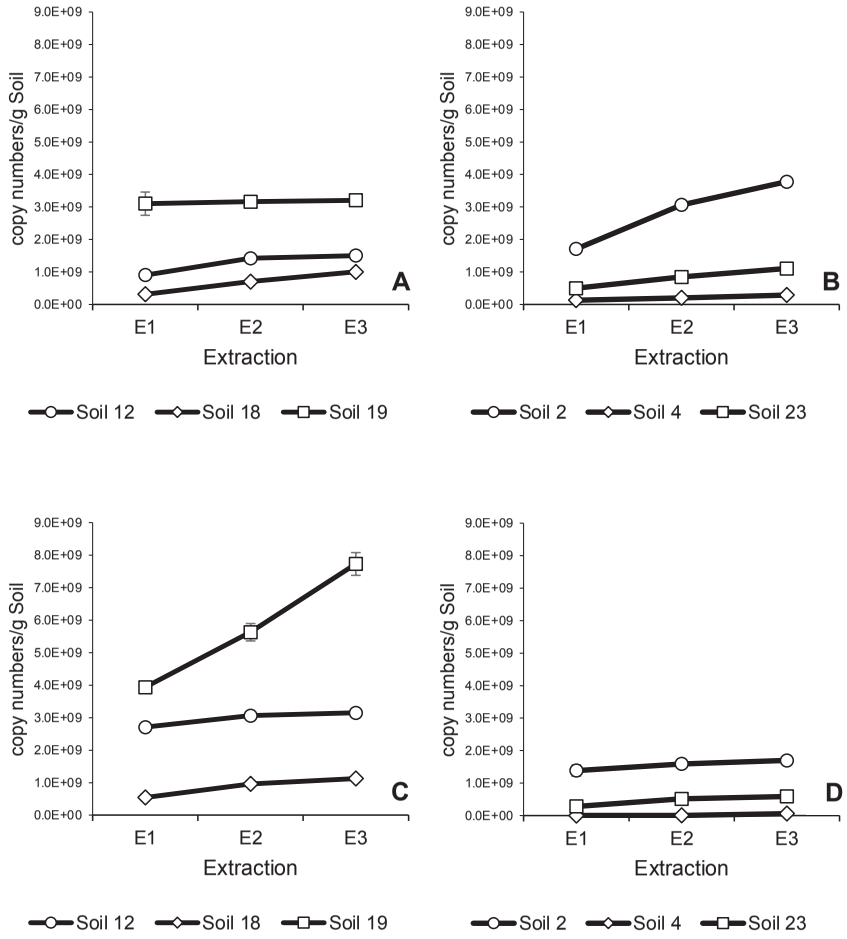

Figure 2. Cumulative 16S rRNA copy numbers in successive extractions determined by qPCR. Average and error bars (SD) of all three technical replicates are presented for each DNA extraction. (A) clay soils extracted with PS; (B) sandy soils extracted with PS; (C) clay soils extracted with FS; (D) sandy soils extracted with FS.

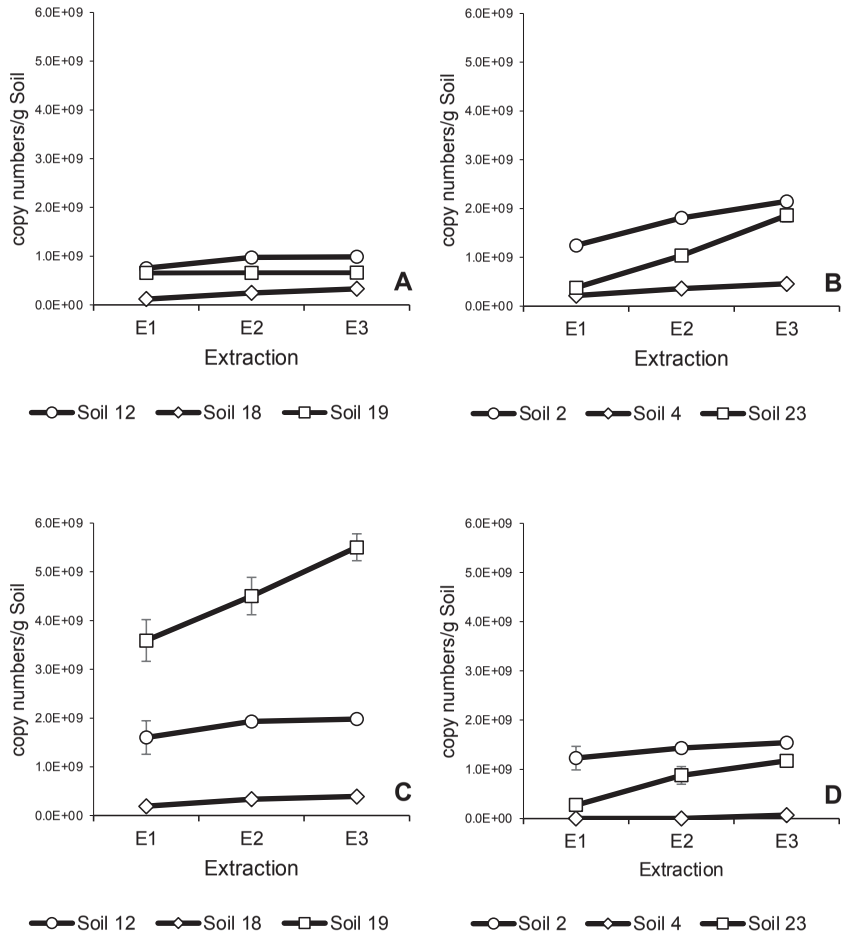

Figure 3. Cumulative 18S rRNA copy numbers in successive extractions determined by qPCR. Average and error bars (SD) of all three technical replicates are presented for each DNA extraction. (A) clay soils extracted with PS; (B) sandy soils extracted with PS; (C) clay soils extracted with FS; (D) sandy soils extracted with FS.

Fungal community analysis by T-RFLP

Fungal community composition was initially measured by T-RFLP analysis, and PCA analysis was used to assess differences among different extractions of a single sample, within a single DNA extraction kit and soil type. Since forward and reverse primers were labelled, both were analyzed to investigate whether results were consistent independently of primer use. Analyses of both primers showed similar results for both DNA extraction kits and both soil types (Figure S3 and S4). Fungal communities detected in clay soils showed to be distinct among different land management types, regardless of the DNA extraction kit used (Figure S3). Overall, successive DNA extractions yielded

different fungal communities in all clay soils. With the use of PS, soils 12 and 18 showed clearly different fungal communities when comparing the three extractions, whereas for soil 19 fungal community composition present in E1 was different from E2 and E3, which were similar (Figure S3A and S3B). Results obtained with FS were slightly different from PS. Soil 12 presented a different fungal community in each successive DNA extraction. However, soil 18 showed a similar fungal community present in E1 and E2, which was different from the community observed in E3. Fungal communities detected for soil 19 were similar for all three extractions (Figure S3C and S3D). As observed for clay soils, fungal communities observed in sandy soils were also distinct among different land management, independently of the DNA extraction kit used (Figure S4). However, differently from clay soils, successive DNA extractions of sandy soils yielded similar fungal communities, especially when using PS (Figure S4A and S4B). Total DNA extraction with PS revealed a very different fungal community when comparing different sandy soils, but not within soils. Successive DNA extraction with FS revealed a different fungal community only for soils 2 and 4, with soil 2 showing a similar community on the first two extractions, whereas soil 4 presented a different community in all three extractions (Figure S4C and S4D).

Prokaryotic and Fungal community analysis by 454-pyrosequencing

To investigate further whether successive DNA extractions of the same soil sample yield different prokaryotic and fungal communities, as well as to confirm T-RFLP results (fungal community), next-generation sequencing was performed on all three extractions obtained from each soil. Chao1 index was used to measure species richness, whereas community diversity was measured by Shannon index. Overall, apparent prokaryotic species richness in clay soils increased in E2 or E3 (Figure 4A), with soil 12 and 19 (PS) being the only two samples that showed a decrease in species richness with successive extractions. Prokaryotic community diversity decreased with successive DNA extractions (Figure 4A), regardless of the DNA extraction kit used. However, for soil 12 (PS) and soil 18 (FS) diversity increased in E3, compared to the previous extraction. Fungal species richness varied considerably across clay soils and DNA extraction kit (Figure 5A). From all samples extracted with FS, soil 18 was the only sample that did not have its highest species richness value in E1. The same trend was observed for samples extracted with PS, where the highest species richness value was observed in E1. Fungal community diversity either increased or remained the same with successive extractions (Figure 5A), regardless of the extraction kit used. Soil 12 (FS) was the only sample, within clay soils, that presented a decrease in fungal diversity with successive DNA extractions (Figure 5A). Overall, prokaryotic species richness in sandy soils decreased with successive extractions, with soil 2 (PS and FS) being the only sandy soil where species richness was highest not in E1 (Figure 4B). Similar to clay soils, prokaryotic diversity in sandy soils decreased with successive extractions (Figure

4B). Soil 23 (PS) was the only exception, where the highest diversity was observed in E2. The highest fungal species richness value in most of the sandy soil samples was obtained only in E2 or E3 (Figure 5B). Soil 2 (FS) and soil 4(PS) were exceptions and had their highest species richness value in E1. Fungal diversity in sandy soils was similar to clay soils, where diversity increased with successive DNA extractions (Figure 5B). The only exception was soil 2 (FS), which showed a decrease in diversity with successive extractions (Figure 5B).

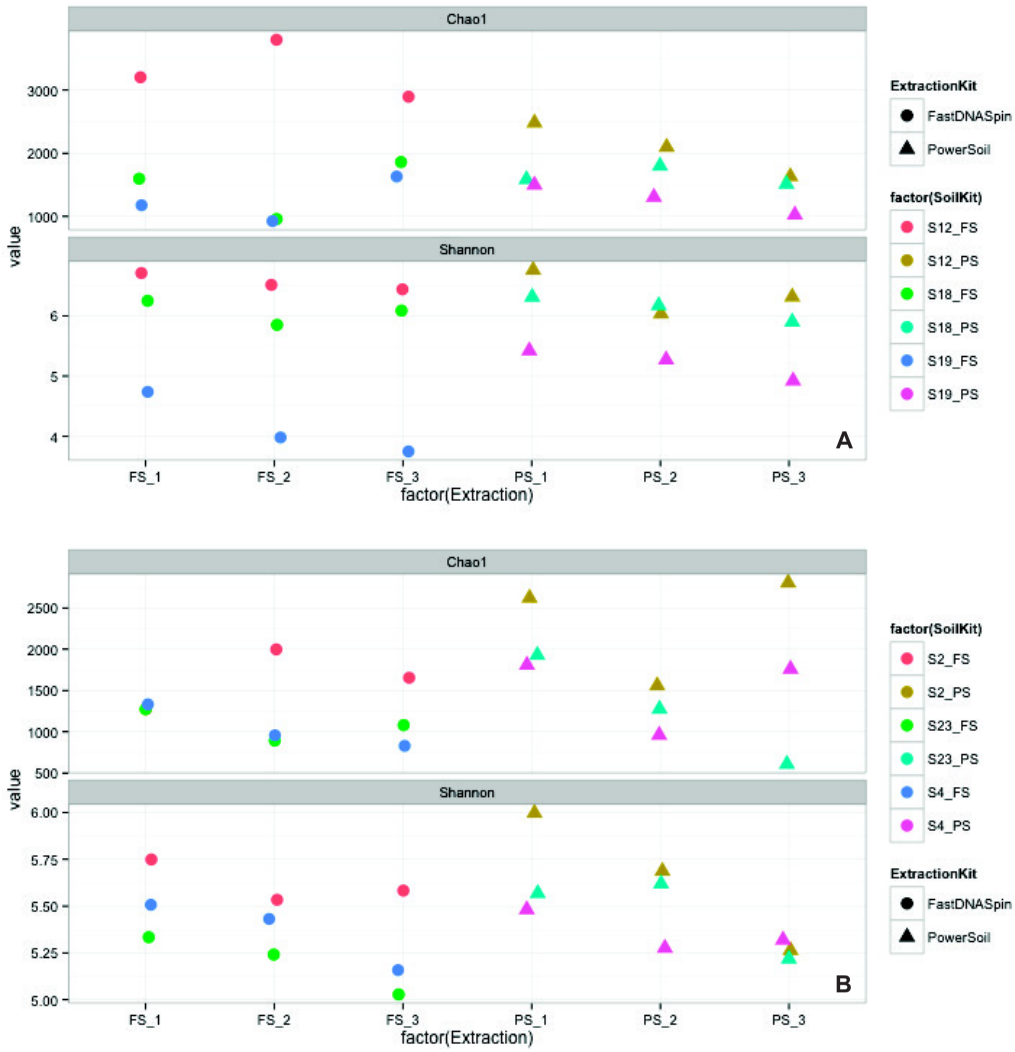

Figure 4. Species richness (Chao1) and diversity (Shannon) of the prokaryotic community in clay (A) and sandy (B) soils.

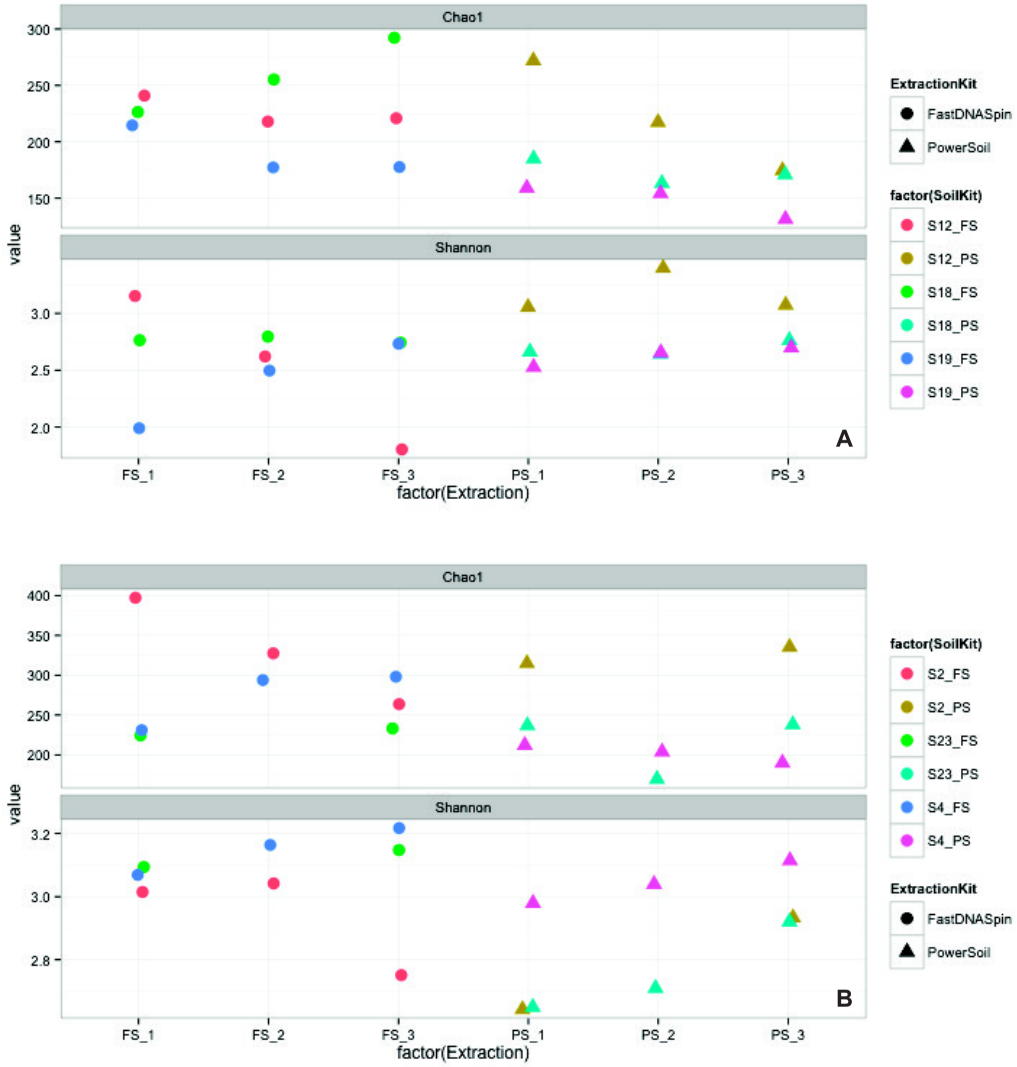

Figure 5. Species richness (Chao1) and diversity (Shannon) of the fungal community in clay (A) and sandy (B) soils.

Multidimensional scaling analysis (MDS), using weighted Unifrac distances (Hamady et al., 2010), was used to compare prokaryotic and fungal community composition in successive DNA extractions in both soil types. Prokaryotic community composition in clay soils showed to be similar in all three successive DNA extractions, regardless of the extraction kit used (Figure 6A). Soil 12 (PS) was the soil sample that presented the most different prokaryotic community among successive DNA extractions, with E1 and E3 being more similar to each other. Despite successive DNA extractions yielding similar prokaryotic community composition, some taxa were clearly enriched when

multiple extractions were performed in a single sample. For instance, a clear increase in relative abundance of the taxa Thaumarchaeota and Firmicutes could be seen with successive DNA extractions in all clay soil samples (Table S3). Other taxa, such as Verrucomicrobia, Choroflexi and Acidobacteria, showed considerably higher relative abundance in E2 and/or E3 for some of the soil samples (Figure 7A). Differently from prokaryotic communities, fungal communities in clay soils were different in successive DNA extractions (Figure 8A). As observed for bacterial and archaeal communities, soil 12 showed the biggest variation in fungal community when comparing successive extractions. Successive extractions promoted the enrichment of a few fungal taxa in some of the clay soils (Figure 9A), however, such enrichment showed to be kit dependent in most of the cases. Sandy soils, as clay soils, presented a similar prokaryotic community composition (Figure 6B). Of all three sandy soils, soil 23 presented the biggest difference in prokaryotic community composition when comparing successive extractions. As observed for clay soils, some taxa were enriched when multiple extractions were performed in a single soil sample. Firmicutes and Planctomycetes increased in relative abundance with successive DNA extractions in all sandy soils (Figure 7B and Table S3). Despite not having an increase in relative abundance in all soil samples, some taxa showed a considerable increase with successive DNA extraction, such as Actinobacteria, Chloroflexi and Proteobacteria (Figure 7B). Fungal community composition in successive DNA extractions on sandy soils was rather similar, contrary to clay soils (Figure 8B). Increase in relative abundance of fungal taxa with successive extraction in sandy soils was also observed, with taxa Ascomycota presenting an increase in all sandy soil samples, regardless the DNA extraction kit used (Figure 9B). A few bacterial and fungal taxa (phylum level) were observed only in E2 and/or E3, indicating that additional taxonomical groups can be identified with successive DNA extractions. However, the relative abundances of such taxa were always very low (lower than 0.05% for prokaryotes and 0.02% for fungi), and the occurrence of additional taxa was not observed for all soil samples (Table 2). Analysis of similarity (ANOSIM) was used to compare microbial communities in both soils, which had been obtained by different DNA extraction strategies. Prokaryotic community composition, in both soils, was not influenced by DNA extraction kit ($p > 0.05$). Fungal community composition, obtained with different DNA extraction kits, was significantly different only in clay soils (ANOSIM, $p < 0.05$, $R = 0.210$).

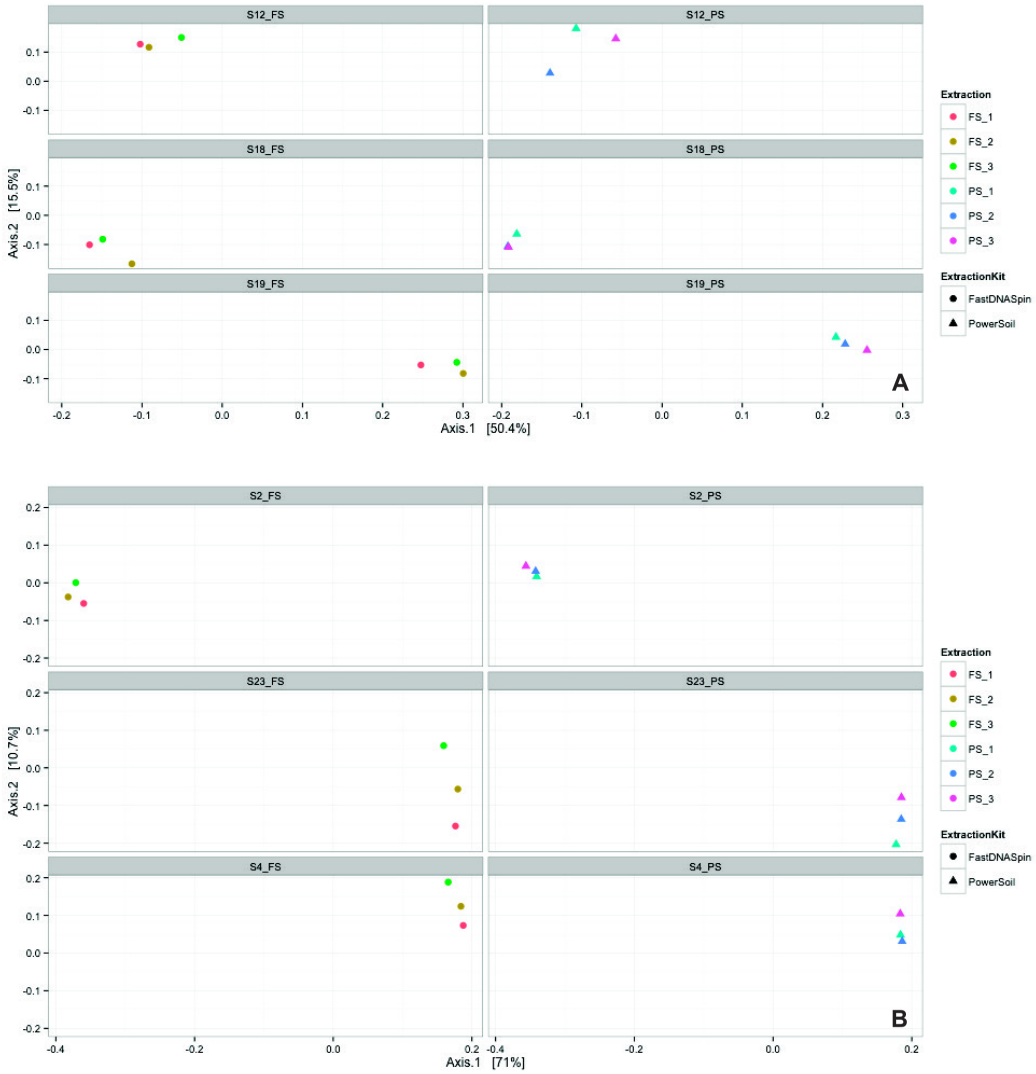

Figure 6. Multidimensional scaling (MDS) analysis of weighted Unifrac values from prokaryotic community in clay (A) and sandy soils (B).

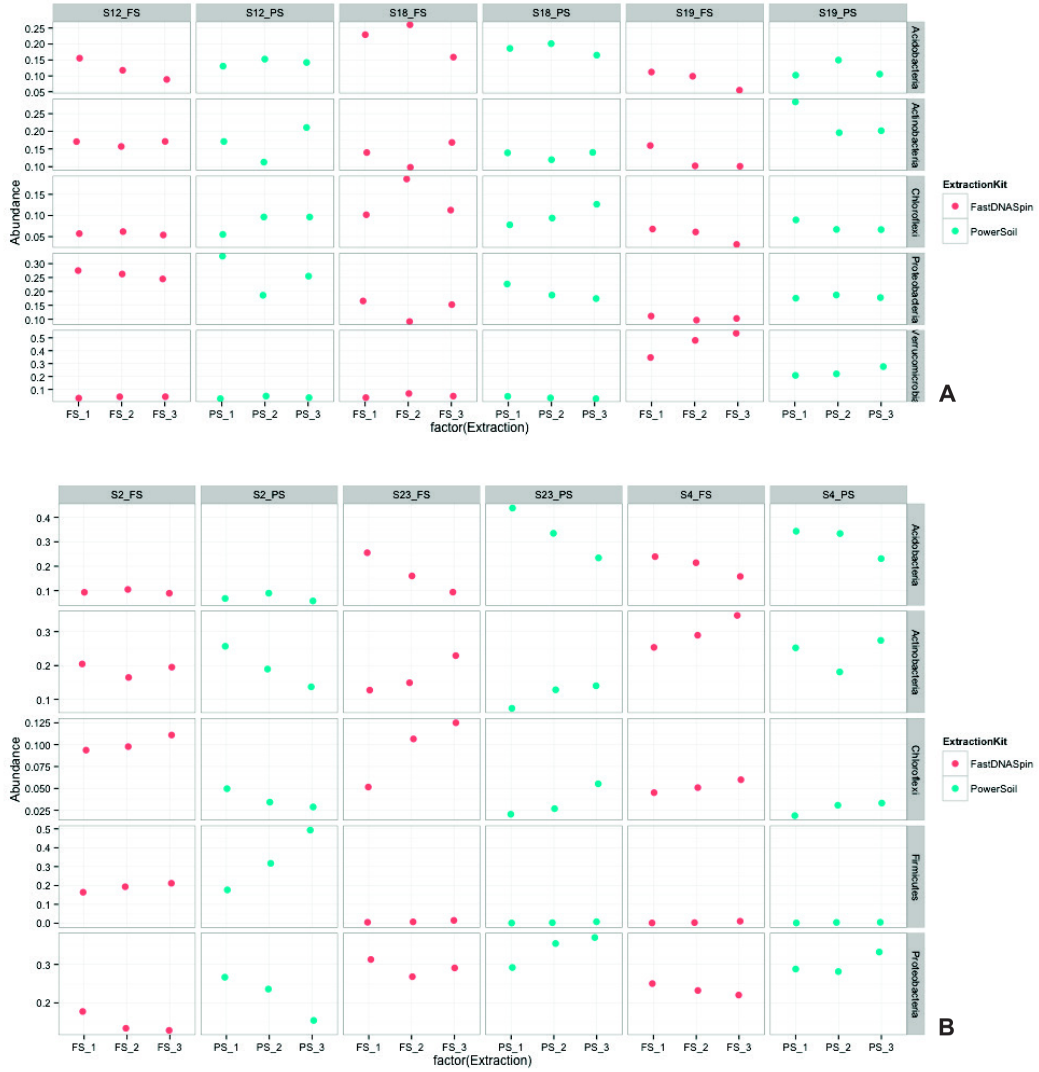

Figure 7. Relative abundance of the five most abundant prokaryotic taxa found in clay (A) and sandy (B) soils. Abundance is depicted as percentage, where one (1.0) corresponds to the sum of all taxa found.

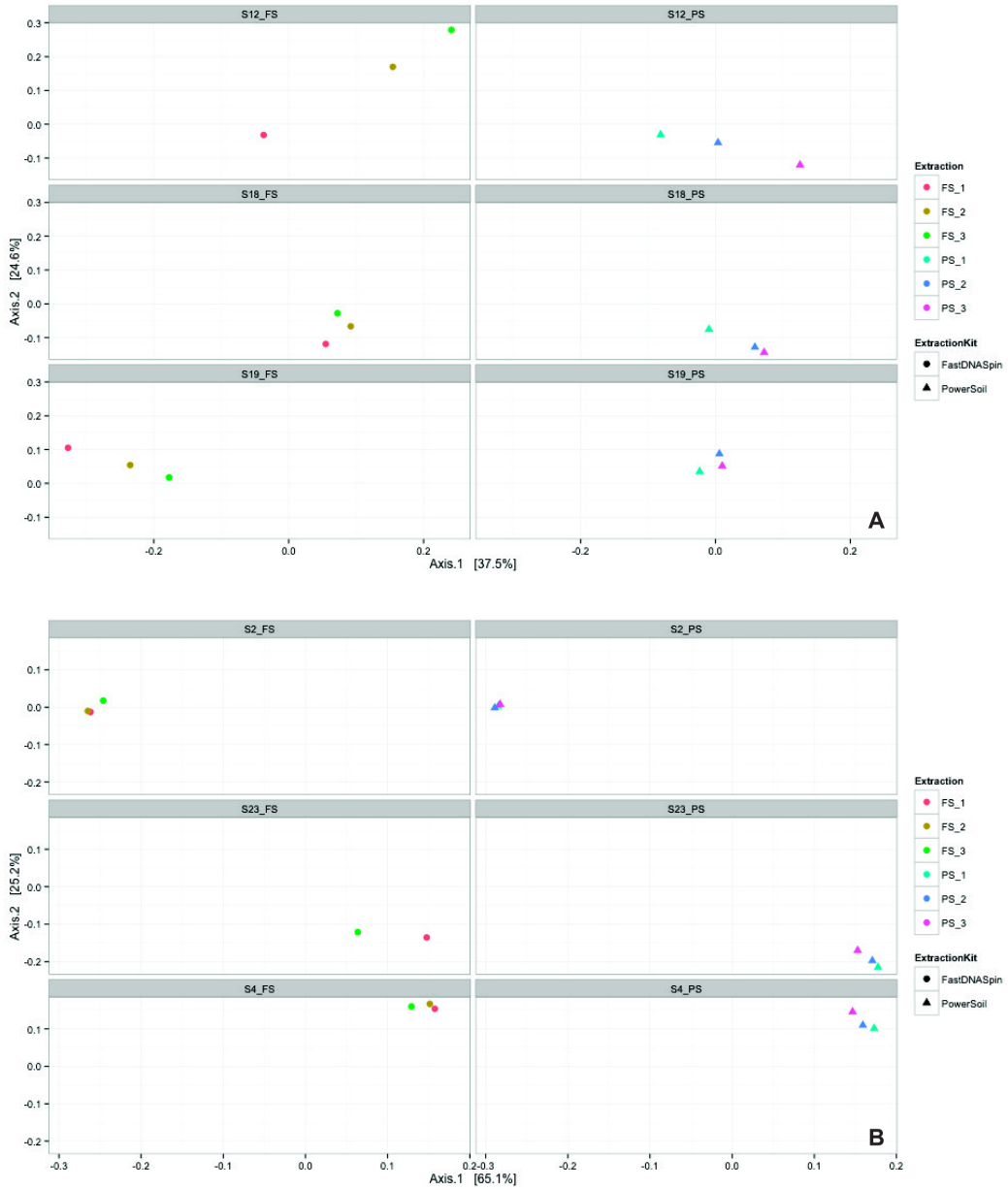

Figure 8. Multidimensional scaling (MDS) analysis of weighted Unifrac values from fungal community in clay (A) and sandy soils (B).

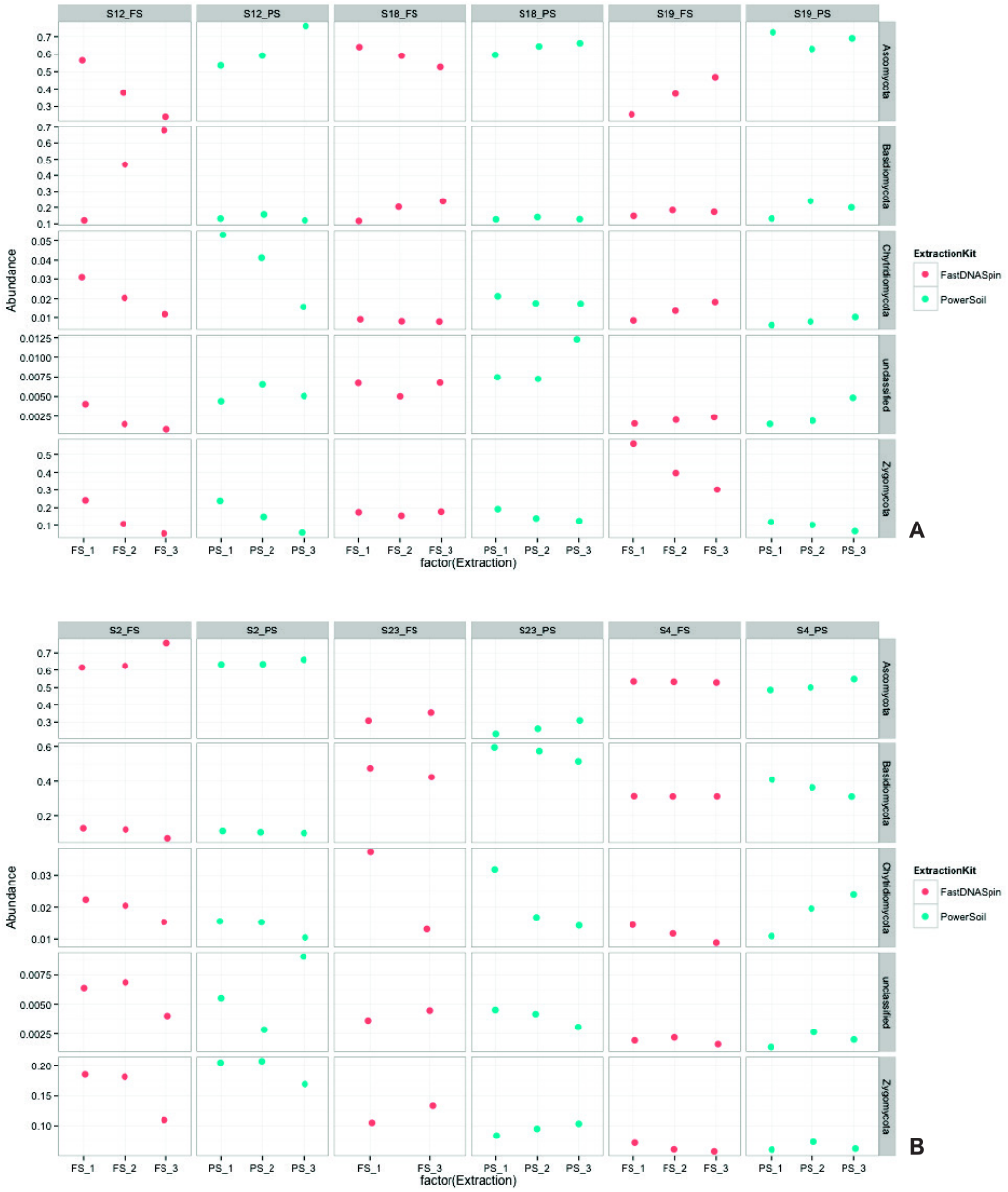

Figure 9. Relative abundance of the five most abundant fungal taxa found in clay (A) and sandy (B) soils. Abundance is depicted as percentage, where one (1.0) corresponds to the sum of all taxa found.

Table 2. Archaeal phylum (A), bacterial phyla (B) and fungal phyla (C) observed only in E2 and/or E3.

A

Phylum	Soil sample	Soil type
Euryarchaeota	12 (PS) and 19 (FS)	Clay

B

Phylum	Soil sample	Soil type
BD1-5	2 (FS)	Sandy
BHI80-139	4 (FS)	Sandy
Candidate_division_TM7	2 (FS)	Sandy
Chlorobi	19 (PS)	Clay
Cyanobacteria	19 (PS)	Clay
Deinococcus-Thermus	12 (PS) and 2 (FS)	Clay and Sandy
Elusimicrobia	19 (PS)	Clay
Fibrobacteres	18 (FS)	Clay
Lentisphaerae	12 (FS), 19 (FS) and 2 (FS)	Clay and Sandy
SHA-109	18 (PS)	Clay
SM2F11	18 (PS) and 12 (FS)	Clay
TM6	18 (PS)	Clay
Tenericutes	12 (PS)	Clay
Thermotogae	4 (PS)	Sandy
WCHB1-60	12 (PS) and 19 (FS)	Clay
WD272	12 (PS)	Clay

C

Phylum	Soil sample	Soil type
Glomeromycota	2 (FS)	Sandy
Other	18 (FS) and 4 (FS)	Clay and Sandy
Unclassified	18 (FS)	Clay

Discussion

Currently, characterization of complex microbial communities such as those present in soil relies heavily on the use of molecular approaches. Such approaches are often used to assess microbial abundance, community composition and diversity. Independently of the approach used, DNA contained in a soil sample needs to be separated from the soil phase as a first step. Therefore, soil DNA extraction is a crucial step, and success of downstream processes used to characterize soil microbial communities will depend largely on how well this first step was performed. Previous studies have shown that not all microbial DNA contained in a soil sample is extracted with a single DNA extraction (Burgmann et al., 2001; Feinstein et al., 2009; Jones et al., 2011). Here, we performed multiple successive DNA extractions on a number of representative soil samples to assess bias related to incomplete DNA extractions, using two widely used commercial soil DNA extraction kits. Moreover, different molecular techniques were applied to determine whether successive DNA extractions would lead to different apparent microbial communities.

DNA yield of all used soils was affected by successive DNA extractions. Soil 19 (PS) was the only soil sample that had more than 90% of all extracted DNA recovered after E1. All other soil samples showed recovery from 30% to 60%, of the total obtained DNA, in E1, which indicates that a significant portion of soil DNA is left behind at the end of the first extraction. This supports results previously described in the literature (Feinstein et al., 2009; Jones et al., 2011). Previous work has shown that DNA yield decreased with successive DNA extraction when, after the first extraction, the soil pellet was washed with extraction buffer; which indicates that DNA obtained in the successive extractions would probably come from newly lysed cells (Feinstein et al., 2009). Overall, DNA yield was significantly higher using FS compared to PS, around four times for clay soils and three times for sandy soils. This is in line with previous studies that have also shown that FS is more efficient than PS in extracting DNA from various soil types (Leite et al., 2014; Vishnivetskaya et al., 2014). Considering that the initial cell lysis step, when using PS, is much more extensive compared to FS (5.5 m s^{-1} for 10 min for PS and 6.0 m s^{-1} for 40 sec for FS), this might be surprising at first sight, however, after cell lysis, released DNA will strongly interact with soil particles, which can influence DNA yield (Lombard et al., 2011). Romanowski et al. (1993) demonstrated that up to 80% of added DNA was found to be adsorbed to sediment in less than 20 min. DNA adsorption to soil particles may be increased by DNA sharing (Pietramellara et al., 2001), however, DNA fragmentation of both kits was very similar (data not shown). Therefore, reasons for a higher DNA yield when using FS may be that this kit promotes not only a better soil homogenization, improving disruption of soil aggregates, but also a better cell lysis and DNA desorption from soil components. Furthermore, FS may have a decreased DNA degradation compared to PS, once DNA is released from cells.

Perhaps, a very extensive cell lysis step, as used for PS, is counterproductive, as it would allow for adsorption of DNA to soil particles for a longer period. Vishnivetskaya et al. (2014) showed that FS was more efficient than PS in genomic DNA recovery from a permafrost soil, which could also be attributed to higher bacterial cell lysis efficiency. Differences in bead to soil ratio has also been found to influence DNA yield while bead-beating is used for mechanical cell lysis (Burgmann et al., 2001). However, DNA extraction kits did not significantly affect DNA yield when recovery percentage was taking into account, which shows that independently of the kit and bead-beating time, more soil DNA was consistently obtained with successive extractions.

Similarly to the DNA yield, cumulative bacterial and fungal abundances increased with successive extractions in all samples, confirming that microbial DNA present in a soil sample is not fully recovered with a single extraction. Similar results have been reported earlier, where for most of the soils analyzed bacterial and fungal abundances levelled off after three successive extractions (Feinstein et al., 2009). Jones et al. (2011) also found that bacterial abundance increased considerably when multiple DNA extractions were performed on the same soil sample. In the present study, however, there was a considerable discrepancy between results of DNA yield and observed abundances of bacteria and fungi. Although DNA extraction kit significantly influenced total DNA yield, it did not influence total bacterial and fungal abundances. Although not significant, bacterial and fungal abundances were always higher on clay soil samples extracted with FS, however, differences were much smaller than those observed for DNA yield, especially for soil 18. That may indicate that using FS more DNA of non-microbial origin could be extracted, or that not all microbial DNA extracted is amplified during qPCR, which could be due to specificity or purity issues. Bacterial abundance in a permafrost soil showed to be around four times higher in samples extracted with FS compared to PS (Vishnivetskaya et al., 2014). The opposite was observed for sandy soil, where bacterial and fungal abundances were always higher on samples extracted with PS, despite DNA yield being higher in samples extracted with FS. Lower microbial abundances in samples extracted with FS may be attributed to a lower DNA purity obtained when using this kit, which could inhibit enzymatic reactions (Wilson, 1997). Both 260/280 and 260/230 ratios, which indicate DNA purity, were consistently lower in sandy soil samples that were extracted with FS (data not shown). Differently from what was reported by Kuramae et al. (2012), pasture and arable soil samples presented higher microbial abundance compared to forest soils.

Fungal communities in successive DNA extractions, which were identified by T-RFLP analysis, varied greatly in clay soils, whereas in sandy soils, fungal communities were more similar to each other, especially for samples extracted with PS. Differences observed between soil types indicate that clay soils might promote a greater degree of protection to fungal cells, which could make cell lysis more difficult. These results

differ from previous findings described on the literature, where fungal communities in successive DNA extractions of three different soil types (organic, clay and sand) were almost identical (Feinstein et al., 2009). Soils used by Feinstein et al. (2009) had a similar sand and clay content to those used here, therefore, other soil properties may play a role. Furthermore, soils analyzed here may have a higher abundance of fungal spores, which could explain the differences observed among successive DNA extractions.

Ecological diversity measures, such as Chao1 and Shannon, were used in order to determine whether apparent prokaryotic and fungal richness and diversity would change in successive DNA extractions. Chao1 is often used to indicate species richness (total number of species in a community), and it relies on the presence of singletons and doubletons, therefore, giving more weight to rare individuals (Hill et al., 2003). The Shannon index on the other hand takes into consideration not only the number of species in a community but also their relative abundance, but also giving more weight to rare than common species (Hill et al., 2003). For both variables, soil type and extraction kit, increase of prokaryotic and/or fungal species richness with successive DNA extraction was observed. Such observation suggests that more taxa/OTUs are obtained when successive DNA extractions are performed, therefore, presenting a more realist picture of the microbial community in those samples. Those taxa/OTUs possibly represent also rare organisms, which can be present in low abundance in the soil for various reasons, such as dormancy. It is known that dormancy is a common life history strategy in microbes (Jones and Lennon, 2010), and it might as well be that metabolic changes caused by such strategy lead to changes in cell structure and morphology that make cells harder to lyse. Increase in diversity with successive DNA extractions was also observed, especially in fungal communities. Increase in Shannon index (diversity) indicated that with successive extractions, not only new taxa/OTUs were being observed, but also that taxa/OTUs observed in these successive extractions appeared at more similar relative abundances. Therefore, both diversity indexes used suggest that extracting soil DNA only once would not promote a realist description of species richness and diversity.

To determine whether successive DNA extractions would yield different prokaryotic and fungal communities in clay and sandy soils, cluster analyses of the 16S and 18S rRNA gene sequencing data were performed. MDS plots revealed that prokaryotic communities obtained in E2 and E3 were similar to E1, for both soils. At phylum level, differences that were observed among successive DNA extractions, of the same soil sample, were in majority shifts in abundance, as observed before (Feinstein et al., 2009; Jones et al., 2011). Various phyla increased in relative abundance with successive DNA extractions, such as Thaumarchaeota and Firmicutes in all clay soils and Firmicutes and Planctomycetes in all sandy soils. Such increase in relative abundance may indicate

that organisms belonging to these phyla are more difficult to lyse, which could be a consequence of their life strategy and/or morphological characteristics. However, Feinstein et al. (2009) found that Firmicutes, gram-positive bacteria that are well known for having the ability to form spores, did not increase in relative abundance with successive DNA extractions in clay and sandy soils. It is important to mention that only the first and the sixth DNA extractions were sequenced in their study. As observed by Feinstein et al. (2009), bacterial phyla that were not identified in E1, but identified in E2 and/or E3 were always in a very low relative abundance. Differently from prokaryotic community, cluster analyses of the 18S rRNA gene data retrieved from successive DNA extractions revealed that communities obtained in E1 were different from E2 and E3 only in clay soils. The opposite has been reported in literature, where fungal communities analyzed by T-RFLP from six successive DNA extractions were very similar to each other (Feinstein et al., 2009). A low number of fungal taxa were identified only in E2 and/or E3, with the majority being unknown taxa. As for prokaryotic communities, major shifts in community composition were due to changes in relative abundance of fungal taxa. The use of both markers, 18S rRNA gene and ITS regions, showed similar results, however, the dissimilarity of fungal communities analyzed by T-RFLP was higher for a few soil samples. A possible reason for that is the higher variability of the ITS regions compared to the 18S rRNA gene, which allow for a better taxonomic differentiation (Anderson and Cairney, 2004).

Successive rounds of DNA extraction from a several representative soil samples, using two widely commercial DNA extraction kits, did result in the identification of additional prokaryotic or fungal phyla in some of the soils analyzed. However, when identified, additional phyla were always present at very low relative abundance. Nevertheless, shifts in relative abundance of well-known groups of soil archaea, bacteria and fungi were observed. In some cases, changes in relative abundance were such that communities originating from the same soil sample, but from a different extraction, were seen as different communities, as indicated by MDS plots. Total bacterial and fungal abundance increased considerably with successive DNA extractions, confirming that not all soil DNA is extracted in a single extraction. Often, in microbiome studies, multiple parallel extractions of a sample are performed, and DNA originating from all extractions is pooled for further experiments. Such strategy attempts to reduce variability between extractions in order to provide a more realistic representation of the microbiome of that particular sample. However, such strategy would still fail to provide an accurate estimation of the relative abundance of microbial groups present in that particular microbiome. Therefore, as Feinstein et al. (2009), we argue that to improve microbial characterization, leading to a more comprehensive analysis, multiple successive extractions of the same soil sample should be done. DNA obtained in multiple extractions should be pooled prior use in further experiments, as indicated by Feinstein et al. (2009).

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Supplementary information

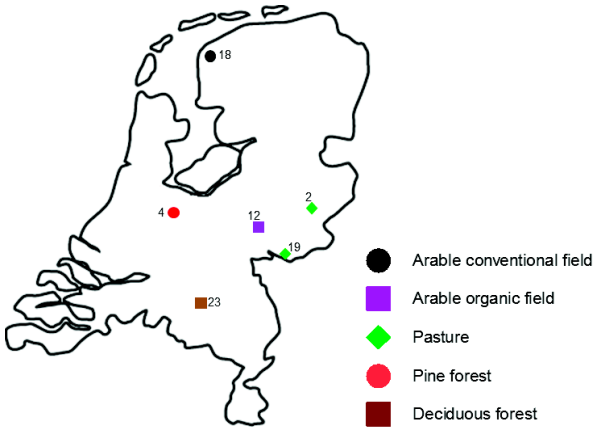


Figure S1. Overview of sampling locations and land management types of the soils used in the present work.

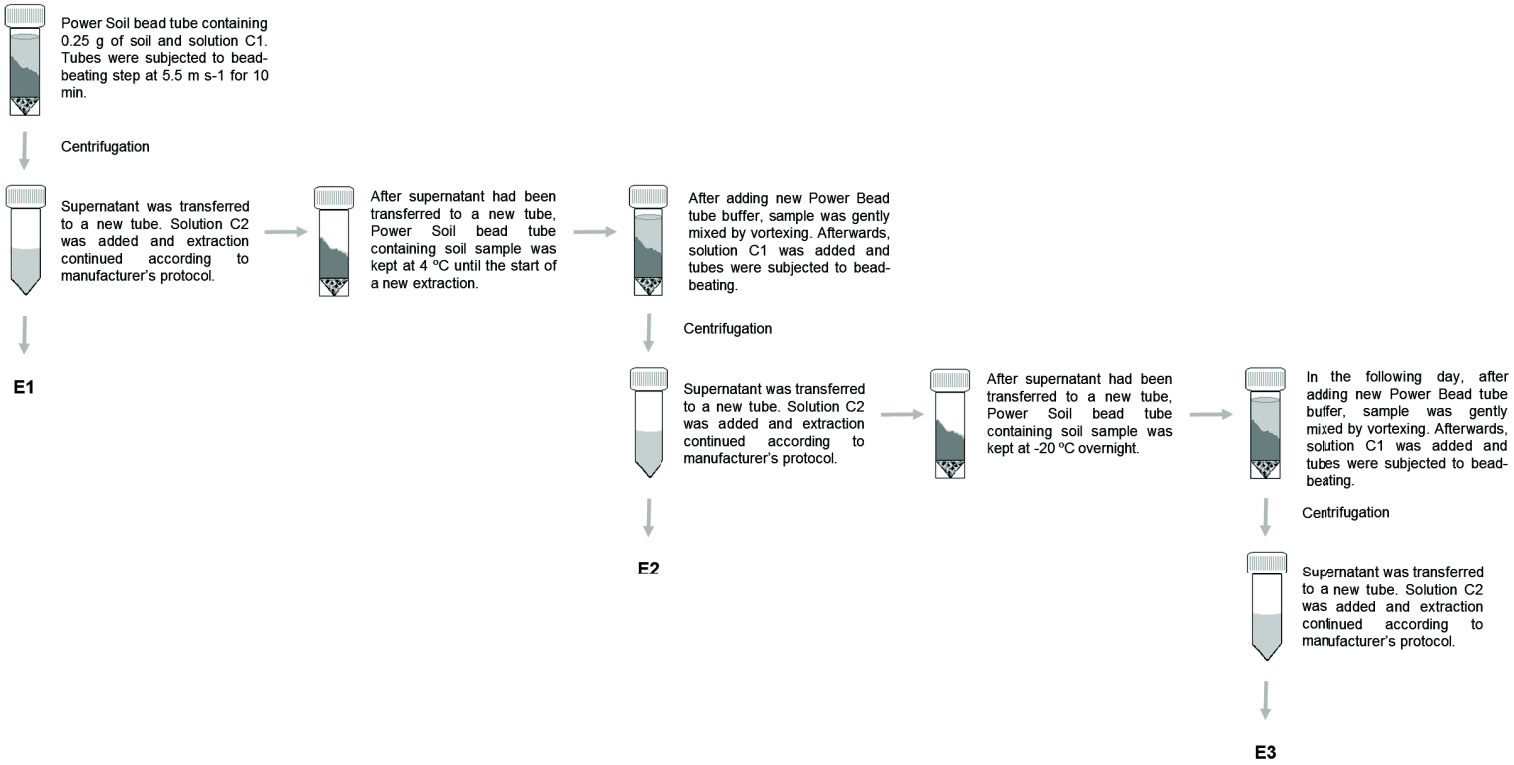


Figure S2. Scheme of the DNA extraction procedure used in the present work. Scheme represents extraction performed with PS. Same procedure was used for FS, however, the amount of soil and bead-beating step were different.

Table S1. Soil physical and chemical charactherists of the samples used in the present work.

Field	Land use	Latitude (N)	Longitude (E)	pH	Total N (%)	Total C (%)	C:N ratio	OM (%)	Total P (mg P_2O_5 $100g^{-1}$)	Clay (%)	Silt (%)	Sand (%)	CaCO3 (%)	Cd (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})	As (mg kg^{-1})	Hg (mg kg^{-1})
sandy soils																					
2	Pasture	52º14"	06º41"	6.1	0.25	2.3	9.2	4	197	1.1	6.8	91.9	0	0.17	6.9	13	0	9.7	28	2.2	0
4	Pine forest	52º08"	05º11"	3.7	0.17	3.8	22.3	6.4	17	0.3	4.3	95.4	0	0.1	0	0	0	15	0	1.8	0.04
23	Deciduous forest	51º32"	05º18"	3.7	0.28	5	17.8	9	49	4.3	9.8	85.9	0	0.13	8.2	0	3.8	23	12	5	0.09
clay soils																					
12	Organic arable field	52º01"	06º12"	6.5	0.13	1.4	11.0	2.7	296	13.4	29.4	57.4	0.1	0.21	19	15	8.4	21	71	10	0.09
18	Conventional arable field	53º12"	05º31"	7.4	0.13	1.2	9.3	1.6	106	16.1	37.9	46	2.4	0.12	29	21	14	14	41	11	0.03
19	Pasture	51º53"	06º17"	6	0.55	4.8	8.7	9.4	310	36.7	51.1	12.1	0.2	0.49	52	27	33	32	130	14	0.08

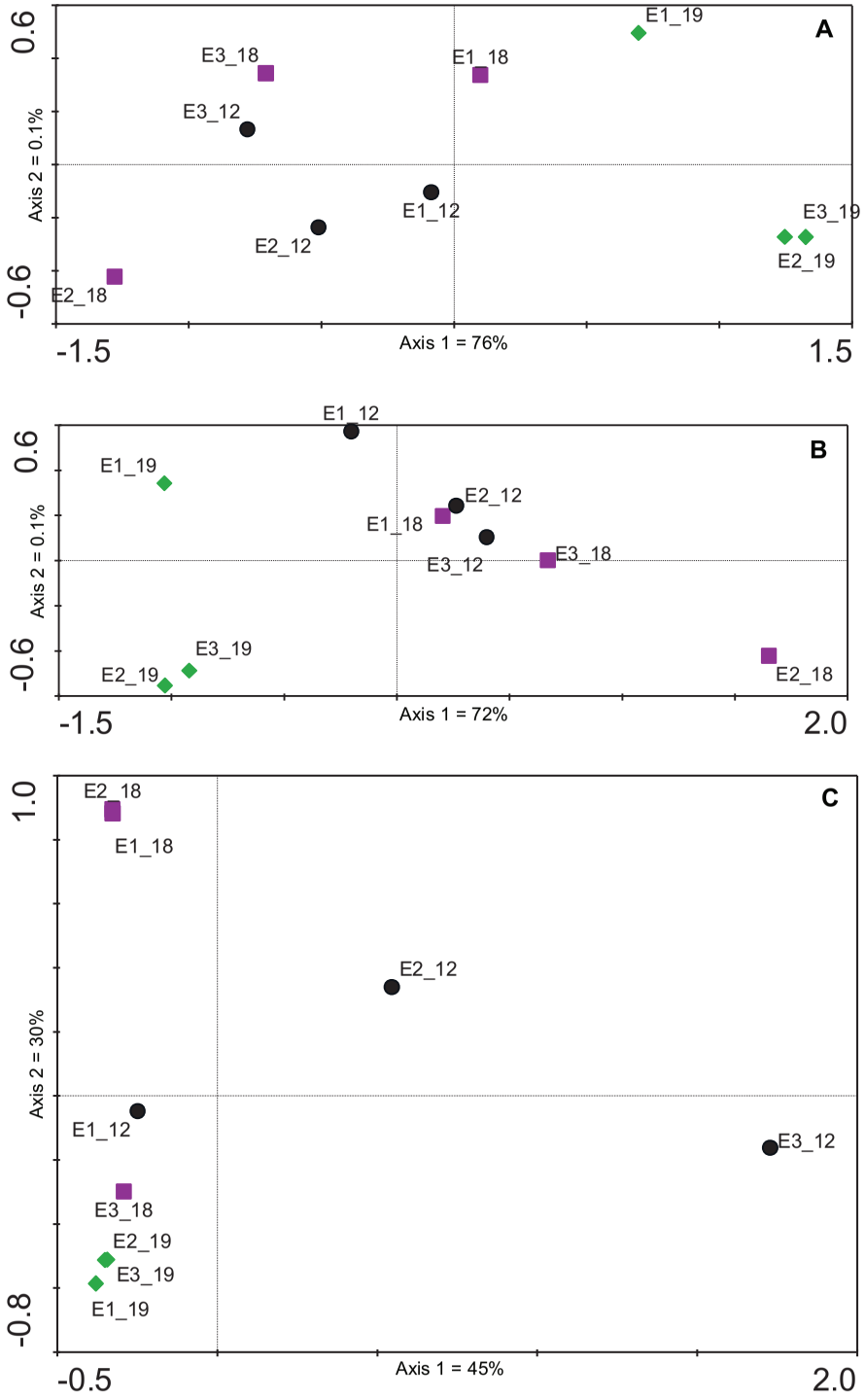
Table S2. DNA yield (µg of DNA/g of soil) (SD) in successive extractions for both kits in clay soils (A) and sandy soils (B).

A

	Soil 12		Soil 18		Soil 19	
Extraction	**PS**	**FS**	**PS**	**FS**	**PS**	**FS**
E1	5.74(0.24)	21.35(14.07)	2.42(0.18)	8.13(0.71)	14.61(8.71)	42.57(8.47)
E2	3.05(0.25)	9.90(4.18)	3.18(0.52)	4.67(3.15)	0.33(0.29)	28.49(2.75)
E3	0.72(0.58)	5.00(0.43)	2.03(0.17)	6.95(1.28)	0.34(0.28)	20.97(17.09)

B

	Soil 2		Soil 04		Soil 23	
Extraction	**PS**	**FS**	**PS**	**FS**	**PS**	**FS**
E1	11.53(1.01)	31.90(8.36)	3.64(0.34)	10.68(2.62)	3.43(0.81)	12.00(8.68)
E2	4.67(1.22)	18.15(0.11)	1.90(0.04)	5.02(1.41)	3.81(0.77)	22.17(15.45)
E3	3.20(0.12)	8.62(0.49)	1.09(0.22)	3.07(0.60)	1.94(0.09)	5.12(1.29)


A
E1_19
E3_18
E1_18
E3_12
E1_12
E3_19
E2_12
E2_19
E2_18
0.6
-0.6
Axis 2 = 0.1%
-1.5
Axis 1 = 76%
1.5
B
E1_12
E1_19
E2_12
E1_18
E3_18
E3_12
E3_19
E2_19
E2_18
0.6
-0.6
Axis 2 = 0.1%
-1.5
Axis 1 = 72%
2.0
C
E2_18
E1_18
E2_12
E1_12
E3_12
E3_18
E2_19
E3_19
E1_19
1.0
-0.8
Axis 2 = 30%
-0.5
Axis 1 = 45%
2.0

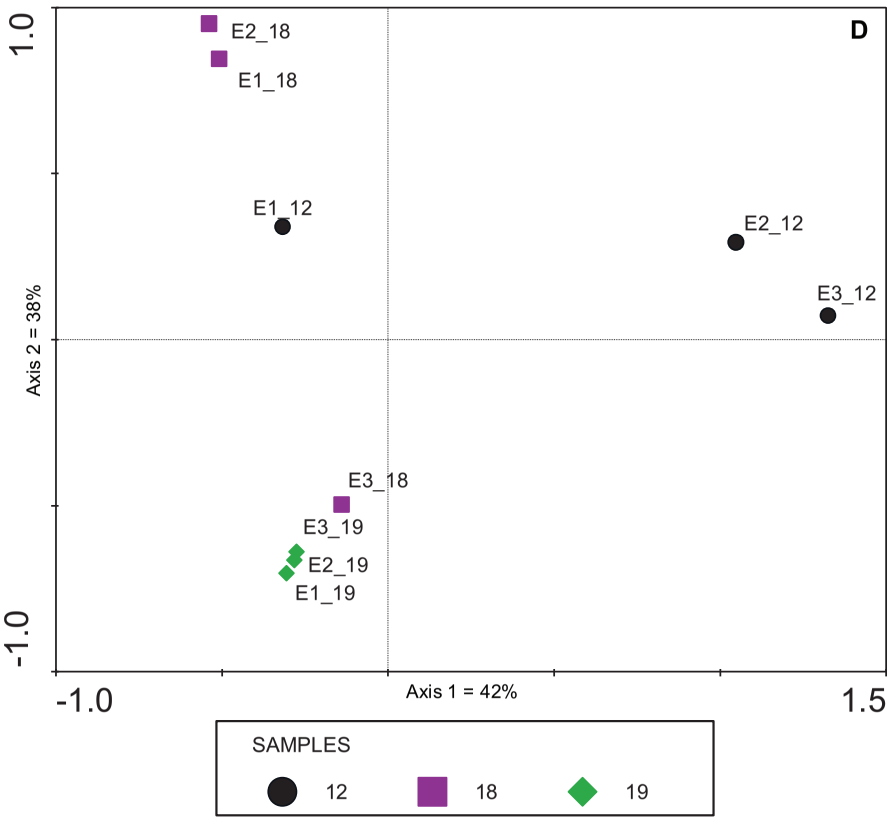
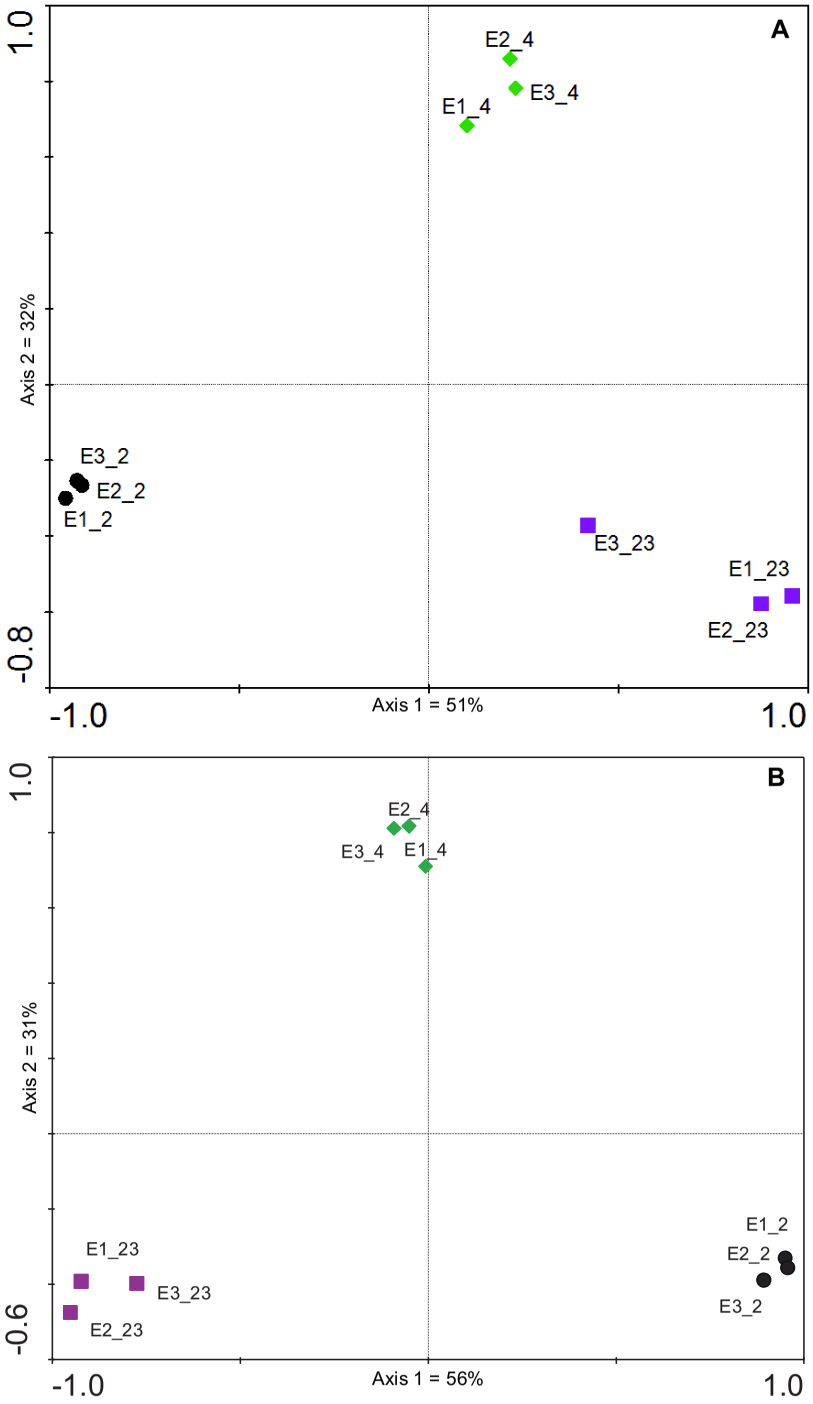


Figure S3. PCA plots of fungal community in clay soils analyzed by T-RFLP. (A) total DNA extracted with PS and analyzed using the forward primer; (B) total DNA extracted with PS and analyzed using the reverse primer; (C) total DNA extracted with FS and analyzed using the forward primer; (D) total DNA extracted with FS and analyzed using the reverse primer.


A
1.0
-0.8
-1.0
1.0
Axis 2 = 32%
Axis 1 = 51%
E2_4
E3_4
E1_4
E3_2
E2_2
E1_2
E3_23
E1_23
E2_23
B
1.0
-0.6
-1.0
1.0
Axis 2 = 31%
Axis 1 = 56%
E2_4
E3_4
E1_4
E1_23
E3_23
E2_23
E1_2
E2_2
E3_2

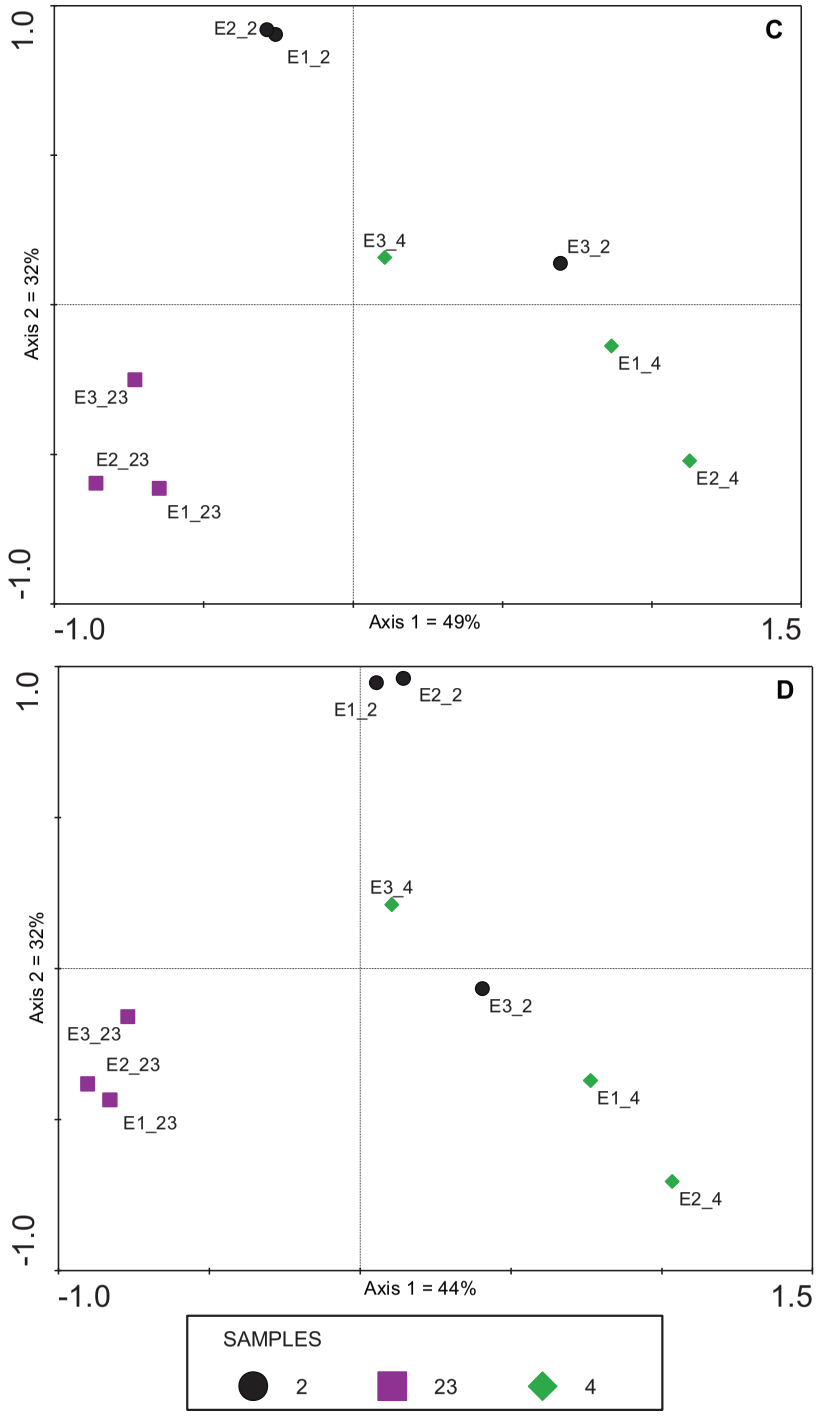


Figure S4. PCA plots of fungal community in sandy soils analyzed by T-RFLP. (A) total DNA extracted with PS and analyzed using the forward primer; (B) total DNA extracted with PS and analyzed using the reverse primer; (C) total DNA extracted with FS and analyzed using the forward primer; (D) total DNA extracted with FS and analyzed using the reverse primer.

Table S3. Relative abundance of the ten most abundant bacterial Phyla in all clay (A) and sandy (B) soils.

A

Taxa	PS									FS								
	E1 Soil12	E2 Soil12	E3 Soil12	E1 Soil18	E2 Soil18	E3 Soil18	E1 Soil19	E2 Soil19	E3 Soil19	E1 Soil12	E2 Soil12	E3 Soil12	E1 Soil18	E2 Soil18	E3 Soil18	E1 Soil19	E2 Soil19	E3 Soil19
Bacteria; Proteobacteria	28.93	17.92	24.58	22.19	18.06	16.75	17.39	18.70	17.66	24.78	24.04	21.85	16.59	9.46	14.67	11.22	10.05	10.49
Bacteria; Actinobacteria	14.09	10.53	19.40	12.74	11.22	13.38	27.17	19.27	20.16	14.62	13.97	15.16	12.48	9.27	15.09	15.58	10.18	10.45
Bacteria; Acidobacteria	12.06	14.88	13.42	17.11	18.96	15.97	10.30	15.01	10.79	14.34	11.69	8.76	21.28	24.24	16.00	11.41	10.14	5.85
Bacteria; Verrucomicrobia	2.59	4.43	3.43	4.50	3.18	2.56	18.91	20.62	26.22	2.95	3.59	3.57	3.35	6.38	4.02	32.72	45.74	50.25
Bacteria; Firmicutes	4.78	5.01	5.80	1.71	2.13	3.02	0.21	0.90	2.36	7.32	14.87	22.29	4.06	3.40	9.81	2.33	2.80	7.47
Bacteria; Chloroflexi	4.80	8.76	8.99	7.60	8.78	11.62	8.41	6.61	6.57	4.98	5.46	4.77	9.72	17.82	10.72	6.72	6.04	3.17
Bacteria; Planctomycetes	4.04	7.03	9.90	8.14	6.34	7.48	6.03	5.64	6.11	3.99	4.34	5.19	6.31	8.80	6.51	5.15	4.50	2.13
Archaea; Thaumarchaeota	3.16	16.32	2.37	6.87	13.12	15.28	0.18	0.76	1.88	4.16	6.32	4.17	7.16	5.72	10.22	3.85	2.93	4.83
Bacteria; Nitrospirae	1.58	1.92	2.11	2.08	2.58	2.19	2.56	5.99	3.40	2.40	1.70	1.01	2.99	5.88	2.14	3.92	3.36	1.08
Bacteria; Gemmatimonadetes	5.39	1.92	2.87	4.52	3.41	2.11	1.61	1.27	0.84	3.46	1.95	1.52	3.43	1.79	1.04	0.96	0.44	0.44

B

Taxa	PS									FS								
	E1 Soil2	E2 Soil2	E3 Soil2	E1 Soil4	E2 Soil4	E3 Soil4	E1 Soil23	E2 Soil23	E3 Soil23	E1 Soil2	E2 Soil2	E3 Soil2	E1 Soil4	E2 Soil4	E3 Soil4	E1 Soil23	E2 Soil23	E3 Soil23
Bacteria; Proteobacteria	25.16	23.19	15.59	27.16	27.76	31.86	27.32	32.87	36.81	17.22	13.37	13.02	24.10	22.29	21.73	30.91	26.69	28.43
Bacteria; Actinobacteria	23.69	18.21	13.35	23.40	17.51	26.17	7.07	12.33	13.72	19.41	15.89	18.99	24.06	27.51	33.33	12.52	14.63	22.08
Bacteria; Acidobacteria	6.67	8.62	5.82	32.28	32.16	21.93	41.28	31.30	22.62	9.08	10.26	8.84	22.76	20.45	15.15	24.94	15.59	8.87
Bacteria; Verrucomicrobia	6.42	4.14	4.57	1.46	1.85	0.75	3.64	2.61	3.52	9.16	15.93	11.10	2.85	1.48	0.88	5.47	4.38	1.52
Bacteria; Firmicutes	15.24	28.27	44.33	0.15	0.45	0.56	0.11	0.34	0.82	14.41	17.79	20.24	0.19	0.40	1.07	0.59	0.89	1.80
Bacteria; Chloroflexi	4.60	3.20	2.87	1.73	2.99	3.05	1.83	2.31	5.28	8.14	9.20	10.70	4.31	4.63	5.68	5.00	10.26	11.56
Bacteria; Planctomycetes	3.36	2.75	3.08	2.56	6.66	4.83	2.06	2.82	5.79	4.80	5.71	8.19	7.36	7.33	8.41	3.95	8.85	9.60
Archaea; Thaumarchaeota	1.86	1.32	1.98	0.38	0.55	0.59	4.54	2.62	1.35	5.92	3.91	3.72	1.43	1.37	0.97	7.06	4.10	2.61
Bacteria; Nitrospirae	0.58	0.72	0.26	0.15	0.18	0.06	0.11	0.14	0.10	0.33	0.18	0.26	0.19	0.02	0.05	0.26	0.45	0.02
Bacteria; Gemmatimonadetes	2.09	1.73	0.93	0.24	0.24	0.29	0.18	0.12	0.10	1.36	0.87	0.97	0.15	0.11	0.12	0.24	0.16	0.08

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Chapter 3

Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms

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Abstract

Aquatic ecological risk assessment of fungicides in Europe under Regulation 1107/2009/EC does not currently assess risk to non-target bacteria and fungi. Rather, regulatory acceptable concentrations based on ecotoxicological data obtained from studies with fish, invertebrates and primary producers (including algae) are assumed to be protective to all other aquatic organisms. Here we explore the validity of this assumption by investigating the effects of a fungicide (tebuconazole) applied at its "non-microbial" HC5 concentration (the concentration that is hazardous to 5% of the tested taxa and derived from acute single species toxicity tests on fish, invertebrates and primary producers (including algae) on the community structure and functioning of heterotrophic microbes (bacteria and aquatic fungi) in a semi-field study, using novel molecular techniques. In our study, a treatment-related effect of tebuconazole (238 µg/L) on either fungal biomass associated with leaf material or leaf decomposition or the composition and biomass of the fungal community associated with sediment could not be demonstrated. Moreover, treatment-related effects on bacterial communities associated with sediment and leaf material were not detected. However, tebuconazole exposure did significantly reduce conidia production and altered fungal community composition associated with leaf material. An effect on a higher trophic level was observed when *Gammarus pulex* were fed tebuconazole-exposed leaves, which caused a significant decrease in their feeding rate. Therefore, tebuconazole may affect aquatic fungi and fungally-mediated processes even when applied at its "non-microbial" HC5 concentration.

Introduction

Microorganisms play a crucial role in the functioning of all ecosystems by providing food for other organisms, decomposing organic material, cycling and transforming nutrients and degrading contaminants (Barlocher, 1985; Duarte et al., 2010; Singh and Walker, 2006; Veraart et al., 2014). Heterotrophic microbes (e.g., bacteria and fungi) are responsible for much of the material and energy flow in fresh waters, and their role in the decomposition of organic material has been particularly well studied, especially in streams (Findlay, 2010; Wurzbacher et al., 2010). Whereas bacteria dominate fine particulate organic material and sediments, fungi dominate on larger particulate organic material such as leaf litter (Fischer et al., 2006). Hyphomycetes, the dominant aquatic fungal group associated with leaf litter, decompose plant material and improve its nutritional quality and palatability to macroinvertebrate consumers (Gessner et al., 2007). Antagonism between fungi and bacteria decomposing leaf litter has been demonstrated, with bacterial abundance being reduced in the presence of aquatic fungi (Gulis and Suberkropp, 2003). There also appears to be a trade-off between fungal growth and either the ability to tolerate, or to compete with, bacteria suggesting that this antagonism is driven by competition (Mille-Lindblom et al., 2006). Synergistic interaction between bacteria and fungi decomposing leaf litter has also been demonstrated (Bengtsson, 1992). Both bacteria and fungi are important in the decomposition of leaf litter (Schneider et al., 2010), however, compared to aquatic hyphomycetes we know very little about the diversity of bacteria associated with leaf material (Duarte et al., 2010).

Many freshwater food webs are fuelled by allochthonous organic matter, and heterotrophic microbes are essential for converting the energy locked in detrital material into animal biomass (Bärlocher, 2005; Gessner et al., 2007; Webster and Meyer, 1997). Consequently, pollutant-induced changes in the composition and/or functioning of microbial communities could have far-reaching ecological consequences. Several studies have investigated the effect of metal pollution and eutrophication on the diversity and functioning of freshwater microbial communities (Bermingham et al., 1996a; Deanross and Mills, 1989; Lecerf and Chauvet, 2008; Sridhar et al., 2001; Suberkropp et al., 2010). However, relatively little is known about their response to fungicide exposure (Maltby, 1992b; Maltby et al., 2009; McMahon et al., 2012; van den Brink et al., 2007), although effects of fungicides on bacterial community structure and functioning in soils and sediments have been reported (Bending et al., 2007; Milenkovski et al., 2010; Widenfalk et al., 2008). Furthermore, effects of the fungicide tebuconazole on the conditioning of leaf litter have been demonstrated (Bundschuh et al., 2011; Zubrod et al., 2011).

Fungicides are widely used in modern agriculture (Battaglin et al., 2011; Wightwick et al., 2010) and are regulated in Europe under Regulation 1107/2009/EC (Commission, 2009). Regulatory risk assessment of fungicides requires toxicity data for a standard

set of test species including a vertebrate (fish), invertebrate (crustacean) and primary producer (alga), but neither fungi nor bacteria (Commission, 2013). Assessment factors are applied to data obtained from standard toxicity tests in order to protect non-tested species, including microbes. However, the extent to which these assessment factors account for the uncertainties associated with extrapolating from standard single-species laboratory toxicity data to microbial communities in natural environments is unknown. One approach to reduce this uncertainty has been to characterize interspecific variation in toxicant sensitivity by constructing species sensitivity distributions (SSDs) (Posthuma et al., 2002).

Maltby et al. (2009) constructed SSDs based on acute single-species toxicity data for 39 fungicides and derived threshold concentrations based on the concentration that was hazardous to either 5% (HC5) or 1% (HC1) of species. Most of the fungicides investigated were general biocides, and data from all taxonomic groups were used to derive SSDs and assess risk. For 12 of the fungicides, it was possible to compare threshold values with effects in semi-field studies, and in all cases the lower-limit HC5 and HC1 values were protective of adverse ecological effects. A limitation of this analysis was that no appropriate toxicity data were available for aquatic bacteria and fungi and only one microbial-relevant endpoint (i.e. leaf decomposition) was measured in the semi-field studies, and then only for 4 of the 12 fungicides. Recent studies by Dijksterhuis et al. (2011), Bundschuh et al. (2011) and Zubrod et al. (2011) reported NOEC (no observed effect concentration) values for aquatic non-target fungi and bacteria at tebuconazole concentrations lower than the HC5 value derived from a SSD and that was constructed with L(E)C50 data for aquatic algae, invertebrates and fish (Maltby et al., 2009). For practical reasons we refer to these SSDs as "non-microbial" SSDs, although available toxicity data for planktonic algae were used in the SSDs constructed by Maltby et al. (2009). This raises the possibility that threshold values derived from "non-microbial" SSDs may not be fully protective of natural microbial communities. Less direct, but supporting evidence, is also provided by the observation of reduced microbially-mediated leaf decomposition at propiconazole concentrations lower than the HC5 value derived from an SSD (Rasmussen et al., 2012). Propiconazole has a similar toxic mode-of-action than tebuconazole.

Here we examine further the validity of using non-microbial acute toxicity data to assess the ecological risk of fungicides by investigating the effects of a fungicide applied at its "non-microbial" HC5 value on microbial community structure and functioning in a semi-field study. The fungicide selected was tebuconazole, a sterol biosynthesis inhibitor, which is widely used and has been regularly detected in surface water (Kahle et al., 2008). In the present paper we elaborate on the reported responses of aquatic microbes to tebuconazole exposure as described in Bundschuh et al. (2011), Zubrod

et al. (2011) and Artigas et al. (2012) by using novel molecular techniques to identify potential effects on microbial communities not only on leaf material but also in the sediment. By relying on the presence of nucleic acids, molecular techniques allow a more comprehensive characterization and a deeper understanding of pollutant-induced effects on microbial communities when compared to traditional techniques (i.e. fungal identification by conidia observation) (Barlocher, 2010; Ekblom and Galindo, 2011; Nikolcheva et al., 2003; Van der Zaan et al., 2010).

Materials and Methods

Microcosm set up

Eight microcosms, each consisting of polycarbonate, cylindrical enclosures (diameter: 1.05 m; surface area 0.865 m^2; height: 0.9 m) were established in an experimental ditch located at the Sinderhoeve Experimental Station, Renkum, the Netherlands (Drent and Kersting, 1993) on 16 July 2010 (i.e. 25 days before fungicide application). Experimental ditches at the Sinderhoeve Experimental Station were constructed in the late 1980s and the sediment used was a loamy-sand sediment collected from an uncontaminated mesotrophic aquatic ecosystem. Therefore, the sediment community present in the experimental ditch used in this study had been well established already before experimental set-up and did not require any pre-conditioning. A lentic model system was chosen since in Dutch agricultural landscapes edge-of-field surface waters subject to fungicide contamination predominantly are drainage ditches. Each enclosure was pushed approximately 0.15 m into the sediment of the ditch, had a water depth of approximately 0.5 m and contained macrophytes, phytoplankton, zooplankton and benthic invertebrates. The sediment consisted of loamy sand with a few centimetres detritus layer on top. The loamy sand prevented percolation of water, therefore, test systems could be considered hydrologically isolated from the surroundings. Furthermore, the water layer in the enclosures was kept similar to that outside the enclosures, also preventing seepage. The only additional organisms included were *Gammarus pulex* (Crustacea, Amphipoda) (30 individuals/enclosure) and *Asellus aquaticus* (Crustacea, Isopoda) (28 individuals/enclosure), both of which play an important role in the breakdown of leaf material.

Two hundred and sixty leaf bags were constructed, being 130 coarse mesh bags (pore size 0.5 cm x 0.5 cm) and the remaining 130 fine mesh bags (pore size 600 µm diameter). Each mesh bag contained either 8 g (coarse mesh bags) or 5 g (fine mesh bags) of alder (*Alnus glutinosa*) leaf material. All leaf bags were conditioned in an experimental ditch for 4 weeks prior to allocation to individual enclosures. At the end of the conditioning period (4 days prior to fungicide application), ten coarse mesh bags and 10 fine mesh bags were randomly selected. Seven bags of each mesh type were used to quantify mass loss during the conditioning period. The remaining 3 mesh bags were used to characterize the aquatic hyphomycete assemblage (conidia identification) colonizing the leaf material post-conditioning but pre-application, and to measure fungal biomass. The remaining mesh bags, fifteen coarse mesh bags and 15 fine mesh bags, were allocated to individual enclosures, 4 days prior to fungicide application.

Fungicide application, water sampling and environmental measurements

Half the enclosures were randomly assigned as 'control' and the other half 'treatment'. Tebuconazole was applied to enclosures as the commercially available product Folicur® (Bayer Crop Science, Germany), active ingredient content of 25% (w/w). Plant protection products are often applied as formulations, entering the environment together with their additives. Therefore, the use of a commercially available and widely used product becomes ecologically more relevant. A single application of 238 µg tebuconazole/L (representing the HC5 using non-microbial acute toxicity data) (Maltby et al., 2009) was applied to the treatment enclosures on August 10th, 2010. The fungicide was applied by pouring approximately 2 L of dosing solution over the water surface and gently stirring the compound in the water column. The control enclosures received water only.

Water samples for tebuconazole measurements were taken from each enclosure on day -1, day 0 (1-2h after fungicide application) and days 1, 6, 13, 20, 34 and 59. Approximately 100 mL of a depth-integrated water sample (but excluding the water layer near the sediment to avoid sampling of detritus and sediment particles) was collected from each enclosure on each sampling date. Of this well-mixed sample, approximately 2 mL was used for each tebuconazole analysis using LC-MS/MS. Tebuconazole in the leaf material collected from litter bags was first extracted by shaking a mixture containing 1.6 g of dry leaf material, 10 g of glass beads and 25 mL of extraction solution (75% acetone and 25% ultrapure water) for 30 min. Subsequently, a sub-sample of the extract was filtered through a 0.45 µm filter and analyzed by LC-MS/MS (for methods see supplementary information). Tebuconazole analyses were duplicated. On the same sampling dates, temperature, pH and dissolved oxygen (DO) were measured at approximately 25 cm water depth using an HQ40D oxygen-acidity meter (Hach Lange, Germany), equipped with a luminescence-based dissolved oxygen probe, and electrical conductivity was measured using a WTW LF191 meter. On days -1, 17 and 59 the alkalinity of a 100 mL unfiltered water sample was measured by titration with 0.02 N HCl to pH 4.2, and on days -1 and 59 the nutrient status was determined by analysing a 100 mL filtered sample (mesh size 1.2 µm) for total nitrogen, nitrate/nitrite, ammonium, ortho-phosphate and total phosphate, following standard procedures.

Reponses of phytoplankton and zooplankton to tebucanazole treatment were studied as well. Since the test systems were treated with concentration resembling the "non-microbial" HC5, it was anticipated that treatment-related effects on planktonic algae and invertebrates would be small, which indeed was the case. Material and methods information to study phyto- and zooplankton, as well as information on the treatment-related effects observed at the population and community level, are presented in the Supporting Information section.

Microbial community composition and abundance

Sediment samples

Three cores of the upper 3 cm of sediment were taken from each enclosure on days -4, 3, 10, 17, 24, 31, 48 and 59. The cores from each enclosure were mixed thoroughly and stored at -80 °C until use. Total DNA was isolated using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) (Mincer et al., 2005) and polymerase chain reaction (PCR) amplifications performed. The bacterial community was targeted by partial amplification of the 16S rRNA gene (V1 and V2 regions), whereas fungal community was targeted by both partial amplification of 18S rRNA gene (V7 and V8 regions) and partial amplification of the internal transcribed spacer (ITS) region (5.8S and 23S regions partially, and ITS2 region completely), using the primers and conditions listed in Table 1. PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using methods described in (Lin et al., 2012).

Bacterial diversity in sediment samples from days -4, 24 and 59 was also investigated by 454-pyrosequencing (Roche Diagnostics, Germany). Amplicons from the 16S rRNA gene were generated by PCR using primers described in Table 1. Each forward primer was appended with the titanium sequencing adaptor A (Table 1) and an "NNNNNNNN" barcode sequence at the 5' end, where the barcode sequence of eight nucleotides was unique for each sample. The reverse primer carried the titanium adaptor B (Table 1) at the 5' end. PCR products were purified using the High Pure PCR Cleanup Micro Kit (Roche) and concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). Purified PCR products were then mixed in equimolar amounts with a final DNA concentration of 1 μg/μL. The pooled amplicons were pyrosequenced using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Germany).

Leaf material

Three fine mesh leaf bags were retrieved on days -4, 3, 10, 17, 31 and 52. Thirty 1-cm diameter leaf discs were cut per fine mesh bag, of which 15 discs were used for fungal identification and 15 discs for fungal biomass measurements. The remaining leaf material was used to determine leaf decomposition. Tebuconazole concentration of leaf material collected from treatment enclosures was determined for all sampling dates except day -4 (for details regarding tebuconazole measurements see supplementary information).

Two sets of five discs from each fine mesh bag were each placed in 30 mL glass bottles containing 10 mL sterile distilled water, and agitated on a shaker for 4 days to stimulate sporulation. Conidia of aquatic hyphomycetes were stained with lactophenol cotton

blue, identified using a microscopy and several identification keys (Gulis et al., 2005; Ingold, 1976; Nilsson, 1964). Total number of conidia per species was assigned to one of five abundance categories (i.e. conidia production score): 0 (conidia absent), 1 (1-3 conidia), 2 (4-9 conidia), 3 (10-15 conidia), 4 (> 15 conidia).

One set of five discs from each mesh bag was pooled and used for DNA extraction using the same procedure used for sediment samples. The remaining leaf material was oven-dried at 60 °C for 3 days and weighed to determine the leaf decomposition on the basis of dry-weight. Leaf-associated fungal and bacterial community structure and composition were analyzed by PCR-DGGE and 454-pyrosequencing, with three randomly selected control and treatment replicates being analyzed for each time period. Pyrosequencing and DGGE analysis were performed as described above and using primers and conditions listed in Table 1.

Fungal biomass on leaf material was determined using an ergosterol assay modified from Newell and Fell (1992), therefore, determining only the biomass of true fungi. Briefly, ergosterol was extracted in alkaline methanol and purified using pentane. The pentane extraction sample was evaporated under nitrogen, and the dried sample was then reconstituted in high performance liquid chromatography (HPLC) – grade methanol. Extracted samples were analyzed for ergosterol by HPLC and a UV detector set at 282 nm. Ergosterol was converted to biomass using a general conversion factor of 5.5 mg/g fungal dry mass (Gessner and Chauvet, 1993).

Table 1. Adaptors and primers used for targeting bacterial and fungal community present in sediment and leaf samples.

Primers	Sequence 5'– 3'	Target	Application	Cycle conditions	References
F968-GC	CGCCCGGGGCGCGCCCCGG GCGGGGCGGGGGCACGGGG GGAACGCGAAGAACCTTAC	Bacteria	DGGE	95 °C – 2min; 35 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C - 60 sec	Nubel et al. (1996)
1401R	CGGTGTGTACAAGACCC	Bacteria	DGGE		Nubel et al. (1996)
NS1	GTAGTCATATGCTTGTCTC	Fungi	DGGE	95 °C – 2min; 35 cycles of 95 °C – 30 sec, 55 °C – 45 sec, 72 °C – 60 sec	White et al. (1990)
GCfung	CGCCCGCCGCGCCCCGCGC CCGGCCCGCCGCCCCCGCC CCATTCCCCGTTACCCGTTG	Fungi	DGGE		May et al. (2001)
ITS3-GC	CGCCCGCCGCGCCCCGCGC CCGGCCCGCCGCCCCCGCC CCGCATCGATGAAGAACGCA GC	Fungi	DGGE	95 °C – 2min; 35 cycles of 95 °C – 30 sec, 55 °C – 45 sec, 72 °C – 60 sec	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	Fungi	DGGE		White et al. (1990)
Adaptor A	CCATCTCATCCCTGCGTGTCT CCGACTCAG	–	Sequencing	–	Provided by GATC-Biotech
Adaptor B	CCTATCCCCTGTGTGCCTTGG CAGTCTCAG	–	Sequencing	–	Provided by GATC-Biotech
27F-DegS	GTTYGATYMTGGCTCAG	Bacteria	Sequencing		van den Bogert et al. (2011)
338R-I	GCWGCCTCCCGTAGGAGT	Bacteria	Sequencing	95 °C – 2min; 30 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C – 60 sec	Daims et al. (1999)
338R-II	GCWGCCACCCGTAGGTGT	Bacteria	Sequencing		Daims et al. (1999)
FF390	CGATAACGAACGAGACCT	Fungi	Sequencing	95 °C – 2min; 30 cycles of 95 °C – 30 sec, 50 °C – 45 sec, 72 °C – 60 sec	Vainio and Hantula (2000)
FR1	AICCATTCAATCGGTAIT	Fungi	Sequencing		Vainio and Hantula (2000)

Leaf litter decomposition

At the end of the conditioning period, seven bags of each mesh type were used to quantify mass remaining from the conditioning period (i.e. 4 days prior to fungicide application). Alder leaf decomposition, expressed as dried mass loss of leaf material deployed in fine mesh bags (i.e. microbial decomposition) or coarse mesh bags (i.e. microbial decomposition plus invertebrate consumption), was calculated for a 56 day period (i.e. 4 days prior to application to 52 days post application). Some of the leaf material in the mesh bags sampled 52 days post application was used for fungal identification and biomass measurements (Section Leaf material). However, as all leaf material used for these procedures was quantified, the total mass of leaf material in each mesh bag could be calculated. Leaf litter decomposition was determined as the difference between leaf mass remaining after the conditioning period and leaf mass remaining 52 days post application.

Shredder feeding

Adult male *Gammarus pulex* (Crustacea, Amphipoda) were collected from Crags Stream Derbyshire, UK (National Grid Reference SK 497 745) and maintained and acclimatized to experimental conditions prior to use in feeding experiments. There were two treatments ('control' and 'tebuconazole') and five exposure periods (3, 10, 17, 31, 52 days), and feeding rate was determined for each treatment and exposure period combination. For each combination, a known mass of leaf material (L_1, initial leaf dry mass, mg) was added to each of 28 glass jars (60 mL), containing 45 mL of Artificial Pond Water (APW) (Naylor et al., 1989), and left to rehydrate for 3-5 days. Control treatment received an average (± SE) of 190 ± 7.3 mg, while tebuconazole treatment received and an average (± SE) of 212 ± 2.8 mg. One adult male gammarid was then added to each of 24 jars. The other 4 jars, which contained leaf material only, were used to control for mass loss due to processes other than invertebrate feeding. Jars were placed in a controlled temperature room at 15 °C with a 12 h light, 12 h dark cycle, and each jar was continuously aerated via a sterile 21 gauge hypodermic needle for 6 days. After a 6-day feeding period, all remaining leaf material was removed from each jar, rinsed with distilled water, and oven-dried at 60 °C for 3 days before being re-weighed (L_2, final leaf dry mass, mg). Animals were also rinsed, dried and weighed (W, animal dry mass, mg) and feeding rate (FR, mg leaf material/mg animal/d) calculated using Equation 1 (Maltby and Crane, 1994):

$$FR = \frac{(L_1 \times C_1) - L_2}{W \times 6} \qquad \text{Equation 1}$$

Where C_1 was the mean proportional leaf mass remaining after 6 days in the absence of invertebrate feeding, calculated using Equation 2

$$C_1 = \frac{\sum A_2/A_1}{N} \qquad \text{Equation 2}$$

Where A_1 and A_2 referred to initial and final mass of control leaves and N was the number of control jars.

Data analysis

The effects of tebuconazole exposure on conidia production, fungal biomass and leaf mass loss were analyzed using ANOVA, and two-sample t-tests were used to assess the difference in feeding rate between animals fed leaf material deployed in control or tebuconazole treated enclosures at each of the five sampling times.

DGGE band detection and quantification of band intensity were performed using the Bionumerics software version 4.61 (Applied Maths, Belgium) (Tzeneva et al., 2009). Effects on the bacterial and fungal communities, in the sediment as well as on leaf

material, were analyzed by the principle response curves (PRC) method (Van den Brink and Ter Braak, 1999), and significance was checked with Monte Carlo permutation test (499 permutations), using Canoco 4.5 (ter Braak and Šmilauer, 2002). PRC analyses were performed using relative band intensity (relative abundance) values obtained from DGGE analysis, which takes into account not only presence or absence of bands, but also their relative abundance. Prior to statistical analysis, DGGE data were Ln (ax+1) transformed, where x stands for the abundance value and a is 2 divided by the lowest positive abundance value in each data set (i.e. bacteria sediment, bacteria leaf material, fungi sediment, fungi leaf material). This transformation was performed to down-weigh high abundance values and approximate a normal distribution for the data (Van den Brink et al., 2000). Effects on the number of bacterial and fungal OTUs (i.e. bands) found on the DGGEs and relative abundance of taxa found with sequencing, were also analyzed by two-sample t-tests. For all statistical tests, statistical significance was accepted when $p \leq 0.05$.

Sequencing information was processed and sorted using default parameters in the QIIME pipeline version 1.6.0 (Caporaso et al., 2010). USEARCH version 5.2.236 (Edgar, 2010) was used to cluster high-quality sequences into operational taxonomic units (OTUs) at 97% sequence identity, whereas UCHIME (Edgar et al., 2011) was used for chimera removal. Taxonomy assignment of the rRNA sequences was done using the QIIME pipeline. RDP classifier (Wang et al., 2007) was used for the assignment of the 16S rRNA sequences, while BLAST (Altschul et al., 1990) was used for the assignment of the 18S rRNA sequences. Greengenes 12_10 release and Silva 108 release (compatible with QIIME) were used as reference databases for taxonomy assignments. The Silva 108 database was supplemented with 50 aquatic hyphomycete 18S rRNA gene sequences from the PHYMYCO database (Mahe et al., 2012) (Supplementary Information Table S1). Rarefaction curves were generated using the alpha_rarefaction.py workflow script available through QIIME. In order to avoid potential bias introduced by sampling depth, all samples were rarefied to an equal number of sequences.

Results

Treatment-related responses reported

The present paper has a focus on treatment-related responses of aquatic bacteria and fungi. However, the responses of phytoplankton and zooplankton populations were studied in detail as well. A summary of the treatment-related effects on phytoplankton and zooplankton is presented as Supporting Information. As expected, an exposure concentration reflecting the median "non-microbial" HC5 resulted in minor effects on a few taxa only (see supplementary information).

Exposure concentrations and environmental measurements in water

Two hours after the fungicide had been applied, the mean (± SE) tebuconazole concentration in water samples from treated enclosures was 300.03 ± 16.3 µg/L, which was 126% of the intended exposure concentration. Tebuconazole concentrations declined during the course of the study, but the fungicide was fairly persistent with the average concentration in treated enclosures being 58.45 ± 15.22 µg/L after 59 days, 19.5% of the initial measured concentration (Fig. S1A). In the treated enclosures, the mean water dissipation time 50% (DT50) from day 1 after treatment onwards was 32 days (range 15 – 39 days).

During the course of the study, the mean (± SE) conductivity in control enclosures was 130 ± 2.85 µS/cm, while in treated enclosures was 132 ± 3.53 µS/cm. Mean (± SE) pH was 7.9 ± 0.1 in control enclosures and 8.2 ± 0.1 in treated enclosures. Mean (± SE) temperature was the same for both treatment, 16 ± 0.5 °C. Mean (± SE) dissolved oxygen from control enclosures was 7.64 ± 0.4 mg/L and 7.55 ± 0.4 mg/L from treated enclosures. Mean (± SE) alkalinity value in control enclosures was 1.16 ± 0.03 mM and 1.18 ± 0.03 mM in treated enclosures (Fig. S2). Except for alkalinity measurements on day 17, no statistically significant treatment effect was detected for any of these variables. On day 17, alkalinity in the treated enclosures was slightly but significantly higher (1.31 mM) than in the control enclosures (1.17 mM). Aqueous nutrient concentrations were generally below detection limits in both the control and treated enclosures (i.e. nitrate/nitrite <0.03 mg/L; ammonium <0.04 mg/L; ortho-phosphate <0.02 mg/L; total phosphate <0.1 mg/L). The exception was total nitrogen concentration, which ranged from 0.5 to 1.0 mg/L (mean ± SE = 0.74 ± 0.07 mg/L) in control enclosures and from 0.6 to 1.1 mg/L (mean ± SE = 0.79 ± 0.07 mg/L) in treated enclosures. However, there was no treatment-related statistical difference in total nitrogen concentration.

Tebuconazole concentrations in Alnus leaf litter

Tebuconazole concentrations in the leaf material deployed in litter bags reached a mean level (± SE) of 82 ± 6.6 µg/g dry weight on day 3. The highest mean (± SE) tebuconazole concentration in leaf material was detected on day 10, 92 ± 4.6 µg/g dry weight. After day 10, tebuconazole concentrations slowly declined in leaf litter and on day 52 the mean (± SE) concentration was 64 ± 10 µg/g dry weight (Fig. S1B). In the treated enclosures the estimated mean leaf litter DT50 of tebuconazole from day 10 onwards was 90 days (range: 32 – 141 days).

Microbial Communities

Sediment

Analysis of the DGGE profiles obtained from sediment samples showed no significant effect of tebuconazole on the number of OTUs (i.e. bands) for either bacterial or fungal communities. The average number (± SE) of bacterial OTUs detected on DGGE profiles of sediment control samples was 40 ± 1, ranging from 37 to 42; and 41 ± 1, ranging from 36 to 43, on sediments samples from tebuconazole treated enclosures. For fungi, the average number (± SE) of fungal OTUs detected using ITS primers was 21 ± 1, ranging from 16 to 25 on control sediment samples, and 20 ± 1, ranging from 14 to 25 on sediment samples from tebuconazole treated enclosures. More fungal OTUs were detected using 18S rRNA primers for both control (31 – 61 OTUs, mean ± SE = 42 ± 2) and tebuconazole treated (23 – 51 OTUs, mean ± SE = 38 ± 2) sediment. PRC analysis of both bacterial and fungal DGGE data sets, which compares communities based on the number and relative abundance of taxa, did not detect significant differences between control and treated enclosures, indicating that tebuconazole had no detectable impact on the microbial communities associated with sediment.

Regarding bacteria a total of 90565 16S rRNA gene sequences with an acceptable quality were obtained with an average (± SE) of 3775 ± 60 reads per sample, being 3280 reads the lowest and 4308 reads the highest number (average read length = 300 base pairs). Based on 97% sequence similarity as threshold, a total of 2735 OTUs was found. Rarefaction curves generated using QIIME showed that diversity of bacterial communities present in the sediment was not fully captured, since curves did not present a plateau. Sequence analysis of the sediment-associated bacterial 16S rRNA gene pool revealed that Proteobacteria was the major bacteria phylum present in the sediment (Fig. 1, Fig. S4), with Betaproteobacteria, Deltaproteobacteria and Alphaproteobacteria being the most abundant classes (Fig. S5). At day 24, some bacterial phyla had a slightly different relative abundance when control and treated units were compared (i.e. Cyanobacteria; Fig. 1). However, because this difference was not consistent in all replicates (Fig. S4), a two-sample t-test was used to determine whether the treatment

significantly affected any specific phylum. No significant differences between control and treated enclosures were found, indicating that the variance was likely due to factor(s) other than the tebuconazole treatment. No sequencing data were obtained for the fungal community associated with sediment.

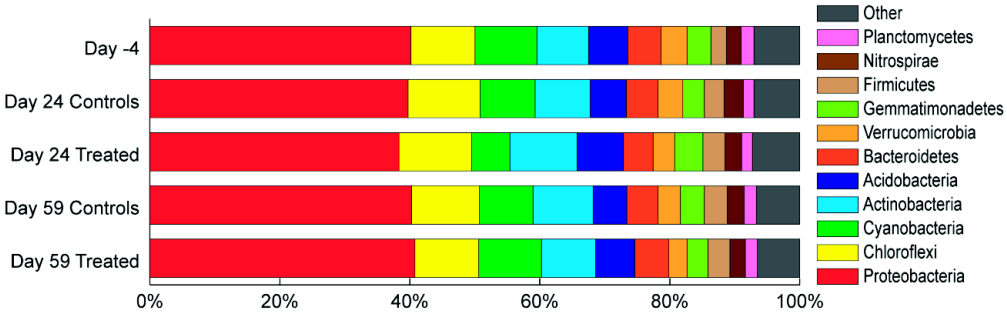

Figure 1. Relative abundance of bacterial phyla detected in sediment samples based on 454 pyrosequencing of 16S rRNA gene fragments. Data from all enclosures were pooled on day -4, on days 24 and 59 data from individual enclosures were pooled according to treatment. All phyla contributing to less than 1% of the total bacteria were grouped as 'Other'. See Figure S2 for profiles of individual microcosms.

Leaf Litter

Fungi

No significant treatment effect was found on fungal biomass present on leaf material, mean value (± SE) in the control was 2.7 ± 0.1 µg/mg dry mass leaf and from 2.9 ± 0.3 µg/mg dry mass leaf in the tebuconazole treatment over the 52 days exposure period. Analysis of the number of fungal OTUs (bands) present on the DGGE profiles indicated that tebuconazole had a significant effect on fungal community on days 17 and 31 after treatment. A two-sample t-test showed that treated enclosures had a significantly decreased number of fungal OTUs compared to control units on day 17 (only for primer set targeting the 18S rRNA gene) and on day 31 (for both primer sets) after the treatment. The total number of fungal OTUs detected depended on the primer set used. For leaf material from control enclosures, the average number (± SE) of OTUs was 7 ± 1, varying from 3 to 7, when the 18S rRNA gene was the amplification target; and 23 ± 1, ranging from 19 to 27 when the ITS region was the target. For leaf material from tebuconazole treated enclosures the average number (± SE) of OTUs was 5 ± 1, varying from 2 to 9; and 25 ± 1, ranging from 23 to 28, for the 18S rRNA gene and ITS region, respectively. PRC analysis of the fungal 18S rRNA gene based DGGE data, indicated that tebuconazole had a significant effect on fungal community structure, but that recovery had occurred by Day 52 (Fig. 2A). Analysis of the ITS region showed a similar pattern, albeit with only borderline significance ($p = 0.06$; Fig. 2B).

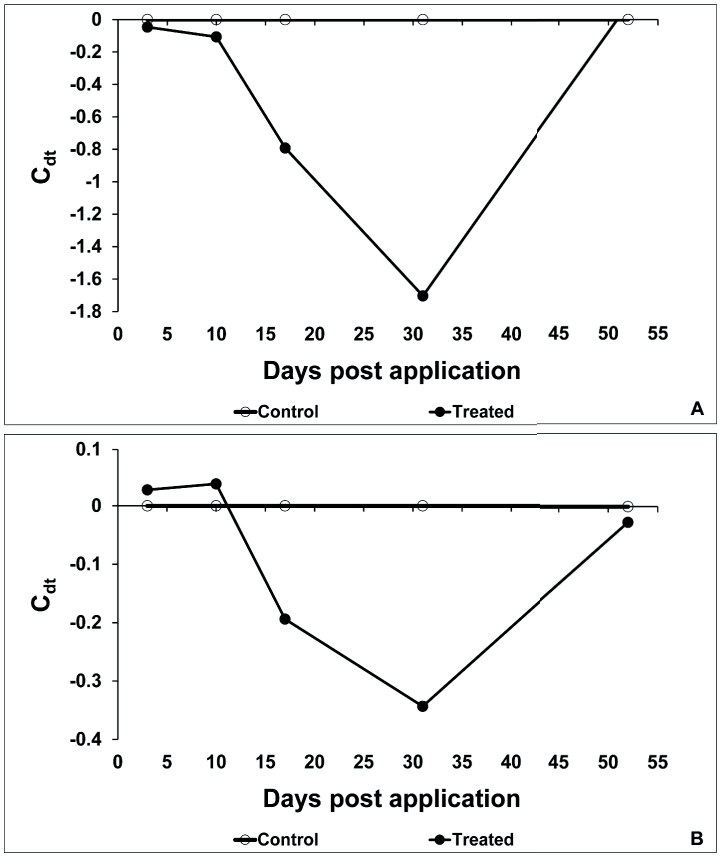

Figure 2. Principal Response Curve diagram of the fungal community structure on leaf litter. Diagrams are based on DGGE profiles of PCR amplified 18S rRNA gene (A) and ITS region (B). A 44% of all variance could be attributed to sampling date and 13% to treatment level, 49% of which is displayed on the vertical axis. B 25% of all variance could be attributed to sampling date and 13% to treatment level, 37% of which is displayed on the vertical axis. C_{dt} = Canonical coefficient showing the difference between treatments and control over time.

A total of 215361 18S rRNA gene sequences with an acceptable quality were obtained with an average (± SE) of 7179 ± 317 reads per sample, being 5019 reads the lowest and 12685 reads the highest number (average read length = 320 base pairs). Based on 97% sequence similarity as threshold, a total of 715 OTUs was found. Based on 18S rRNA gene sequencing, the fungal community on leaf material was dominated by taxa belonging mainly to the Chitridiomycota and Ascomycota phyla (Fig. 3, Table S2), and the dominant genera were *Nowakowskiella*, *Cladochytrium*, *Anguillospora* and *Pestalotiopsis*. *Tetracladium* was also found on leaf material from both control and treated enclosures, but it had a low relative abundance (0.2% of the total fungi in average). Sequencing data indicated that the fungal community on tebuconazole-exposed leaf material exhibited

an increase in the relative abundance of *Anguillospora* and a decrease in the relative abundance of *Pestalotiopsis* over the duration of the experiment (Fig. 3).

In terms of conidia production, *Anguillospora longissima* and *Tetracladium setigerum* were the dominant aquatic hyphomycete species colonising leaf material irrespective of treatment, however, conidia production by *Anguillospora longissima* was significantly reduced by exposure to tebuconazole at all-time points (Fig. 4). The abundance of *Tetracladium setigerum* conidia was not affected by tebuconazole exposure (Fig. 4).

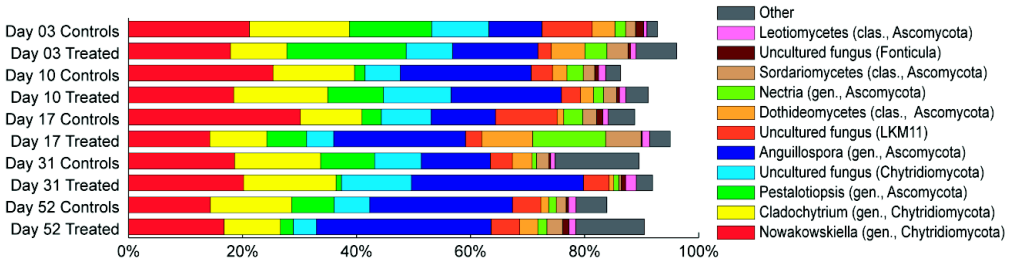

Figure 3. Relative abundance of fungal taxa detected on Alnus leaf material. Data from individual enclosures were pooled according treatment. All taxa contributing to less than 1% of the total fungi were grouped together and called 'Other'.

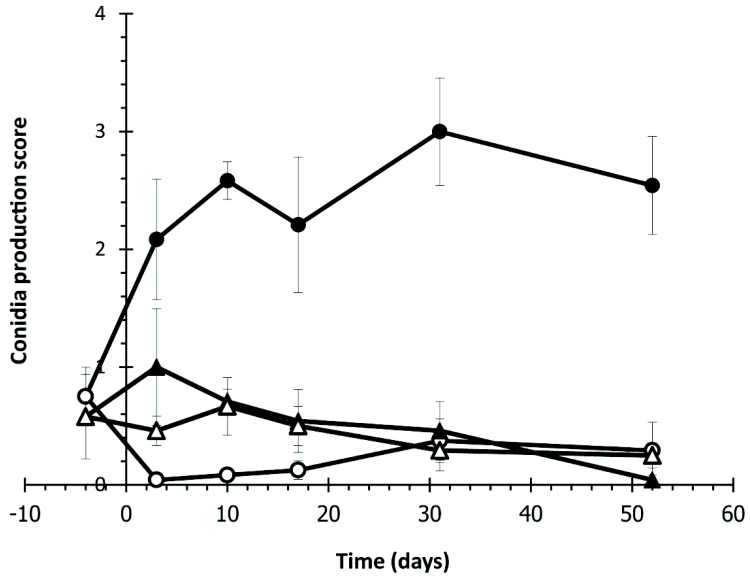

Figure 4. Mean (± SE) conidia production scores for Anguillospora longissima (circles, solid line) and Tetracladium setigerum (triangles, dashed line) on leaf material from control enclosures (black symbols) or enclosures treated with tebuconazole (white symbols).

Bacteria

A total of 133438 16S rRNA gene sequences with an acceptable quality were obtained with an average (± SE) of 4448 ± 148 reads per sample, being 3085 reads the lowest and 6032 reads the highest number (average read length = 300 base pairs). Based on 97% sequence similarity as threshold, a total of 1871 OTUs was found. As with sediment, 16S rRNA gene sequence analysis revealed Proteobacteria as the dominant bacterial phylum from leaf material (Fig. 5), however, its relative abundance was greater on leaf material (63 - 75%) than in sediment (39 - 41%). Alphaproteobacteria was the most abundant class, followed by Betaproteobacteria and Deltaproteobacteria (Fig. S6). Sediment showed a higher diversity of bacterial phyla when compared to leaf litter. In total 40 different phyla were identified from sediment samples, while 30 were identified from leaf litter. However, the most abundant phyla in both control and treated enclosures were the same.

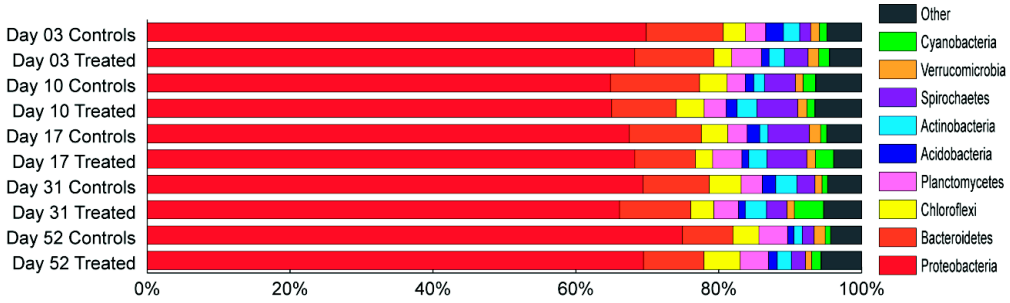

Figure 5. Relative abundance of bacterial phyla detected on Alnus leaf material. Data from individual enclosures were pooled according treatment. All phyla contributing to less than 1% of the total bacteria were grouped together as 'Other'.

Rarefaction curves generated using leaf litter sequencing data showed that either bacterial or fungal communities diversity were not fully obtained here (Fig. S3). Curves did not present a plateau in either case, however, complete fungal diversity was closer to be obtained than bacterial. While bacterial and fungal diversity were not entirely captured, predominance of a few bacterial and fungal taxa was realized (Fig.1, Fig.3, and Fig.5).

Leaf litter decomposition

Leaf mass loss (g dry mass) was significantly greater from coarse mesh than fine mesh bags, but there was no siginificant effect of treatment on leaf mass loss. By the end of the study (day 52), mean (± SE) total mass loss was 1.43 ± 0.06 g of which approximately 40 % (0.56 ± 0.03 g) was due to microbial decomposition.

Shredder feeding

The mean (± SE) feeding rate of *Gammarus pulex* offered leaves previously deployed in the control enclosures was 0.47 ± 0.04 mg/mg dried animal/day and 0.37 ± 0.02 mg/mg dried animal/day for animals offered leaves previously deployed in the tebuconazole-treated enclosures. Animals fed tebuconazole-exposed leaves had consistently lower feeding rates than those fed with non-exposed leaf material (Fig. 6). This treatment effect increased with exposure time and was statistically significant from 17 days exposure to the end of the experiment at 52 days.

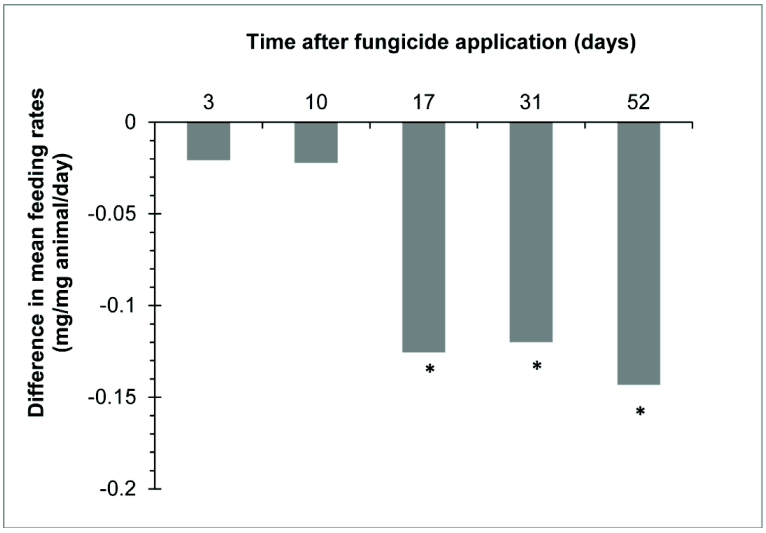

Figure 6. Differences in mean feeding rates (treatment minus control) of G. pulex fed leaves previously deployed in tebuconazole treated or control enclosures. Asterisks denote a significant difference in feeding rate between control and treatment.

Discussion

The aim of this study was to evaluate the extent to which a fungicide concentration resembling its acute "non-microbial" HC5 (established using acute toxicity data for aquatic animals and plants, including algae), is protective of heterotrophic microbial community structure and functioning. We exposed microbial communities to 238 µg tebuconazole/L (i.e. the "non-microbial" HC5) in outdoor enclosures containing macrophyte, algal and invertebrate communities representative of Dutch ditches. Based on laboratory studies, tebuconazole is expected to be relatively persistent in water (degradation DT50: 365 days for test system; 42.6 days for water phase (FOOTPRINT, 2013) and to adsorb to organic material, including leaf material (log K_{ow}: 3.7) (Kahle et al., 2008). The mean dissipation DT50 of tebuconazole in study enclosures was 32 days for the water phase and 90 days for leaf material. It seems plausible to assume that the freely dissolved tebuconazole fraction in water is bioavailable to microbes. To what extend the fraction of tebuconazole present in organic matter and plant litter is directly bioavailable to associated bacteria and fungi remains an important research question.

A greater number of bacterial phyla were associated with sediment than with leaf material. The total number of bacterial phyla detected by sequencing was 43 for sediment and 30 for leaf material, and all bacterial communities were dominated by Proteobacteria; dominant classes being Alphaproteobacteria, Betaproteobacteria and Deltaproteobacteria. High abundance of Proteobacteria, associated with freshwater sediments, has already been found in previous studies (Besemer et al., 2012; Spring et al., 2000; Tamaki et al., 2005). The relative abundance of Proteobacteria was 39 - 41% on sediment and 64 - 73% on leaf material. This dominance of Proteobacteria is in line with data presented by McNamara and Leff (2004) who found that Proteobacteria accounted for 30-60% of total bacteria present in biofilms obtained from maple leaves. Hutalle-Schmelzer et al. (2010) reported that after the introduction of leaf litter in limnetic systems Proteobacteria increased, suggesting that they are able to grow on, and process, recalcitrant compounds originating from leaf litter.

The maximum number of fungal OTUs detected by DGGE, targeting the 18S rRNA gene, was 61 for sediment and 9 for leaf material. Such difference might be explained by the higher eukaryotic diversity in the sediment, since finding a primer set that covers the full breadth of the fungi kingdom while excluding non-fungal sequences is a very difficult, if not impossible task (Anderson and Cairney, 2004; Anderson et al., 2003). When the ITS region was targeted, the maximum number of OTUs was 25 for sediment samples and 28 for leaf material. The sediment fungal community was not sequenced. However, sequencing of the fungal community associated with leaf material indicated that the dominant groups were Chytridiomycota (zoosporic fungi) and Ascomycota (includes hyphomycetes) and that the dominant genera were *Anguillospora*, *Cladochytrium*,

Nowakowskiella and *Pestalotiopsis*. Previous studies have also detected a high abundance of the zoosporic fungi (e.g. *Cladochytrium*, *Nowakowskiella*) associated with decomposing leaves (Marano et al., 2011), indicating the potential importance of these fungal groups in leaf decomposition. Most studies of leaf decomposition have focused on the role of aquatic hyphomycetes. Only four genera of aquatic hyphomycetes were detected by sequencing: *Anguillospora*, *Tetrachaetum*, *Tumularia* and *Tetracladium* (Table S2).

There was no evidence of a significant effect of tebuconazole treatment on the structure of the bacterial community associated with sediment or leaf material. We are not aware of other studies that have addressed the impact of tebuconazole on sediment bacteria, but the effect of tebuconazole on soil bacteria has been studied. However, the results of these studies are inconsistent: using the same concentration of tebuconazole, Bending et al. (2007) found no effect on soil bacterial biomass or community structure, whereas Muñoz-Leoz et al. (2011) reported a decrease in soil microbial biomass and activity. A study with the fungicide metiram (108 and 324 μg/L), which used the same outdoor system as used here for tebuconazole, did not detect any consistent treatment-related effects on the sediment bacterial community (Lin et al., 2012). Widenfalk et al. (2008) found that captan, applied to a freshwater sediment at environmentally relevant Maximum Permissible Concentrations (1.3 μg/kg dw) (Crommentuijn et al., 2000) did not affect microbial activity or biomass, but did result in significant shifts in the microbial community; suggesting the presence of functional redundancy in the captan-stressed microbial community. Milenkovski et al. (2010) investigated the toxicity of eight fungicides, including captan, to a bacterial community isolated from a wetland; toxic effects only being seen at fungicide concentrations much higher than would normally would occur in the aquatic environment.

Several studies have investigated the effects of tebuconazole on bacteria associated with decomposing leaf material. Under laboratory conditions, bacterial cell numbers on conditioned leaves were not affected when exposed to 65 μg tebuconazole/L (Zubrod et al., 2011) but were significantly decreased when exposed to 500 μg tebuconazole/L (Bundschuh et al., 2011). Artigas et al. (2012) analyzed the effects of a chronic tebuconazole exposure (6 weekly pulses of 20 μg/L) in experimental laboratory channels and found that bacterial biomass temporally decreased on conditioned *Alnus* and *Populus* leaves, followed by full recovery. Furthermore, using molecular techniques they demonstrated effects of repeated tebuconazole application on bacterial community structure, particularly on decomposing *Populus* leaf material. This contrasts with our results, since we did not find treatment-related effects. Major differences between our study and Artigas et al. (2012) are exposure regime, flow regime and the source communities used. We colonised leaves in static ditches and dosed standing water systems once with 238 μg tebuconazole/L. Artigas et al. (2012) colonised leaves in a

stream and dosed flowing water systems six times with 20 µg/L at weekly intervals. The persistent nature of tebuconazole in our study resulted in an average water concentration of 58 µg/L after 59 days, suggesting that the difference in effect is not due to exposure concentration. It is highly probable that the composition of the microbial communities on leaf material differed between the two studies and therefore the difference could be due to the greater sensitivities of stream bacterial communities to tebuconazole exposure. However, comparison between microbial communities described here and those found by Artigas et al. (2012) is not possible, since molecular techniques used in the Artigas et al. (2012) study did not include sequencing analysis, and thus did not provide information on the identity of bacteria.

Tebuconazole had no effect on the fungal community in sediment, but did affect the fungal community associated with leaf material. We are not aware of other studies that have addressed the impact of tebuconazole on sediment fungal communities, although there have been studies on soil organisms, individual fungal species and fungal communities on leaves. Tebuconazole (5 mg/kg) had a limited effect on the eukaryotic soil community (Bending et al., 2007) and four of the seven fungal species exposed to tebuconazole in single-species laboratory tests had NOEC values below 238 µg tebuconazole/L (Dijksterhuis et al., 2011). Although none of these four species were found in the present study, above data indicate that we could have expected effects on fungi in our enclosure experiment. One possible explanation for why we could not demonstrate such an effect might be that exposure conditions in the laboratory tests did not reflect exposure conditions in the upper sediment layer of our field test systems, where the bioavailable fraction of tebuconazole may be lower. Another reason for the less sensitive response might be that the fungi present in the sediment of our field enclosures were genetically and/or physiologically more tolerant to tebuconazole-stress than the isolates used in the laboratory experiment.

Tebuconazole exposure resulted in a change in the total number of OTUs and relative abundance of fungi on leaf material, as well as sporulation, but no change in biomass. Previous studies have reported a reduction in fungal biomass when leaf material, which had been colonized by using pre-conditioned leaves in streams, is exposed to tebuconazole (Artigas et al., 2012; Bundschuh et al., 2011; Zubrod et al., 2011) suggesting that stream fungal communities may be more sensitive to tebuconazole than ditch fungal communities. In the present study, fungal biomass on leaf material was lower than that reported for leaf material in streams (i.e. 2.5 – 3.1 µg fungus/mg dry leaf mass compared to 21 - 104 µg fungus/mg dry leaf mass) (Gessner and Chauvet, 1993; Sridhar et al., 2009). Baldy et al. (2002) compared fungal biomass on leaf litter between a pond and a river and reported that the fungal biomass associated with decomposing leaves in the river was approximately 2.5 - 4.0 times larger than in the

pond. Furthermore, sequencing results showed that Chytridiomycota was dominant in the leaf material, which is a group of fungi that does not contain ergosterol (Gessner et al., 1997). Therefore, part of the fungal community present on the leaf material could not have been quantified by the methodology used here. The relatively low fungal biomass on the leaf material, coupled with a difference in community composition, may explain the absence of treatment-related effects on fungal biomass in our study.

Sequencing data demonstrated an increase in the relative abundance of *Anguillospora* and a decrease in the relative abundance of *Pestalotiopsis* on alder leaf material exposed to tebuconazole. The production of conidia by *Anguillospora longissima* was reduced by tebuconazole exposure, indicating that sporulation was sensitive to this fungicide. This is consistent with previous studies that have demonstrated a significant inhibition of fungal sporulation in the presence of tebuconazole (65 µg/L) (Zubrod et al., 2011) and other stressors, including heavy metals (Bermingham et al., 1996b; Duarte et al., 2009; Lecerf and Chauvet, 2008). In contrast, eutrophication had a positive effect on conidia production (Lecerf and Chauvet, 2008), and Lin et al. (2012) could not demonstrate treatment-related effects of exposure to the fungicide metiram (up to 324 µg/L) on conidia production. Lecerf and Chauvet (2008) have argued that of a suite of measures of fungal performance (i.e. microbial leaf decomposition, conidia production, fungal biomass and species richness), conidia production was the most sensitive indicator of anthropogenic stress in streams.

Fungal community composition and abundance are important for microbially-mediated leaf decomposition and the utilization of leaf material by shredder invertebrates (Barlocher, 1985; Maltby, 1992a, b; Suberkropp and Arsuffi, 1984). Despite a change in fungal community structure, there was no evidence of an effect of tebuconazole on microbial decomposition, but there was a significant effect on shredder feeding rate. *Gammarus pulex* feeding was significantly reduced when fed leaf material that had been exposed to tebuconazole for at least 17 days. This finding is in line with a study of Bundschuh et al. (2011) that reported that *G. fossarum* significantly preferred leaf material that had not been exposed to tebuconazole over leaf material previously exposed to concentrations of either 50 or 500 µg/L tebuconazole. Zubrod et al. (2010) also reported a significant reduction in the feeding rate of *G. fossarum* when offered leaf material previously exposed to a tebuconazole concentration of 600 µg/L. Reduction in feeding rate of *G. pulex* could be due to either a toxic effect of tebuconazole accumulated in leaf material, a reduction in food quality through tebuconazole-induced changes in microbial composition or due to a toxic effect of the tebuconazole in the water phase. *Alnus* leaf material did accumulate tebuconazole (mean values 64-82 µg/g) and it is known that *G. pulex* exhibits preferences for particular species of aquatic hyphomycete (Arsuffi and Suberkropp, 1989; Graca et al., 1994). Tebuconazole

exposure did alter fungal community composition, with the maximum effect being detected after 31 days exposure, followed by recovery. However, it is unclear how the changes detected in community structure would alter shredder feeding preferences. Interestingly, tebuconazole-induced reductions in feeding rate observed in laboratory studies did not translate into reduction in invertebrate-mediated leaf decomposition in the microcosms. A probable explanation for that might be the low density of shredders in the experimental units, relative to the total amount of leaf material present.

In conclusion, tebuconazole at its "non-microbial" HC5 concentration (238 μg/L) derived from acute toxicity tests with fish, invertebrates and primary producers, had no effect on fungal biomass, leaf decomposition or bacterial communities associated with either sediment or leaf material in microcosms. Tebuconazole exposure did, however, reduce conidia production by a dominant aquatic hyphomycete species and alter the fungal community associated with *Alnus* leaf material; although the effects on the fungal community were short-term and recovery occurred by 52 days post-application. The feeding rate of *Gammarus pulex* fed leaf material exposed to tebuconazole for at least 17 days was also significantly reduced. Despite tebuconazole application have caused a limited ecological impact, such impact should not be ignored; instead, should alarm for the necessity of further research. Consequently, regulatory risk assessment of azole fungicides in Europe under Regulation 1107/2009/EC, which requires toxicity data for a standard set of test species including a vertebrate (fish), invertebrate (crustacean) and primary producer (algae) only, may not be adequate to avoid effects on the structure and function of microbial communities in freshwater ecosystems. This may be also true for stream (lotic) ecosystems, where although duration of exposure may be reduced, fungi play a more prominent role in decomposition and the microbial community may be more sensitive to fungicide exposure.

Acknowledgements

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Supplementary information

Table S1. Aquatic hyphomycete taxa added to the Silva 108 release database. Sequences were retrieved from the PHYMYCO database (Mahe et al., 2012).

GenBank accession number	Taxa
AY204598.2	*Anguillospora longissima* strain ccm-F00980
AY204599.1	*Anguillospora longissima* strain ccm-F11891
AY204600.1	*Anguillospora longissima* strain CBL22
AY178822.1	*Anguillospora crassa* strain F-05584
AY178823.1	*Anguillospora crassa* strain CCM F-15283
AY204601.1	*Anguillospora furtiva*
AY178824.1	*Anguillospora filiformis* strain CCM F-19787
AY178825.1	*Anguillospora filiformis* strain CCM F-20687
AY357268.1	*Articulospora tetracladia* strain Boss Brook 2
AY357269.1	*Articulospora tetracladia* strain 104-300
AY357271.1	*Articulospora tetracladia* strain UMB 22.01
FJ804122.1	*Clavariopsis aquatica* strain WD(A)-00-1
AY357279.1	*Lunulospora curvula* strain 94-228
AY204605.1	*Lemonniera aquatica*
AY357280.1	*Tetrachaetum elegans* strain 30-426
AY357281.1	*Tetrachaetum elegans* strain 105-326
AY204629.1	*Tricladium splendens* strain ccm-F11989
AY204630.1	*Tricladium splendens* strain ccm-F12386
AY204631.1	*Tricladium splendens* strain ccm-F16599
AF388576.1	*Tetracladium marchalianum*
AY204613.1	*Tetracladium marchalianum* strain ccm-F19399
AY204614.1	*Tetracladium marchalianum* strain ccm-F26199
AY204615.1	*Tetracladium marchalianum* strain ccm-F26299
AY204616.1	*Tetracladium marchalianum* strain ccm-F26399
AY204617.1	*Tetracladium marchalianum* strain CB-ELBE90
AY204618.1	*Tetracladium marchalianum* strain CBELBE50
AY204619.1	*Tetracladium marchalianum* strain CBL27
AF388578.1	*Tetracladium furcatum*
AY357287.1	*Tumularia aquatica* strain CCM F-02081
AY357273.1	*Dimorphospora foliicola* strain GWM-07-9
AY357274.1	*Dimorphospora foliicola* strain UMB 172.01
AY357276.1	*Geniculospora grandis* strain UMB 198.01
AF388575.1	*Tetracladium apiense*
AY204603.1	*Nectria lugdunensis* strain ccm-F245
AY204604.1	*Nectria lugdunensis* strain CBE98
AY204586.1	*Alatospora acuminata strain CBL8*
AB072234.1	*Bullera taiwanensis*
DQ645513.1	*Cryptococcus gastricus* isolate AFTOL-ID 1887
AB075546.1	*Filobasidium globisporum*
DQ419918.1	*Kriegeria eriophori isolate* AFTOL-ID 886
AB586076.1	*Powellomyces sp.* NBRC 105427
AB021670.1	*Sporobolomyces falcatus*
AB000956.1	*Taphrina pruni*
AB085808.1	*Trimorphomyces papilionaceus*
AB126645.1	*Rhodotorula marina*
FJ517752.1	*Rhodotorula sp.*
AF346553	*Pestalotiopsis jester*
AY275185.2	*Sirococcus conigenus*
FJ176844.1	*Pleosporales sp.* CBS 536.93
FJ716243.1	*Hypocrea sp.*

Table S2. Relative abundance (%) of all fungal taxa found on the leaf litter samples. Replicates were combined for every sampling date and treatment.

Fungal taxa	Day 03 Controls	Day 03 Treated	Day 10 Controls	Day 10 Treated	Day 17 Controls	Day 17 Treated	Day 31 Controls	Day 31 Treated	Day 52 Controls	Day 52 Treated
Nowakowskiella (gen., Chytridiomycota)	21.220	17.899	25.352	18.459	30.128	14.268	18.600	20.184	14.356	16.727
Cladochytrium (gen., Chytridiomycota)	17.552	9.870	14.272	16.476	10.806	9.979	15.080	16.218	14.276	9.905
Pestalotiopsis (gen., Ascomycota)	14.407	20.936	1.837	9.789	3.410	6.998	9.479	0.956	7.418	2.291
Uncultured fungus (Chytridiomycota)	10.115	8.250	6.547	11.990	9.202	4.956	15.111	12.969	7.920	5.033
Anguillospora (gen., Ascomycota)	9.378	14.958	22.916	19.376	11.266	23.134	12.144	30.192	25.029	30.644
Uncultured fungus (LKM11)	8.765	2.300	3.747	3.302	10.802	2.824	3.838	4.501	4.967	4.959
Dothideomycetes (clas., Ascomycota)	4.028	5.955	2.539	2.308	1.164	8.924	3.464	0.797	1.382	3.225
Nectria (gen., Ascomycota)	1.834	3.859	2.858	1.752	3.326	12.816	0.784	0.911	1.331	1.587
Sordariomycetes (clas., Ascomycota)	1.761	3.740	2.014	2.302	2.443	6.216	2.183	0.441	1.742	2.746
Uncultured fungus (Fonticula)	1.440	0.380	0.678	0.491	1.070	0.210	0.292	0.748	0.404	1.127
Leotiomycetes (clas., Ascomycota)	0.546	0.980	1.238	1.107	0.972	1.274	0.774	1.842	1.304	1.159
Monoblepharis (gen., Chytridiomycota)	0.512	0.079	0.260	0.041	1.074	0.223	0.384	0.015	0.590	0.032
Agaricomycetes (clas., Basidiomycota)	0.264	4.561	0.034	0.953	0.660	0.505	8.019	0.550	1.169	8.718
Tetrachaetum (gen., Ascomycota)	0.248	1.162	0.246	0.331	0.218	1.122	0.364	0.149	0.344	0.322
Zoophagus (gen., Zygomycota)	0.208	0.221	0.142	0.083	0.196	0.067	0.102	0.030	0.024	0.032
Tumularia (gen., Ascomycota)	0.073	0.096	0.133	0.160	0.058	0.094	0.051	0.149	0.111	0.129
Lecanoromycetes (clas., Ascomycota)	0.068	0.034	0.010	0.006	0.027	0.054	0.000	0.015	0.030	0.023
Uncultured fungus (LKM15)	0.107	0.164	0.840	1.852	1.511	0.282	0.548	0.441	0.553	0.823
Blastocladiales (ord., Blastocladiomycota)	0.051	0.085	0.020	0.030	0.067	0.036	0.184	0.064	0.054	0.032
Hypocrea (gen., Ascomycota)	0.039	0.108	0.187	0.030	0.143	0.617	0.077	0.030	0.034	0.069
Pezizomycetes (clas., Ascomycota)	0.039	0.023	0.034	0.030	0.009	0.009	0.000	0.025	0.007	0.005
Eurotiomycetes (clas., Ascomycota)	0.023	0.017	0.010	0.077	0.004	0.004	0.036	0.154	0.030	0.005
Uncultured fungus (Fungi)	0.028	0.006	0.103	0.036	0.036	0.009	0.102	0.035	0.165	0.124
Rozella (gen., Chytridiomycota)	0.023	0.023	0.010	0.047	0.009	0.273	0.246	0.401	0.222	0.488
Uncultured fungus (LKM11)	0.028	0.085	0.034	0.041	0.067	0.080	0.236	0.099	0.047	0.198
Tetracladium (gen., Ascomycota)	0.011	0.028	0.005	0.000	0.156	0.000	0.067	0.050	0.570	0.092
Taphrinomycetes (clas., Ascomycota)	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.005
Hyaloraphidium (gen., Blastocladiomycota)	0.011	0.000	0.000	0.000	0.004	0.000	0.010	0.000	0.000	0.009
Catenaria (gen., Blastocladiomycota)	0.006	0.011	0.025	0.000	0.000	0.000	0.005	0.010	0.017	0.023
Mortierellales (ord., Zygomycota)	0.006	0.000	0.010	0.000	0.000	0.000	0.005	0.000	0.024	0.005
Uncultured Zygomycota (Zygomycota)	0.006	0.278	0.020	0.024	0.000	0.063	0.518	0.064	0.310	0.124
Pleosporales (gen., Ascomycota)	0.000	0.062	0.005	0.000	0.000	0.022	0.000	0.000	0.000	0.014
Uncultured fungi (Blastocladiomycota)	0.000	0.000	0.025	0.006	0.000	0.004	0.000	0.030	0.000	0.028
Rhizidium (gen., Chytridiomycota)	0.000	0.000	0.103	0.041	0.103	0.000	0.138	0.005	0.027	0.041
Kickxellaceae (fam., Zygomycetes)	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.005	0.013	0.009
Tremellomycetes (clas., Basidiomycota)	0.000	0.000	0.015	0.000	0.004	0.000	0.020	0.005	0.067	0.014
Entorrhizomycetes (clas., Basidiomycota)	0.000	0.023	0.020	0.065	0.013	0.027	0.010	0.040	0.121	0.037
Pucciniomycetes (clas., Basidiomycota)	0.000	0.006	0.083	0.006	0.009	0.004	0.031	0.020	0.024	0.014

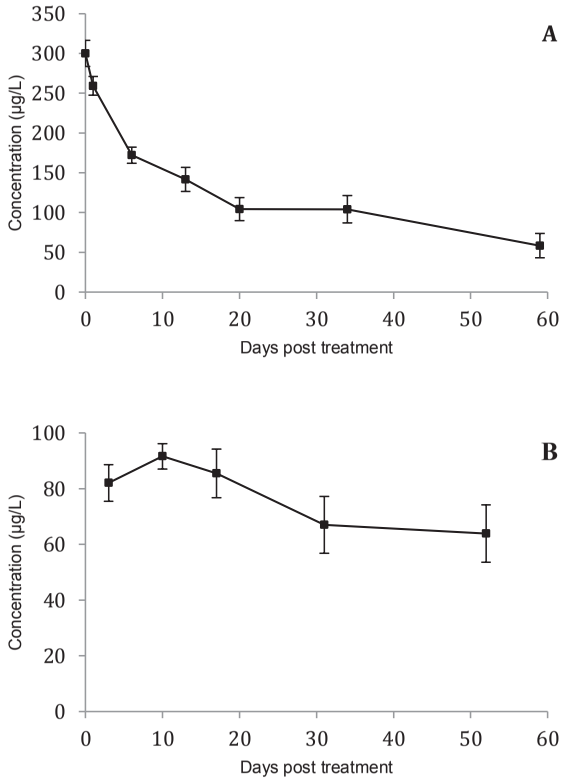


Figure S1. Variation in mean (±SE) of the tebuconazole concentration over time in water phase (A) and leaf litter (B). Average values of all treated microcosms are shown (n=4).

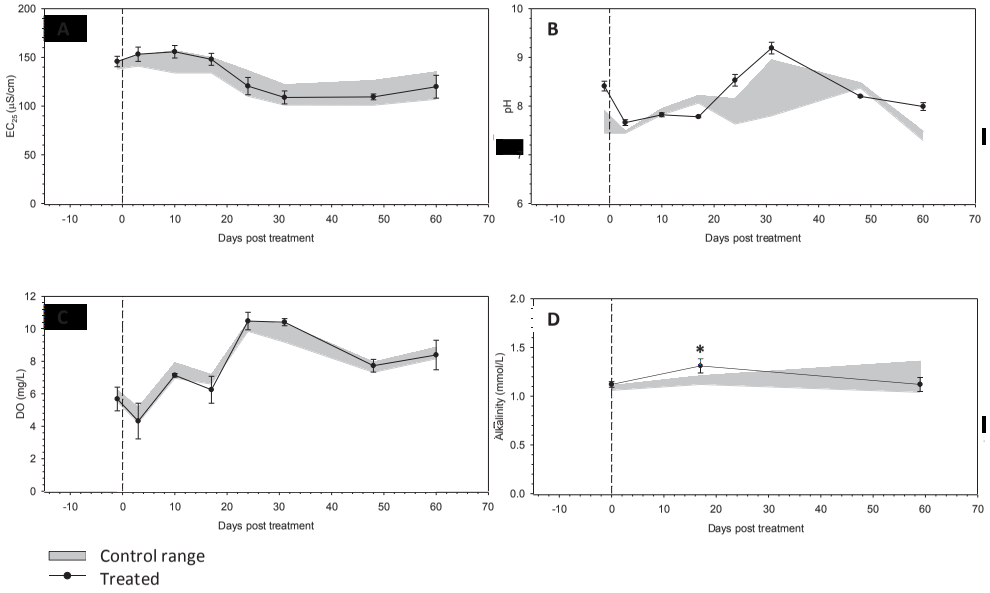
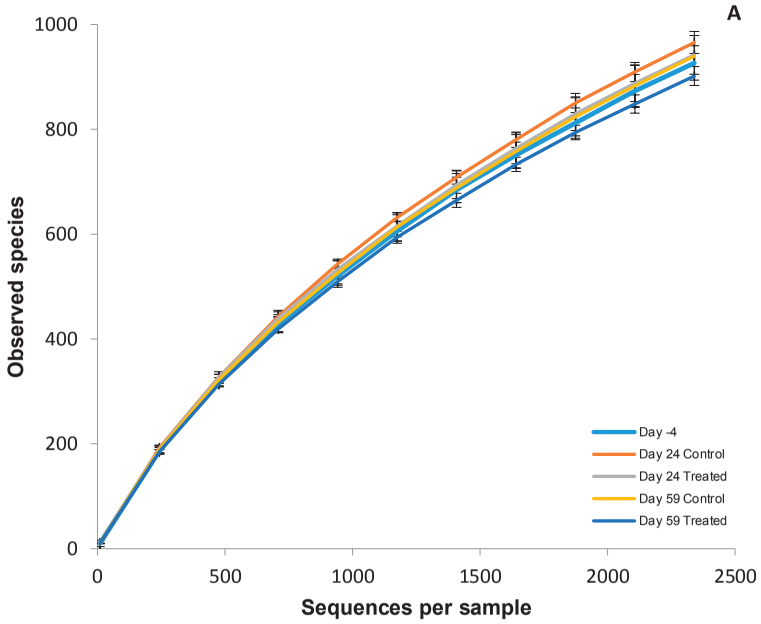
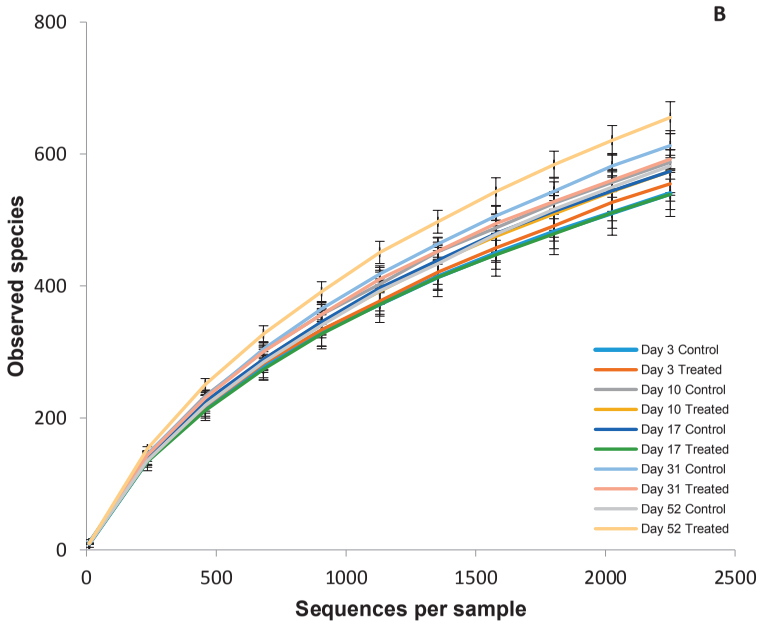


Figure S2. Variation in mean (± SD) conductivity (A), pH (B), dissolved oxygen (C) and alkalinity (D) in tebuconazole treated enclosures. Range of conditions in control enclosures are indicated by the shaded area. The vertical broken line indicated when the fungicide was applied and the asterisk denotes a significant difference between control and treated enclosures.


A
1000
800
600
400
200
0
Observed species
0
500
1000
1500
2000
2500
Sequences per sample
Day -4
Day 24 Control
Day 24 Treated
Day 59 Control
Day 59 Treated


B
800
600
400
200
0
Observed species
0
500
1000
1500
2000
2500
Sequences per sample
Day 3 Control
Day 3 Treated
Day 10 Control
Day 10 Treated
Day 17 Control
Day 17 Treated
Day 31 Control
Day 31 Treated
Day 52 Control
Day 52 Treated

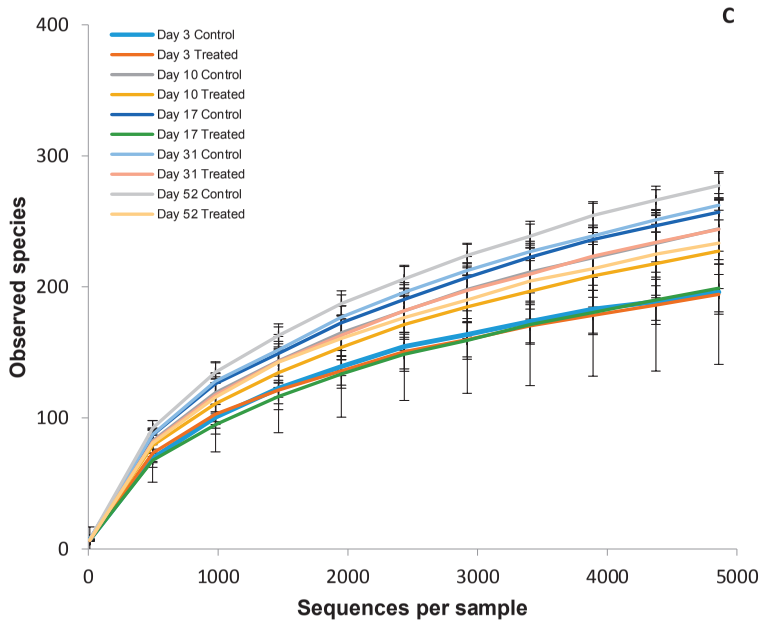


Figure S3. Rarefaction curves of observed bacterial species in the sediment (A), bacterial species on the leaf material (B) and fungal species on the leaf material (C). Error bars represent standard error.

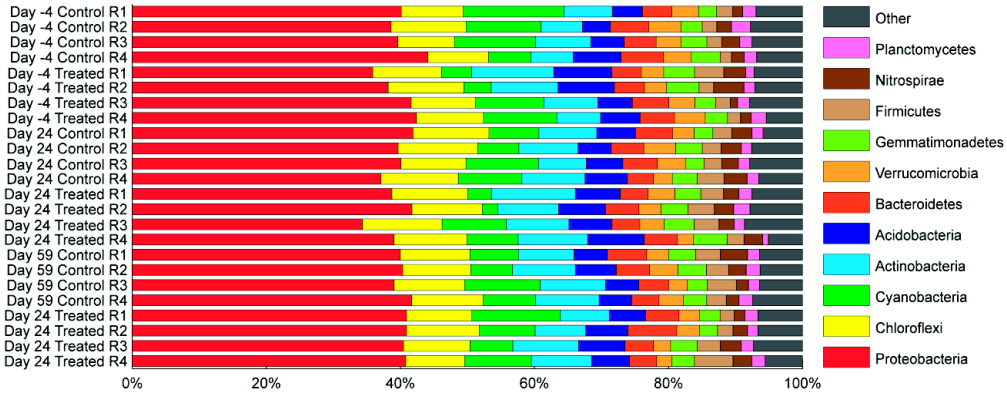


Figure S4. Phyla found in the sediment samples based on 454 pyrosequencing of 16S rRNA gene fragments. All units are represented and 'R' indicates replicates. All phyla contributing to less than 1% of the total bacteria were grouped together and called 'Other'.

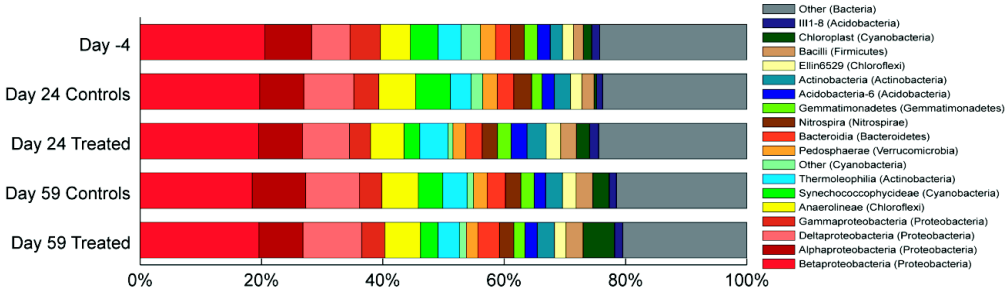


Figure S5. Bacterial classes found in the sediment samples. All units were grouped on day -4; on days 24 and 59 units were grouped according treatment. All classes contributing to less than 1% of the total bacteria were grouped together and called 'Other'. Bacterial phylum to which each class belongs in shown in between brackets.

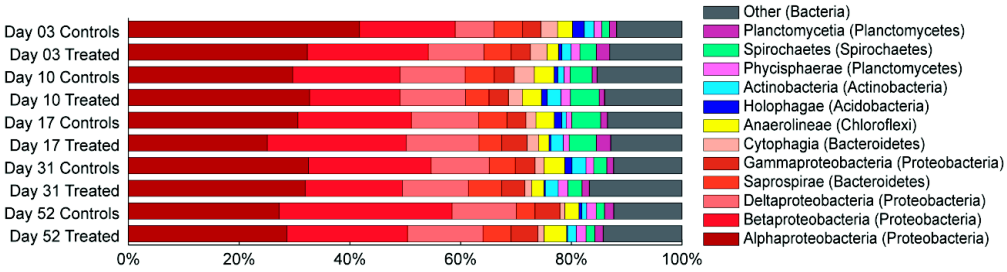


Figure S6. Bacterial classes found on the leaf material. Experimental units were grouped according treatment. All classes contributing to less than 1% of the total bacteria were grouped together and called 'Other'. Bacterial phylum to which bacterial classes belong to is shown between brackets.

Chemical analysis of tebuconazole in water and plants

The analytical methods here described were used for extraction and quantification of tebuconazole in the water phase and leaf material used in the present work. These methods were developed by the Environmental Risk Assessment Team at Alterra Institute - Wageningen University.

Tebuconazole standard stock solutions were prepared using a reference material with purity of 98.8 %. Firstly, 19 mg of the reference material was dissolved in 29.7 g of acetone, producing a stock solution of 500 mg/L. An aliquot of this stock solution was further diluted (100-fold) in acetone to produce a second stock solution of stock 5 mg/L). Both stock solutions were stored at -20 °C until further use.

Prior analysis, calibration standards with concentrations ranging from 0.5 to 250 ng/mL were freshly prepared by diluting the stock previously prepared. Dilutions were made

directly into GC vials using a mixture of acetone and Milli-Q water (75/25 % v/v), using a Hamilton 500 dilutor. The extracts were analyzed by LC-MS/MS (see Table S3).

Table S3: LC-MS/MS-Conditions for tebuconazole analysis

Instrument	
Autosampler:	Agilent G1329A
Pump:	Agilent G1312A (binary pump)
Detector:	Agilent G63110A QQQ
Source:	Agilent G1948 Electrospray
Column thermostat:	Agilent G1316A
Separation (leaf samples)	
Eluent A:	10% Milli-Q water (Advantage A10) + 0.1 % v/v formic acid
Eluent B:	90% Methanol + 0.1 % formic acid
Injection Volume:	75 µL
Flow Rate:	0.7 mL/min.
Column:	Agilent Zorbax Eclipse XDB C18 (4.6 mm x 150 mm, 5 micron)
Column temperature:	40 °C
Separation (water samples)	
Eluent A:	20% Milli-Q water (Advantage A10) + 0.1 % v/v formic acid
Eluent B:	80% Methanol + 0.1 % formic acid
Injection Volume:	50 µL
Flow Rate:	0.7 mL/min.
Column:	Agilent Zorbax Eclipse XDB C18 (4.6 mm x 150 mm, 5 micron)
Column temperature:	40 °C
Detection:	
Ionization Mode:	Positive
Heater Gas Temperature:	350 °C
Spray Voltage:	3000 V
Nebulizer pressure:	50 psi
Nitrogen flow:	10 L/min
Scan Mode:	Multiple reaction monitoring (MRM)

Compound	Precursor Ion	Product Ion	Fragmentation	Collision Energy
tebuconazole	308.2	125.1	95	42
tebuconazole	308.2	70	95	22

Retention time: about 3.3 min (leaf samples) and 5.6 min (water samples).

Responses of phytoplankton and zooplankton in the tebuconazole microcosm experiment

Materials and methods

Introduction

This tebuconazole outdoor microcosm experiment was conducted in concert with another outdoor microcosm experiment studying the ecological effects of exposure to the fungicide metiram (see Lin et al. 2002). In fact the metiram and tebuconazole experiments shared the same control test systems. The materials and methods to sample and identify phyto- and zooplankton, as well as a more detailed description of the phytoplankton and zooplankton populations in control test systems are described in detail in Lin et al. (2012). Approximately 100 different taxa of phytoplankton were identified in the test systems. For zooplankton this was approximately 30 (see Lin et al. (2012) for further details.

Univariate analysis

Prior to univariate and multivariate analyses, abundance data of zooplankton were Ln($Ax+1$) transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero (see Van den Brink et al. (2000) for rationale). This was done to down weigh high abundance values and to approximate a log-normal distribution of the data. The zooplankton and phytoplankton data were respectively, Ln(10x+1) and Ln(1.47x+1) transformed before analysis. All other variables were tested using untransformed values. No Observed Effect Concentrations (NOECs) at parameter or taxon level were calculated using the ANOVA test. The analyses were performed with the Community Analysis computer program (Hommen et al., 1994). The analysis resulted in an overview of possible statistically significant differences in population densities of phytoplankton and zooplankton taxa between the control and the treated replicates for each sampling week.

Multivariate analysis

The response of the zooplankton and phytoplankton communities to the treatment was analyzed using the Redundancy Analysis (RDA) ordination technique and by Monte Carlo permutation testing (Van Wijngaarden et al., 1995). RDA was used to obtain an overview of the effects of the pesticide at the community level. This technique produces a diagram which summarizes the data set, while still showing species composition for all samples (see Fig. S8 as example). In the diagram, samples with nearly identical species composition lie close together, while samples with very different species composition lie far apart. A species which is relatively abundant in a sample will be situated close to this sample. This diagram allows effects at the community level to be distilled. To check

whether the treatment related differences shown in the RDA-diagrams were statistically significant, Monte Carlo permutation tests were performed. Both RDA and Monte Carlo permutation tests were performed using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002). RDA diagrams are only shown when significant differences were indicated.

Results

Zooplankton

Multivariate analyses

Table S4: Results of Monte Carlo permutation tests showing the significance of the effects of tebuconazole on the zooplankton community for each sampling date.

day	P-value
-1	0.434
3	0.224
10	0.664
17	0.053
24	0.729
31	0.184
48	1
59	0.909

Since no statistical differences (all p values > 0.05) were indicated in Table S4 (Monte Carlo permutaion), the RDA diagram is not shown.

Univariate analyses

For three zooplankton taxa more or less consistent treatment-related responses were observed in the tebuconazole microcosm experiment (see Figure S7).

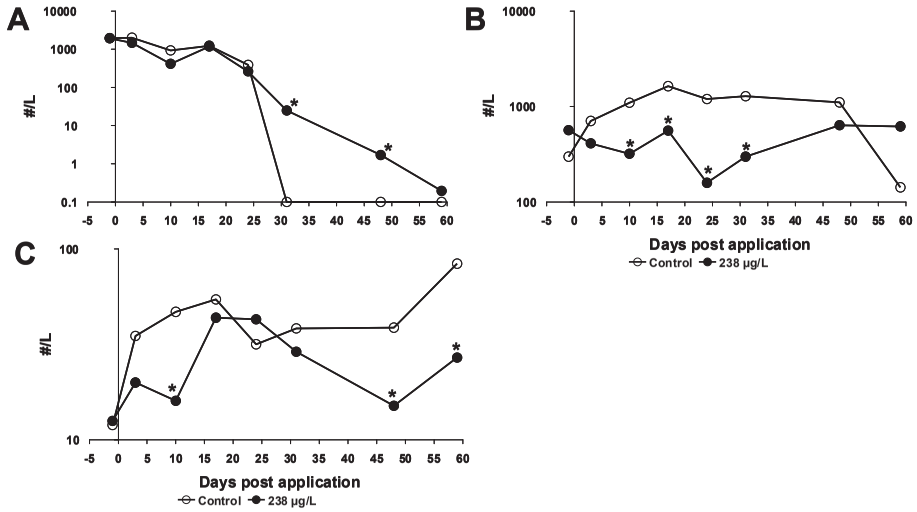


Figure S7. Dynamics in numbers for (A) Anuraeopsis fissa, (B) nauplii and (C) Cyclopoida. Only taxa that showed a consistent response to the treatments are shown (asterisk indicate significant differences with the control).

The rotifer *Anuraeopsis fissa* showed a treatment-related increase at the end of the study (indirect effect) while Copepod nauplii and Cyclopoida showed a treatment-related decrease on several samplings.

Phytoplankton

Multivariate analyses

Table S5: Results of Monte Carlo permutation tests showing the significance of the effects of tebuconazole on the phytoplankton community for each sampling date.

day	P-value
-1	0.152
3	0.06
10	0.128
17	0.185
24	0.026
31	0.146
48	0.057
59	0.42

A statistical significant difference in phytoplankton community composition (p-value < 0.05) could be demonstrated on the isolated sampling day 24 only (Table S5). On the RDA biblot (Figure S8) it can indeed be seen that on sampling day 24 (D24*C and D24*T) the trajectory of the control test systems (green line; D*C data points) deviated most from that of the tebuconazole-treated test systems (red line; D*T data points).

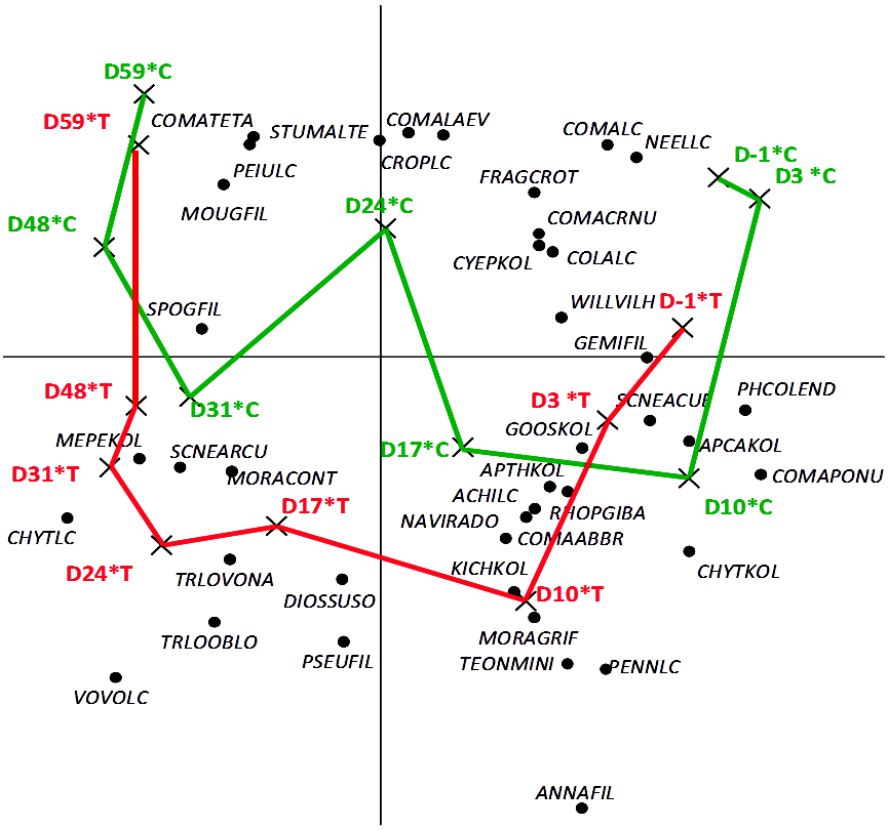


Figure S8. RDA biplot showing the differences in phytoplankton species composition between the control and the tebuconazole treatment. Treatment-time interaction explained 39% of the total variation in species composition between all samples of which 38% is displayed on the horizontal axis and another 17% on the vertical axis.

Univariate analyses

For one phytoplankton population only (*Trachelomonas* gr *oblonga*) a consistent and statistically significant treatment-related response could be demonstrated (Figure S9). Tebuconazole application caused an increase in abundance of this taxon at the end of the tebuconazole microcosm study.

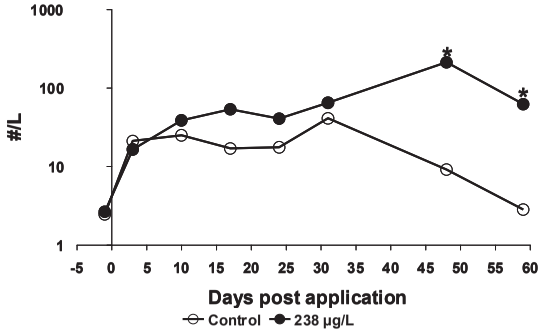


Figure S9. Dynamics in numbers for Trachelomonas gr oblonga (asterisk indicates significant difference between treated and control test systems).

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Chapter 4

Molecular assessment of bacterial community dynamics and functional endpoints during sediment bioaccumulation tests

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Abstract

Whole sediment toxicity tests play an important role in environmental risk assessment of organic chemicals. It is not clear, however, to what extent changing microbial community composition and associated functions affect sediment test results. We assessed the development of bacterial communities in artificial sediment during a 28 day bioaccumulation test with polychlorinated biphenyls, chlorpyrifos and four marine benthic invertebrates. DGGE and 454-pyrosequencing of PCR-amplified 16S rRNA genes were used to characterise bacterial community composition. Abundance of total bacteria and selected genes encoding enzymes involved in important microbially-mediated ecosystem functions were measured by qPCR. Community composition and diversity responded most to the time course of the experiment, whereas organic matter (OM) content showed a low but significant effect on community composition, biodiversity and two functional genes tested. Moreover, OM content had a higher influence on bacterial community composition than invertebrate species. Medium OM content led to the highest gene abundance and is preferred for standard testing. Our results also indicated that a pre-equilibration period is essential for growth and stabilization of the bacterial community. The observed changes in microbial community composition and functional gene abundance may imply actual changes in such functions during tests, with consequences for exposure and toxicity assessment.

Introduction

Sediment microbial communities play an important role in ecosystem functions like nutrient cycling, primary production and decomposition.[1] Microbial communities have a large influence on abundance and diversity of benthic invertebrates by controlling carbon dynamics[1] and providing a food source.[2-5] On the other hand, benthic invertebrates can affect microorganisms for instance by bioturbation, i.e. reworking of sediments by animals and plants.[6-9]

Microorganisms influence the degradation and bioavailability of contaminants that accumulate in aquatic sediments[10-12] by adsorption,[13] bioaccumulation[14] and biodegradation.[15-18] In turn, chemicals that enter the environment might affect microbial community structure and function[14,19-22] and thereby cause effects at higher trophic levels.[23-25] Hence, microbial communities constitute an important endpoint in sediment quality assessment,[26,27] since they are ecologically relevant,[28] might affect environmental transformation of chemicals[27] and are sensitive to chemicals.[20]

Effects of contaminants in aquatic sediments can be assessed by sediment toxicity testing.[26] Natural sediments are highly complex and heterogeneous in time and space. Therefore, artificial sediments are often used to standardize toxicity test procedures and to allow for more comparable outcomes. Microbial communities, however, are poorly developed in artificial sediments compared to natural sediments.[29,30] Nevertheless, the presence of microbial communities in artificial sediment, even when poorly developed, still might directly or indirectly influence the quality of sediment and water,[30] chemical behaviour, food availability, symbioses and other processes (Figure S1). Such processes may already start during the sediment equilibration period, which is a common stage of sediment preparation, following spiking. Eventually, microbes may affect the outcome of standard tests with higher organisms.[29] For instance, the bioavailability of chlorpyrifos for *Chironomus riparius* increased with the presence of microbes and biofilms.[14]

Ideally, benthic invertebrate toxicity tests should be performed with single species, in order to avoid interactions that might influence test outcomes. However, it is difficult to exclude microorganisms during an invertebrate test or during any sediment test. Absence of microorganisms would also make such tests less ecologically relevant, since sediment microbial communities play an important role in ecosystem functions.[1] Here we argue that because unavoidable microorganisms might influence test results, there is a need to understand microbial community development in artificial sediments during sediment tests.[29] Moreover, toxicity tests using sediment microorganisms often focus on evaluating effects on single species[31] or on global microbial endpoints, such as microbial community density.[32] Such approaches may fail to detect effects on microbial community composition, structure and/or function. Therefore, measurements of

ecologically relevant endpoints associated with benthic microbial communities in standardised sediment toxicity tests are also needed.[26]

The aim of the present study was to assess the development of bacterial communities and selected genes involved in important microbially mediated ecosystem functions, during pre-equilibration and exposure stages of a whole-sediment test. A 28-day bioaccumulation experiment was conducted with four marine benthic invertebrates on artificial sediment spiked with six polychlorinated biphenyls (PCBs) and chlorpyrifos (CPF) at concentrations non-toxic for invertebrates.[33] Denaturing gradient gel electrophoresis (DGGE) and 454-pyrosequencing of PCR-amplified bacterial 16S ribosomal RNA (rRNA) gene fragments were used to investigate bacterial community structure and composition in the artificial sediment. Bacterial abundance was measured by quantitative polymerase chain reaction (qPCR), as well as abundance of selected genes encoding enzymes involved in important microbially mediated ecosystem functions, such as nitrogen-fixation, ammonia-oxidation, denitrification, sulphate-reduction and degradation of organophosphate compounds like CPF. To our knowledge, this is the first study to describe bacterial community dynamics during a bioaccumulation test using a complementary set of state of the art molecular tools.

Materials and Methods

Sediment bioaccumulation experiment

Sediment samples for microbial analyses were taken during different phases in a bioaccumulation experiment, which has been described before.[33] Here, a brief summary is provided. Visual representation of the experiment (Figure S2) and details on methods and chemicals used are provided as supplementary information (SI). A 28-day sediment bioaccumulation test was performed in a temperature-controlled room of 14 °C under average (standard deviation (SD)) light conditions of 21 (2) lux with a photoperiod of 16h light: 8h dark. Four marine benthic invertebrate species were used: *Arenicola marina* (Linnaeus, 1758) (annelid; sub-surface deposit feeder), *Corophium volutator* (Pallas, 1766) (crustacean: detritus feeder), *Macoma balthica* (Linnaeus, 1758) (mollusc; facultative suspension feeder) and *Nereis virens* (Sars, 1835) (polychaete; omnivore). These species live buried in the sediment. *A. marina*, *C. volutator* and *M. balthica* were collected from the field and *N. virens* was obtained from a professional bait farm.[33] Four treatments (n=4) were used: enclosed single species at nominal low (1%), medium (5%) and high (15%) organic matter (OM) content and 'mixed species' at medium OM content. In the 'mixed species' treatment, all four species were tested together in the same aquarium (35L´30W´30H cm). For the enclosed single species treatments, direct species interaction was avoided by introducing four enclosures per aquarium, using fine mesh gauze.[33]

Standard sediment was prepared according OECD guideline 218[34] with small modifications. Peat, calcium carbonate (1%) and natural seawater were mixed to obtain a homogeneous slurry, which was spiked with PCBs and CPF and thoroughly mixed with quartz sand (75%) and kaolin clay (20%). Peat was added in different quantities to obtain the aforementioned low, medium and high OM content treatments.[33] Peat was dried (40 °C) and ground before being used for sediment preparation. After grinding, three random samples were taken and kept at -20 °C until further analyses.

Sediment was spiked with six PCB congeners, i.e. 28, 52, 101, 118, 153, 180 and CPF. PCBs were chosen as a representative of legacy compounds (POPs) and as relatively inert chemicals with a dose below toxicity thresholds for invertebrates and therefore an ideal tracer chemical for bioaccumulation. CPF was chosen as a representative of insecticides, which are a contrasting chemical group (e.g. regarding their degradability and usage patterns) as compared to PCBs.[33] The nominal concentration for sum PCBs was 36 μg/kg dry weight and for CPF it was 3.12 μg/kg dry weight. The total chemical concentration was the same for all treatments, however, pore water concentrations differed because of the differences in OM content. To allow for (pseudo-) equilibrium between chemicals and sediment prior to the start of exposure, sediment was agitated for 69 days on a roller bank in the dark at room temperature. Control sediment received

the same amount of solvent, i.e. acetone, as the treated sediment.[33] Test chemicals were extracted from water using Empore disks and biota and sediment samples were soxhlet extracted. The extracts were analyzed by gas chromatography–mass spectrometry, following published procedures.[33]

Unfiltered natural seawater from the Eastern Scheldt, the Netherlands, was used as pore water and overlying water. The volume of overlying water was approximately 25 L and the wet sediment to overlying water volume ratio in the aquaria was kept at 1 to 5 for the enclosed single species test and 1 to 6 for the mixed species test. Water flow was possible through the gauze and was enhanced by aeration to ensure complete mixing of overlying water. Invertebrates were added 7 days after the sediment water system was prepared to allow for better physical-chemical stability as has been recommended by Verrhiest et al.[30] In each aquarium, 5 *A. marina*, 70 *C. volutator*, 25 *M. balthica* and 10 *N. virens* individuals were added in their respective enclosures. In the mixed species treatment, the same numbers of individuals per species were put together in an aquarium without enclosures, to test effects of species-species interaction on bioaccumulation. Invertebrates were fed with spiked ground fish food (TetraMin) suspended in deionised water, three times per week after the first week of the experiment.[33] The water quality variables oxygen, temperature, salinity, conductivity and pH were measured three times a week. Ammonium, nitrate, chlorophyll (cyanobacteria, green algae and diatoms) and turbidity were measured weekly in a mixed sample containing an equal volume of water from each enclosure.

Sediment collection for microbial analysis

Sediment samples for microbial analyses were taken at the start of the pre-equilibration of the sediment (t=-69 days), at start (t=0 d) and at the end (t=35 d) of the bioaccumulation test (Figure S2). Note that the duration of the bioaccumulation experiment was 28 days, starting after a stabilization period of 7 days. Therefore, the end of the bioaccumulation experiment is referred to as t=35 d. Pre-equilibration samples were taken after adding the sediment compounds and mixing them thoroughly on a roller bank for 1 day (t=-69 d) (Figure S2). If more than one container was used for sediment preparation, subsamples from each container were mixed and three random samples were taken. At the end of the pre-equilibration period (t=0 d), which was the start of the experiment, containers with the same sediment were thoroughly mixed and three random samples were taken. At the end of the experiment (t=35 d) invertebrate test species were removed, sediment from each enclosure was mixed and a sample was taken. For the treatments without enclosure, the whole sediment was mixed and a sample taken, after removal of the test species. Samples were stored at -20 °C until further analyses. In addition, samples of control and spiked medium OM sediment were taken during the sediment preparation phase at t=-69 d, t=-62 d, t=-55 d and t=-41 d in a similar way as described above.

Total abundance of bacteria and selected functional genes

Total DNA was isolated from all sediment and peat samples using the FastDNA Spin kit for soil (MP Biomedicals) according to manufacturer's protocol. Sediment samples of all OM contents collected during the pre-equilibration period (t=-69 d) and the start of the bioaccumulation experiment (t=0 d) were used for DNA isolation, yielding in total 18 samples. However, for the sediment samples at the end of the bioaccumulation period (t=35 d), only low and medium OM content samples were extracted, giving rise to in total 36 samples. Analysis of all samples was not feasible; therefore high OM content samples were left out as less chemical effect on the bacterial community was expected because of lower bioavailability. qPCR was used to determine the abundance of total bacteria (16S rRNA gene), nitrogen-fixing bacteria (*nifH* gene), ammonia-oxidizing bacteria (*amoA* gene), denitrifying bacteria (*nosZ* gene), sulphate-reducing bacteria (*dsrA* gene) and bacteria capable of hydrolyzing organophosphate compounds (*opd* gene). For peat samples, only total bacterial abundance was quantified. qPCR reactions were performed in a 384-well plate (Bio-Rad, Veendaal, the Netherlands) using a CFX384 Real-Time PCR Detection system (Bio-Rad, Veendaal, the Netherlands). All samples were analyzed in triplicate and reactions were carried out in a total volume of 10 μL. qPCR reactions targeting total bacteria, nitrogen-fixing and ammonia-oxidizing bacteria were performed according to Rico et al.[35]. Abundance of the denitrification gene *nosZ* was quantified according to Veraart et al.[36]. Abundance of the *dsrA* gene was quantified according to Foti et al.[37]. Abundance of the *opd* gene was quantified using primers 3F and 3R described by Singh et al.[38]. For each qPCR reaction, a standard curve comprising 10-fold serial dilutions of the target gene was included. Standards were obtained by amplifying the target genes from bacterial sources known to harbour one or more genes of interest. Specificity of target gene fragment amplification was checked by melting curve analysis for each qPCR reaction. Primer combinations and cycle conditions are described in Table S1.

Bacterial community structure and composition

In the same sediment samples used for qPCR, bacterial community composition was investigated by 454-pyrosequencing (Roche Diagnostics, Germany) of the 16S rRNA gene. Amplicons were generated by PCR amplification of the V1 and V2 regions of the 16S rRNA gene (Table S2) and sequenced using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Constance, Germany). Preparation of sediment samples for sequencing was done according to Dimitrov et al.[39]. Bacterial community structure of medium OM sediment samples taken during pre-equilibration period of the control and spiked sediments, were furthermore analyzed by DGGE fingerprinting of PCR amplicons. Total DNA extraction, PCR reactions and DGGE were performed according to Lin et al.[40].

Data analyses

Raw 454-pyrosequencing data were processed and sorted using default parameters in the Quantitative Insights Into Microbial Ecology pipeline (QIIME) version 1.7.0[41], according to Dimitrov et al.[39]. Principal Coordinates Analyses (PCoA) were performed using un-weighted and weighted UniFrac distances. Unifrac is a method of calculating distance between microbial communities taking into consideration phylogenetic information, where only presence/absence (un-weighted) or relative abundance (weighted) of operational taxonomic units (OTUs) can be taken into account. PCoA plots were used to visualize similarities or dissimilarities among samples taken at start (t=-69 d) and end (t=0 d) of the pre-equilibration period as well as at the end of the actual bioaccumulation test (t=35 d). Statistical differences between samples taken at different sampling times were tested using analysis of similarity (ANOSIM) by permutation with 999 replicates, as implemented in QIIME. OTUs were defined at a 97% sequence identity threshold. In order to avoid bias introduced by sequencing depth, all samples were rarefied to an equal number of sequences (4557 reads).

DGGE band detection and quantification of band intensity were performed using Bionumerics software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). Multidimensional Scaling (MDS) was performed in order to compare bacterial communities present in sediment samples taken from the pre-equilibration phase, which had been analyzed by DGGE. MDS analysis was performed using Bionumerics software version 4.61.

Bacterial 16S rRNA, *nifH*, *amoA*, *nosZ*, *dsrA* and *opd* gene abundance data and Shannon diversity index (16S rRNA gene) were checked for normality with Q-Q plots and Shapiro-Wilk tests and for equality of variances with Levene's test. Log transformation was used for data that were not normally distributed, however, in case data were still not normally distributed the non-parametric Kruskal-Wallis test with pairwise comparison was used. Data for which assumptions were met were tested either with a t-test or with a two-way ANOVA (factors: OM or time or species) with a significance level α=0.05 using SPSS version 19. The least significant difference (LSD) was used as a post hoc test for main effects. When an interaction effect was detected with two-way ANOVA, an LSD test adjusted for multiple pairwise comparisons was used to detect differences.

Results and discussion

Chemical exposure, survival of benthic invertebrate species and water quality

Results of the bioaccumulation experiment have been described before.[33] In brief, because of the experimental design, concentrations in the sediment were similar for treatments and stayed relatively constant during the experiment for PCBs. PCBs are chemically and biologically stable and can persist in sediments and soils for years.[42,43] In contrast, at the end of the experiment, the concentration of CPF was below the detection limit in all treatments, which might be explained by biologically-mediated and surface-catalysed hydrolysis, oxidation, photolysis and volatilization.[38,44,45] A previously reported halve-life time (DT_{50}) for CPF in water-sediment systems was 36.5 days.[46] Survival of invertebrates ranged from 47% for *C. volutator* to 60% and higher for *A. marina*, *M. balthica* and *N. virens* in all treatments. Survival for *A. marina* in the mixed species was 0% probably due to predation by *N. virens*.[33] A good water quality was maintained during the test, and variation of temperature, pH, DO and conductivity among enclosures was low (Table S3, S4).

Gene abundance during pre-equilibration phase and bioaccumulation test

A selection of genes was used to quantify overall bacterial abundance as well as to target important ecosystem functions mediated by microorganisms in sediments. This enabled us to address to what extent presence and abundance of such genes are affected by the various steps during artificial sediment pre-equilibration and bioaccumulation testing, by varying OM content and by presence of benthic invertebrates.

General patterns

Overall, abundance of all genes targeted here was low or below detection limit (highest 10-fold serial dilution of the qPCR control where amplification was observed) at the start (t=-69 d) and end (t=0 d) of the pre-equilibration period and increased during the pre-equilibration and bioaccumulation period of the experiment, especially for medium OM (Figure 1, S3, S4, Table S5). The total bacterial abundance, as measured by 16S rRNA gene-targeted qPCR, ranged between 7.8×10^6 to 6.6×10^8 copies/g wet sediment for all treatments and time points (Table S5), which lies in the lower range found for natural marine sediment ($2x10^7$ to $3x10^9$ copies/g wet sediment, calculated assuming 3.6 16S rRNA gene copies per cell and an average marine sediment density[47] of 1.7 g/cm^3).[44,45,48,49] Abundances of functional genes in the artificial sediment were up to seven orders of magnitude lower than those found in natural marine sediment (Table S6).[48-53] These findings correspond with the conclusion of Goedkoop et al.[29] and Verrhiest et al.[30] that artificial sediment is a poor replacement for natural sediment. However, if impacts of microbes on test results were to be minimized, then artificial sediments would be

a better choice, even though the ecological relevance decreases. Bacterial communities in artificial sediment originate mainly from the sediment components and any other bacterial source during preparation (e.g. bacteria present in the air) and therefore might differ from a natural sediment bacterial community.[29] After grinding, the total bacterial abundance in peat was higher than the bacterial abundance in the sediment at start of the pre-equilibration period (t=-69 d) (Table S5). Consequently, it can be assumed that peat was the main bacterial source. The seawater that was used to prepare the sediment might have been another main source, however, bacterial abundance in the seawater was not measured.

At the start and end of the pre-equilibration period (t=-69 d and t=0 d), *nosZ* was only detected in some cases (Figure S3D, S4D), whereas *dsrA* and *opd* abundances were all below the detection limit. At the end of the bioaccumulation period (t=35 d), however, these genes were detected, with highest values found for *nosZ* and *dsrA* (Table S5). This suggests that during the testing phase bacterial growth might be stimulated by changing conditions during the experimental period, such as increased concentrations of nutrients in general, as well as specialized feeding of bacteria on the spiked chemical e.g. bacteria capable of hydrolyzing organophosphate compounds (*opd* gene). Studies conducted in soils have demonstrated the importance of microbial activity for the degradation of CPF, where degradation half-lives were significantly longer in sterile soil (abiotic degradation) compared to natural soils (abiotic and biotic degradation).[38,44,54,55] Moreover, DT_{50} for aquatic photolysis (29.6 days) and hydrolysis (25.5 days) are much longer than the total DT_{50} in the aquatic phase (5 days),[46] indicating that biodegradation dominates degradation in sediments.[44] Consequently, it is plausible that the disappearance of the organophosphate CPF during the bioaccumulation test can be explained by an increased abundance of bacteria capable of hydrolyzing organophosphate compounds as quantified by *opd* gene-targeted qPCR. Previously, a similar relationship between functional gene abundances and chemical degradation has been shown e.g. for chloroethenes and hexachlorobenzene,[56-58] which further supports the plausibility of this explanation.

Additionally, bacteria can be introduced either with the added invertebrate test species[55,56] and/or by experimental procedures and environmental surrounding (e.g. air). Moreover, bioturbation by invertebrates may positively influence bacterial abundance and diversity.[6-8] For example, Dollhopf et al.[53] showed that bioturbation delivered oxygen to sediment microorganisms, enhancing coupled nitrification-denitrification in salt marsh sediment, consequently increasing the abundance of genes related to such processes.

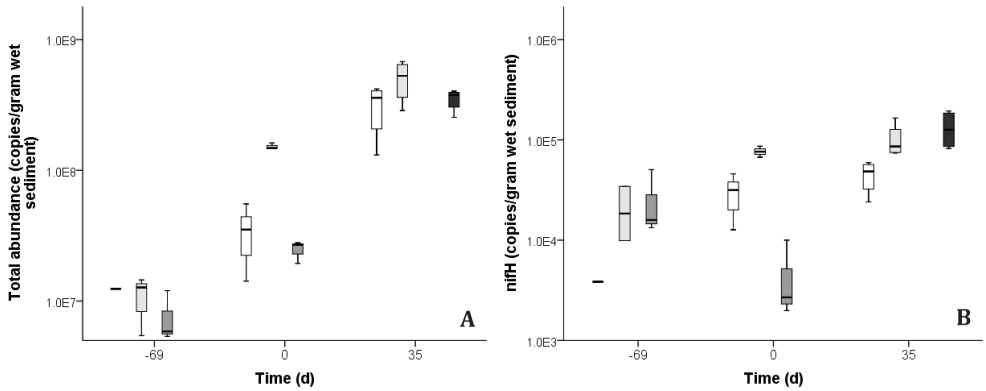

Figure 1. Gene abundances (copies/gram wet sediment) for total bacterial 16S rRNA gene (A) and nifH (B) at the start of the pre-equilibration period (t=-69 d, n=3), at the end of the pre-equilibration period/start of experiment (t=0 d, n=3) and at the end of the bioaccumulation experiment (t=35 d, n=4) for low (white), medium (light grey), high (medium grey) and medium mixed species (dark grey) organic matter content. Note different scales on y-axes. For an overview of the pre-equilibration period only, check Figure S4.

Effect of time, OM and species

At the start of the pre-equilibration period (t=-69 d), no difference was found for total bacteria, *nifH* and *amoA* abundance between the OM treatments except for *nifH* at low OM. At the end of the pre-equilibration period (t=0 d) however, the abundance of all detected genes was higher in the medium OM than in the low and high OM treatment (Figure S3, S4, Table S5). Based on the lower pore water concentrations of PCBs and CPF and the higher nutrient availability at high OM, the highest bacterial abundance would be expected at high OM instead of medium OM.

Total bacterial abundance differed significantly between start (t=0 d) and end (t=35 d) of the bioaccumulation test for both low and medium OM, whereas *nosZ* and *dsrA* abundances were different between start and end for medium OM only (independent t-test; two-tailed $p<0.05$, Table S5, S7, Figure 1A, S3D, S3E). For *amoA* no significant differences could be found in neither of the OM treatments, despite the high numerical increase in abundance (Figure S3C, Table S5, S7). For *nosZ* and *dsrA* for low OM and *opd* for low and medium OM treatments, no statistical tests were performed as values at t=0 d were below detection limit. However, a similar numerical increase in abundance occurred as observed also for *amoA* (Figure S3D, S3E, S3F, Table S5). The gene *nifH* did neither showed significant differences nor a numerical increase in abundance between start and end of the bioaccumulation test (Figure 1B, Table S7).

At the end of the bioaccumulation period (t=35 d), abundance for almost all targeted genes was lower for *A. marina* and *C. volutator* compared to treatments with *N. virens* and *M. balthica* (Figure 2, S5). No significant interaction was detected between the OM content and invertebrate species on total bacterial abundance, neither on any of the targeted functional genes in the sediment (2-way ANOVA, $p>0.05$, Table S8). There was, however, a significant main effect of OM content on the total bacterial, *nifH* and *dsrA* abundance in the sediment at t=35 d (Table S8, $p<0.05$), where low OM content had lower abundance than medium OM.

Moreover, a significant main effect ($p<0.05$) of benthic invertebrate species on *amoA* (Figure S5C) and *nosZ* (Figure S5D) in the sediment was detected. Gene abundances in sediments with *A. marina* and *C. volutator* were more similar to each other than those observed in sediments with *M. balthica* and *N. virens*. The highest difference was observed between *A. marina* with low abundance and *M. balthica* with high abundance. As mentioned before, bioturbation can stimulate bacterial growth, thus leading to increased bacterial abundance. *A. marina* and *C. volutator* share the same bioturbation mechanism: creating and irrigating U-shaped tubes in the whole sediment or in the top 2 cm of the sediment.[59] In contrast, *N. virens* creates and irrigates burrow galleries in the whole sediment,[59] whereas *M. balthica* burrows itself in the first 2-6 cm of the sediment and is a biodiffuser.[60] The type of bioturbation determines the magnitude of the effect[59,60] and explains that species with more similar bioturbation strategies show a greater similarity in bacterial abundance. However, for specific functional processes this might be different. For example, *C. volutator* and *M. balthica* increase the flux of nitrate from sediment to the overlying water, whereas *A. marina* and *N. virens* increase the nitrate flux from overlying water to sediment.[8,60] All species have been reported to increase the flux of ammonium from the sediment to overlying water.[60] Differences in fluxes were explained with the depth distribution of nutrients in pore water, irrigation activity and microbial activity in faecal pellets.[8,60]

At the end of the rolling period (t=0), the medium OM treatment showed no significant difference in abundance between the enclosed single species and mixed species treatment, for any of the genes (independent t-test, $p>0.05$, Table S9). In mixed species systems, it can be expected that the bioturbation activities of the species with the highest impact will dominate the effects of the other bioturbating species, rendering them less visible.[59] In summary, our results show that variables during a sediment test, such as OM content, time and added invertebrate species, affected functional endpoints, such as the abundance of nitrogen-fixing bacteria, ammonia-oxidizing bacteria, denitrifying bacteria, sulphate-reducing bacteria and bacteria capable of hydrolyzing organophosphate compounds. Additional tests will be needed to determine whether the effects found here with respect to effects on microbial composition and general and pollutant-specific functions can be generalized to other chemicals.

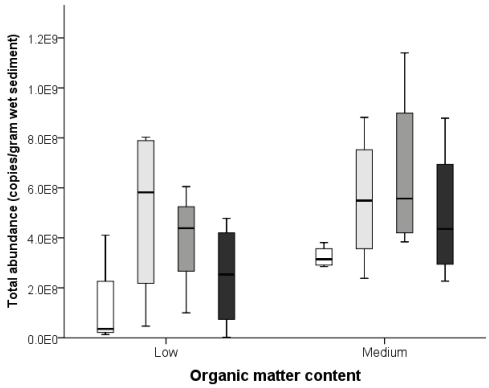

Figure 2. Total bacterial abundance (copies/gram wet sediment) at the end of the bioaccumulation experiment (t=35 d, n=4) at low and medium organic matter content for Arenicola marina (white), Nereis virens (light grey), Macoma balthica (medium grey) and Corophium volutator (dark grey). Lines indicate no significant difference in abundance between species within a treatment. Small letters indicate significant differences in abundance between treatments (α=0.05) determined by two-way ANOVA.

Bacterial community composition during pre-equilibration and bioaccumulation stages of the test

During the pre-equilibration period, control and spiked sediment with medium OM content showed a similar bacterial community structure, based on DGGE profiles (Figure S6). Control and spiked sediment differed most at the beginning of the pre-equilibration period (t=-69 d), becoming more similar at the end of the pre-equilibration period (Figure S6). However, it seems unlikely that PCBs spiked into the sediment could alter the sediment bacterial community so quickly, that is, in such a way that the bacterial communities in the control and spiked sediment would differ already after a single day of mixing. Previous work showed effects of PCBs on structure, composition and function of microbial communities in sediment and soil, however, after a much longer time (1-8 months).[61,62] Therefore, differences between control and spiked sediment at the start of the pre-equilibration period might reflect insufficient mixing of the sediment after all components had been mixed for one day. Bacterial community appeared to develop in a similar way over time in spiked and control sediment, with community structure of both treatments being very similar at the last two sampling dates. No major difference was observed between control and spiked sediment during the pre-equilibration phase (Figure S6).

Samples of PCBs and CPF spiked sediment from the pre-equilibration period and the bioaccumulation experiment, containing low, medium and high concentrations of OM, were subsequently analyzed by 454-pyrosequencing to obtain a more detailed view on

potential changes in microbial community structure than is possible by DGGE analysis. A total of 444304 16S rRNA gene sequences with an acceptable quality were obtained with an average of 8228 reads per sample, being 4557 reads the lowest and 13935 reads the highest number (average read length = 300 base pairs). Based on 97% sequence similarity as threshold, a total of 1632 OTUs was found.

Sequencing analysis revealed that *Proteobacteria* was the major bacterial phylum present in the sediment samples (Figure 3, Table S10, S11). At the start of the pre-equilibration period (t=-69 d), sediment containing low and medium OM content showed a similar relative abundance of *Proteobacteria*, which was higher than that in high OM sediment. Similar relative abundance was also observed for the phyla *Acidobacteria* and *Actinobacteria*, however, sediment with high OM content presented a higher relative abundance of these groups. The phylum *Bacteroidetes* was present at higher relative abundance in low OM content sediment, whereas *Firmicutes* were observed only in the high OM content sediment (Figure 3, Table S10). Despite the fact that peat samples were not included in the sequence-based analysis, the bacterial profiles obtained from sediment samples at the beginning of the pre-equilibration period (t=-69 d) give an indication of the relative abundance of different bacterial phyla in peat. For example, the fact that *Firmicutes* were observed only in sediment with high OM content suggests that this bacterial phylum represents only a minor component in the peat-associated microbial community. Moreover, varying the OM content was enough to produce artificial sediment with significantly different bacterial community compositions, as was demonstrated by ANOSIM (un-weighted UniFrac R=0.85, p=0.001; weighted UniFrac R=0.83, p=0.001). At the end of the pre-equilibration period (t=0 d) the relative abundance of *Proteobacteria* was similar to the initial level observed for sediment samples with low and medium OM content, whereas the sediment with high OM content showed a higher relative abundance compared to its initial value. *Acidobacteria*, *Actinobacteria*, WPS-2, *Planctomycetes* and *Firmicutes* decreased in relative abundance at the end of the pre-equilibration period, whereas *Bacteroidetes* increased considerably in all sediment samples (Figure 3, Table S10). At the end of the pre-equilibration period (t=0 d) bacterial communities in all sediment samples were more similar than at the beginning of the pre-equilibration period, as indicated by ANOSIM (un-weighted UniFrac R=0.30, p=0.005; weighted UniFrac R=0.16, p>0.05), which confirms the results of the DGGE analysis. Observed richness (i.e. number of OTUs) as well as diversity, as indicated by the Shannon index (Figure S7), were consistently higher for sediment samples at the beginning of the pre-equilibration period (t=-69 d) compared to those at the end of pre-equilibration (t=0 d). For low OM content, however, the Shannon index increased significantly during the bioaccumulation test whereas for medium OM the diversity was similar between t=0 d and t=35 d (Figure S7, Table S7). At the end of the bioaccumulation test (t=35 d),

there were no differences in bacterial diversity between the test species but there was a significant difference between low and medium OM content (Figure S7, Table S8).

At the end of the bioaccumulation test (t=35 d), *Proteobacteria* was still the most abundant phylum present in the sediment samples (Figure 3, Table S11). *Bacteroidetes'* relative abundance increased in all sediment samples collected at the end of the bioaccumulation test (t=35 d), compared to relative abundance values at the beginning (t=-69 d) and end of the pre-equilibration period (t=0 d). Values were consistently higher in sediment samples containing medium OM content, compared to low OM content (Figure 3, Table S10, S11). The relative abundance of *Firmicutes* had also increased by the end of the bioaccumulation test (t=35 d). *Acidobacteria* and *Actinobacteria* relative abundances at the end of the bioccumulation test (t=35 d) were similar to values observed at the end of pre-equilibration period (t=0 d) (low and medium OM content) (Figure 3, Table S10, S11). Bacterial community composition of all sampling points was compared using PCoA analysis and un-weighted and weighted UniFrac distances (Figure 4), which showed grouping of samples according to time rather than to OM content, especially for un-weighted UniFrac (ANOSIM, un-weighted UniFrac R=0.81, p=0.001; weighted UniFrac R=0.74, p=0.001). However, when only comparing samples taken at the end of the bioaccumulation test (t=35 d) a clear separation between sediment containing low and medium OM content was observed, indicating that OM content had a direct influence on bacterial community composition or indirectly via chemical concentrations in the pore water, which in turn depend on OM content (Figure 4) (ANOSIM, un-weighted UniFrac R=0.30, p=0.036; weighted UniFrac R=0.53, p=0.007). PCoA analysis also showed that OM content had a higher influence on bacterial community composition than invertebrate species, especially for weighted UniFrac (Figure 4). Diversity decreased during the pre-equilibration period and increased during the bioaccumulation test, reaching similar diversity values observed at the beginning of the pre-equilibration period (t=-69 d). The observed bacterial richness showed the same pattern (Figure S7).

Establishing a direct link between bacterial community composition observed in the sediment samples and results of the qPCRs assays is difficult. Important microbially mediated ecosystem functions, including those targeted here, are often performed by a wide range of microorganisms. Such functional redundancy may also be reflected at the DNA level, meaning that functional genes frequently do not present a completely conserved DNA sequence across different organisms. Furthermore, next-generation sequencing results often do not provide the necessary taxonomical depth for a detailed classification of observed OTUs. An attempt to predict functional composition based on the 16S rRNA gene information obtained by sequencing was done using the software PICRUSt.[63] However, quality control of PICRUSt predictions indicated that results were

not trustworthy for the dataset described here due to insufficient coverage of annotated genomes related to organisms found in this study in the underlying database. Therefore, in order to acquire detailed molecular information about ecosystem functions associated with a certain sediment sample, either a metagenomics or metatranscriptomics study would be required, as these provide direct sequence information with respect to a microbial community's functional capacity and actual activity as reflected in actively expressed genes.[64-67]

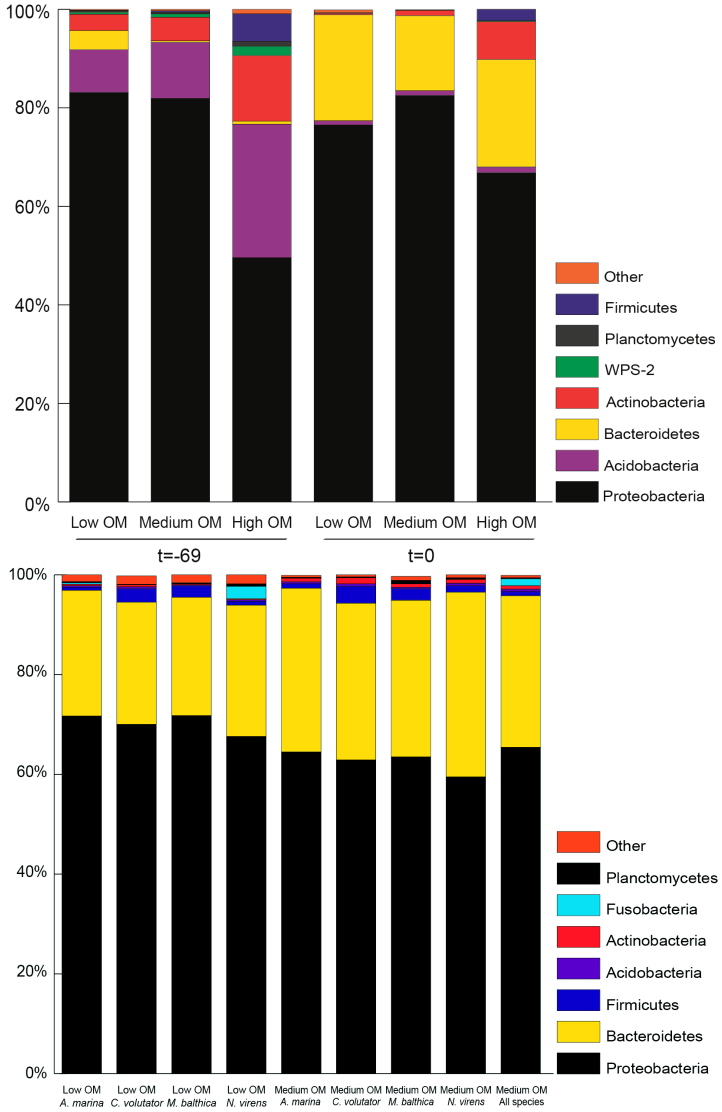

Figure 3. Relative abundance of bacterial phyla detected in sediment samples based on 454 pyrosequencing of 16S rRNA gene fragments, at the beginning (t=-69 d) and end of the pre-equilibration period (t=0 d) (A) and at the end of the bioaccumulation test (t=35 d) (B). All phyla contributing to less than 1% of the total bacteria were grouped as 'Other'.

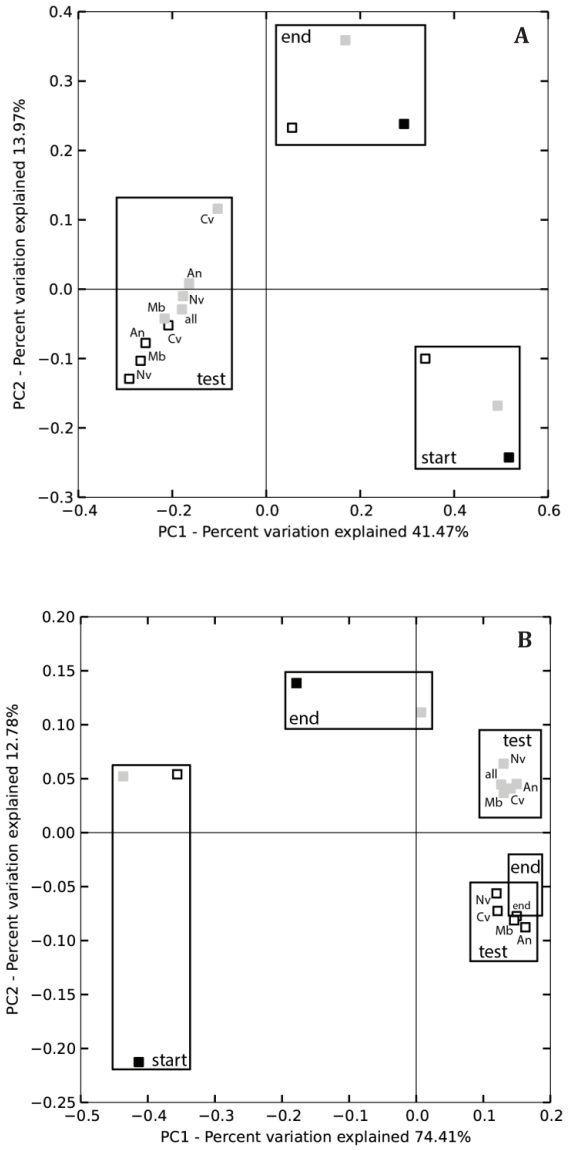

Figure 4. PCoA plots of unweighted (A) and weighted (B) UniFrac distances of sediment samples collected during pre-equilibration phase and bioaccumulation test. Sampling dates are shown as start (t=-69 d), end (t=0 d) and test (t=35 d). OM content is depicted as white (low OM), light grey (medium OM) and black (high OM) squares. Am = Arenicola marina, Nv = Nereis virens, Mb = Macoma balthica, Cv = Corophium volutator and all = all invertebrate species together. ANOSIM, un-weighted UniFrac R=0.81, p=0.001; weighted UniFrac R=0.74, p=0.001.

Implications

This study showed that microbial communities changed as a function of time and as a function of organic matter content. Effects of invertebrate species, however, were only detected for two genes (*amoA* and *nosZ*). OM content more strongly affected bacterial dynamics than invertebrate species. The treatment with medium OM content had the highest gene abundance, and in the light of ecological relevance thus is to be preferred in standard sediment tests, which matches the recommendation by the OECD to use 5% OM by default. Our results also indicated that besides the equilibration of spiked chemicals, a pre-equilibration period is also essential for growth and stabilization of the bacterial community. Therefore, the seven-day pre-equilibration period recommended by the OECD might need to become obligatory, with an extended pre-equilibration period for persistent hydrophobic chemicals with slow sorption kinetics. With the introduction of invertebrate species in the test system, bacterial biodiversity increases, which might change the dynamics of the microbial community already present. Invertebrate species might as well directly contribute to microbial community dynamics by reworking of the sediment via e.g. bioturbation and feeding on bacteria.

We showed that during a bioaccumulation experiment in an OECD set up, the bacterial diversity and community composition as well as functional endpoints such as: the abundance of nitrogen-fixing bacteria, ammonia-oxidizing bacteria, denitrifying bacteria, sulphate-reducing bacteria and bacteria capable of hydrolyzing organophosphate compounds were significantly affected by the test conditions. This is especially important (a) for functions that affect chemical exposure, like in the present case the ability to hydrolyze organophosphate compounds and (b) for functions that affect the water quality variables driving the performance of the test species. After all, such changes can affect the outcomes of the tests for the target species in an unpredictable manner and limit the reliability of the subsequent steps in the risk assessment. A similar test set up without invertebrates could be used to assess microbial endpoints from which community level dose response relationships could be derived. For instance, a standard inoculum could be applied to standard sediment, after which community composition and gene abundance patterns are assessed as a function of chemical dose. In terms of ecological relevance, however, having a mixed species system that includes microbes as well as invertebrates remains closer to reality.

Acknowledgement

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Supplementary information

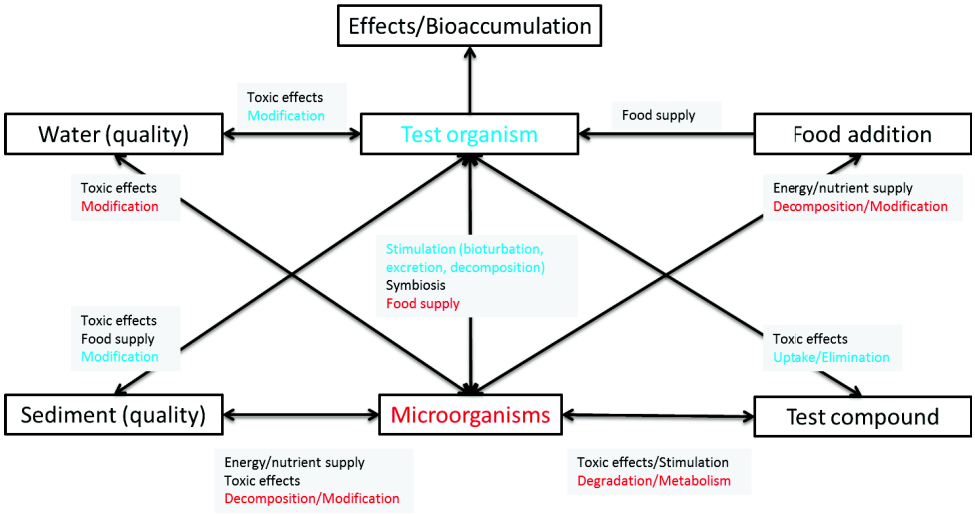

Figure S1. Illustration depicting possible influences that microorganisms might have on toxicity and bioaccumulation test results (adapted from Goedkoop et al.[29]).

Material and methods

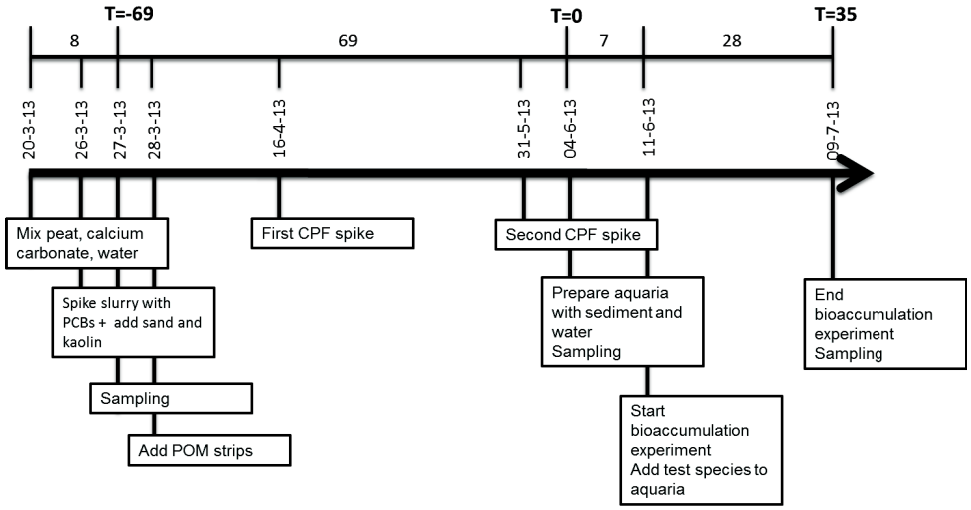

Figure S2. Experimental time (days) scheme of steps during pre-equilibration (from t=-69 d until t=0 d), stabilizing period (from t=0 d until t=7 d) and bioaccumulation test (from t=7 d until t=35 d).

Chemicals

PCBs standards IUPAC numbers 28, 52, 101, 118, 153, 180, chlorpyrifos (CPF) (purity 98.0 %) and chlorphyrifos-D10 (internal standard) were obtained from the company Dr. Ehrenstorfer, Germany. For OECD sediment peat from Klasmann Deilmann Benelux BV, CaCO3 powder from Sigma Aldrich, Germany, quartz sand from Geba 0.06-0.25 mm, Eurogrid, The Netherlands and kaolin from Sigma Aldrich, German was used.

Water quality

The water quality variables oxygen, temperature, salinity, conductivity and pH were measured with a Hach (HQ40d) portable multi-meter using the Luminescent Dissolved O2 probe (LDO101), the conductivity probe (CDC401) and the gel filled pH electrode (PHC 101) or pH meter (SG8-ELK) by Mettler Toledo (bioaccumulation experiment, test 3). Temperature was measured with the O2 probe. Ammonium concentrations were measured with the ammonium cell test by Merck with a range of 0.20 - 8.00 mg/l NH4-N and nitrite concentrations with the colorimetric nitrite test by Merck with a range 0.025-0.5 mg/L NO2-. Phytoplankton concentrations (µg/L) were measured with the Algal Lab Analyzer using a spectrofluorometer (bbe). Turbidity (NTU) was measured with a turbidity meter (TN100; Eutech instruments).

Extraction and analyses

Extraction and analysis followed previously published procedures.[68] Water samples (n=3) of natural seawater were taken to determine background concentrations. Water samples were extracted using C18 Empore disks. 200 µL of internal standard solution (PCB112, 80 ng/mL) was added to 200 mL of sample after which the sample was introduced onto the disk and subsequently eluted with 20 mL dichloromethane. The samples were concentrated to 200 µL and transferred to sample vials for analysis.

Invertebrate analysis used mixed samples of surviving individuals per treatment. Biota, sediment and fish food (Tetramin) samples were dried using sodium sulphate (Merck) and extracted by soxhlet extraction using a mixture of pentane/dichloromethane (50:50 v/v). Internal standard solution (1 mL) (PCB112, 80 ng/mL) was added to each sample. For biota samples, half of the extract was dried to gravimetrically determine the fat content. Extracts were then concentrated to 2 ml using a rotavap (Heidolph) and cleaned up on a 25 g florisil column. The extract was run into the column and subsequently eluted using 200 ml of 7% diethyl-ether in pentane. The extract was then concentrated to 1 mL for sediments and 0.5 mL for the biota samples under a gentle flow of nitrogen and transferred to a vial for analysis.

Moisture content was determined gravimetrically after drying for 3 hours at 105 °C. Sediment organic matter content was determined gravimetrically after drying at 550 °C for 2 hours.

Analyses

Analytical procedures were published before (e.g.[68]). Invertebrate, sediment, water and fish food (1 µL) were injected on a Shimadzu GCMS2010 (GC) coupled to a GC-MS-QP2010 Ultra (MS) detector (Shimadzu's Hertogenbosch, the Netherlands). Column used was a 30m x 0.25 mm i.d. HT8 with a film thickness of 0.25 µm. Analysis was performed using Electron Impact (EI) in single ion monitoring (SIM) mode. Injection port and source temperatures were 250 and 200 °C respectively. Oven temperature program started at 90 °C, hold for 3 minutes, increased by 20 °C/min to 170 °C followed by an increase by 2.5 °C/min to 292. At the end of the program, a column was heated to 320 °C for 10 minutes. The following quantifier and qualifier ions were monitored respectively, 256 and 258 for PCB 28, 292 and 290 for PCB 52, 326 and 324 for PCB 101, PCB 112 and PCB118, 360 and 362 for PCB 153, 394 and 396 for PCB 180 and 197 and 314 for chloropyrifos.

Quality assurance

Recovery was between 80-110% for all compounds. Calibration curves consisted of 9 points within a range of 1-650 ng/mL. $R2 \geq 0.999$ was achieved for each calibration curve for all compounds. Limit of quantification of the PCBs and CPF depended on sample intake, which was typically <1 ng/L for water, <0.1 ng/L for sediment, <0.1ng/g fish food and between <0.03 ng/L and <10 ng/L for biota. Spiked concentrations ranged from 20% to 65% of the nominal concentrations. Water background concentrations were below <1 ng/L.

Table S1. Primers and cycle conditions used in the quantitative PCR reactions.

Target gene	Primers	Cycle conditions	qPCR Standards	References
16S rRNA	BACT1369F PROK1492R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec	*Escherichia coli* (genomic DNA)	69
nifH	nifHF nifHR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 63 °C – 45 sec, 72 °C 60 sec	*Pseudomonas stutzeri* DSM 4166 (genomic DNA)	70
amoA	amoA-1F amoA-2R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 55 °C – 45 sec, 72 °C 60 sec	*Nitrosospira multiformis* ATCC25196 (cloned gene fragment)	71
nosZ	nosZ2F nosZ2R	95 °C – 3min; 40 cycles of 95 °C – 15 sec, 65 °C – 30 sec, 72 °C 30 sec	*Pseudomonas nitroreducens* DSM 1650 (genomic DNA)	72
dsrA	DSRp2060F DSR4R	95 °C – 3min; 40 cycles of 95 °C – 40 sec, 55 °C – 40 sec, 72 °C 60 sec	*Desulfitobacterium sp.* (cloned gene fragment)	37
opd	3F 3R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 57 °C –3sec, 72 °C 60 sec	*Sphingomonas sp.* DSM 16637 (genomic DNA)	38

Table S2. Primers and cycling conditions used for targeting bacterial community present in sediment samples.

Primers	Sequence 5'– 3'	Cycle condition	References
27F-DegS	GTTYGATYMTGGCTCAG		73
338R-I	GCWGCCTCCCGTAGGAGT	95 °C – 2min; 30 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C – 60 sec	74
338R-II	GCWGCCACCCGTAGGTGT		74

Table S3. Average (SD) water quality values for dissolved oxygen (DO), salinity, conductivity, pH, temperature and ammonium with their range (min-max) for the bioaccumulation test for systems with and without enclosure over the 28d experimental period.

	DO (mg/L)	Range (mg/L)	Salinity (‰)	Range (‰)	Cond (mS/m)	Range (mS/m)	pH	Range	Temp (°C)	Range (°C)	Ammonium (mg NH_4-N /L)	Range (mg NH_4-N /L)
Enclosed single species	9.00 (0.66)	4.18-9.87	34.4 (0.4)	33.4-35.7	40.4 (0.7)	29.6-41.9	8.09 (0.32)	7.15-9.95	12.8 (0.2)	12.4-14.1	1.85 (2.07)	0.1-10.14
Mixed species	9.39 (0.43)	7.16-9.94	34.4 (0.5)	33.5-35.3	40.7 (0.48)	40.1-42.0	8.07 (0.10)	7.89-8.32	13.2 (0.2)	12.9-13.6	2.60 (2.25)	0.1-6.73

* Values outside detection range were not used for calculation.

Table S4. Average (SD) phytoplankton concentration (µg/L) and turbidity (NTU) values and range (min-max) for the bioaccumulation test for systems with and without enclosure over the 28d experimental period.

	Cyanobacteria (µg/L)	Range (µg/L)	Green algae (µg/L)	Range (µg/L)	Diatoms (µg/L)	Range (µg/L)	Turbidity (NTU)	Range (NTU)
Test 3	0.25 (0.23)	0-1.39	0.18 (0.30)	0-1.72	0.78 (0.99)	0.27-3.44	7.04 (6.61)	0.86-30.1
Test 3 mixed	0.27 (0.11)	0.05-0.5	0	0-0	0.71(0.31)	0.32-1.33	81.72 (114.65)	1.05-302.00

Table S5. Average and standard deviation (SD) of total bacterial, nifH, amoA, nosZ, dsrA and opd abundance in copies/gram wet sediment and percentages of the specific genes compared to the total bacteria at start of the pre-equilibration time (t=-69, n=3), at the end of the pre-equilibration time/start of experiment (t=0, n=3), pure peat after grinding, and at the end of the bioaccumulation test (t=35, n=4) at low, medium, and high organic matter content for Arenicola marina, Nereis virens, Macoma balthica, Corophium volutator and mixed species.

			Total bacteria		*nifH*			*amoA*		
Time	**OM content**	**Species**	**Average (copies/g wet sediment)**	**SD**	**Average (copies/g wet sediment)**	**SD**	**% of total abundance**	**Average (copies/g wet sediment)**	**SD**	**% of total abundance**
-69	Low		1.24E+07		3.85E+03		0.03	BDL[a]		
-69	Medium		1.09E+07	4.80E+06	2.21E+04	1.73E+04	0.20	2.51E+02	1.53E+02	0.0023
-69	High		7.75E+06	3.71E+06	2.66E+04	2.08E+04	0.34	2.71E+02	1.52E+02	0.0035
0	Low		3.49E+07	2.06E+07	3.00E+04	1.66E+04	0.09	3.37E+01		0.0001
0	Medium		1.52E+08	8.53E+06	7.67E+04	9.78E+03	0.05	7.47E+02	2.06E+02	0.0005
0	High		2.48E+07	4.66E+06	4.89E+03	4.44E+03	0.02	5.46E+01	1.31E+01	0.0002
0	Pure peat		2.54E+07	2.23E+07						
35	Low	*Arenicola marina*	1.24E+08	1.92E+08	1.99E+04	2.84E+04	0.02	1.33E+03	1.90E+03	0.0011
35	Low	*Nereis virens*	5.03E+08	3.58E+08	5.91E+04	4.46E+04	0.01	1.47E+04	1.03E+04	0.0029
35	Low	*Macoma balthica*	3.96E+08	2.12E+08	6.74E+04	4.56E+04	0.02	1.72E+04	1.27E+04	0.0043
35	Low	*Corophium volutator*	2.47E+08	2.14E+08	2.87E+04	2.16E+04	0.01	8.83E+03	5.14E+03	0.0036
35	Medium	*Arenicola marina*	3.24E+08	4.29E+07	9.22E+04	5.40E+04	0.03	2.46E+03	1.21E+03	0.0008
35	Medium	*Nereis virens*	5.54E+08	2.70E+08	9.27E+04	2.94E+04	0.02	5.81E+03	2.50E+03	0.0010
35	Medium	*Macoma balthica*	6.59E+08	3.39E+08	8.48E+04	2.08E+04	0.01	1.46E+04	2.10E+04	0.0022
35	Medium	*Corophium volutator*	4.94E+08	2.81E+08	1.43E+05	1.78E+05	0.03	6.49E+03	5.48E+03	0.0013
35	Medium	Mixed species	3.52E+08	6.73E+07	1.35E+05	5.75E+04	0.04	7.42E+03	3.35E+03	0.0021

[a] BDL=Below Detection Limit.

Table S5 continued.

			nosZ			*dsrA*			*opd*		
Time	**OM content**	**Species**	**Average (copies/g wet sediment)**	**SD**	**% of total abundance**	**Average (copies/g wet sediment)**	**SD**	**% of total abundance**	**Average (copies/g wet sediment)**	**SD**	**% of total abundance**
-69	Low		BDL[a]			BDL			BDL		
-69	Medium		BDL			BDL			BDL		
-69	High		1.01E+04	6.01E+03	0.13	BDL			BDL		
0	Low		BDL			BDL			BDL		
0	Medium		1.37E+06	1.31E+05	0.90	2.33E+03	7.68E+02	0.002	BDL		
0	High		3.77E+03		0.02	BDL			BDL		
35	Low	*Arenicola marina*	7.20E+05	1.31E+06		1.56E+04	2.45E+04		3.96E+04		
35	Low	*Nereis virens*	5.43E+06	3.89E+06	0.58	2.28E+05	3.49E+05	0.01	6.82E+04	3.52E+04	0.032
35	Low	*Macoma balthica*	7.84E+06	4.95E+06	1.08	2.09E+05	1.44E+05	0.05	1.22E+05	9.16E+04	0.014
35	Low	*Corophium volutator*	4.38E+06	3.37E+06	1.98	2.56E+04	2.00E+04	0.05	3.00E+04	1.58E+04	0.031
35	Medium	*Arenicola marina*	4.26E+06	2.33E+06	1.77	7.63E+05	2.54E+05	0.01	3.76E+04	2.56E+04	0.012
35	Medium	*Nereis virens*	5.60E+06	3.15E+06	1.31	6.68E+05	2.57E+05	0.24	2.71E+04	1.56E+04	0.012
35	Medium	*Macoma balthica*	9.59E+06	2.09E+06	1.01	4.72E+05	3.07E+05	0.12	1.13E+05	1.45E+05	0.005
35	Medium	*Corophium volutator*	4.91E+06	1.99E+06	1.46	9.21E+05	1.27E+06	0.07	4.60E+04	3.06E+04	0.017
35	Medium	Mixed species	3.38E+06	1.61E+06	0.99	2.98E+06	4.86E+05	0.19	2.29E+04	8.99E+03	0.009

[a] BDL=Below Detection Limit.

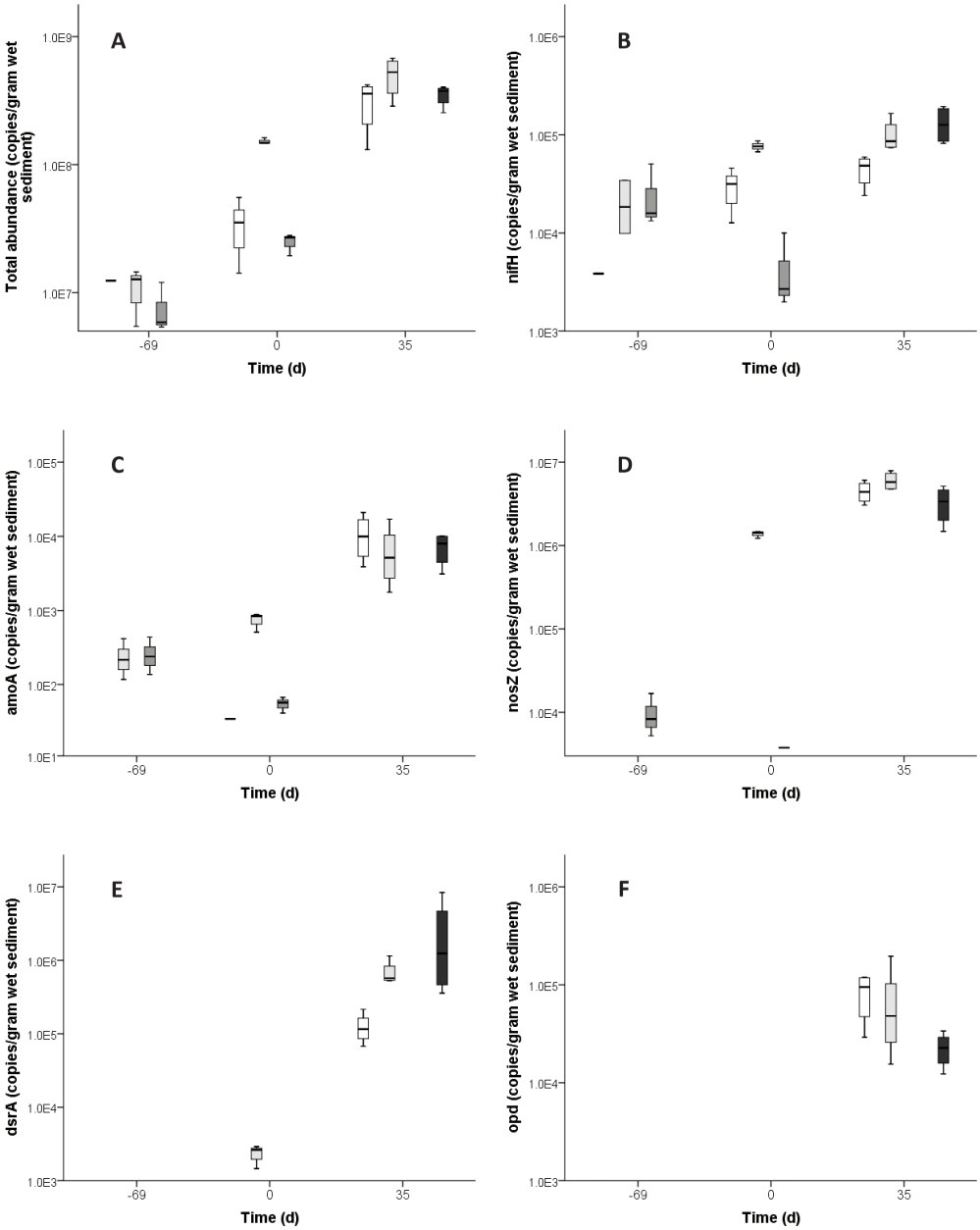

Figure S3. Gene abundances (copies/gram wet sediment for (A) total bacterial 16S rRNA gene (B) *nifH*, (C) *amoA*, (D) *nosZ*, (E) *dsrA* and (F) *opd*, at start of the pre-equilibration period (t=-69 d, n=3), at the end of the pre-equilibration period/start of experiment (t=0 d, n=3) and at the end of the bioaccumulation experiment (t=35 d, n=4) for low (white), medium (light grey), high (medium grey) and medium mixed species (dark grey) organic matter content. Y-axes is on log scale and note different scales on y-axes. For an overview of the pre-equilibration period only, check Figure S4.

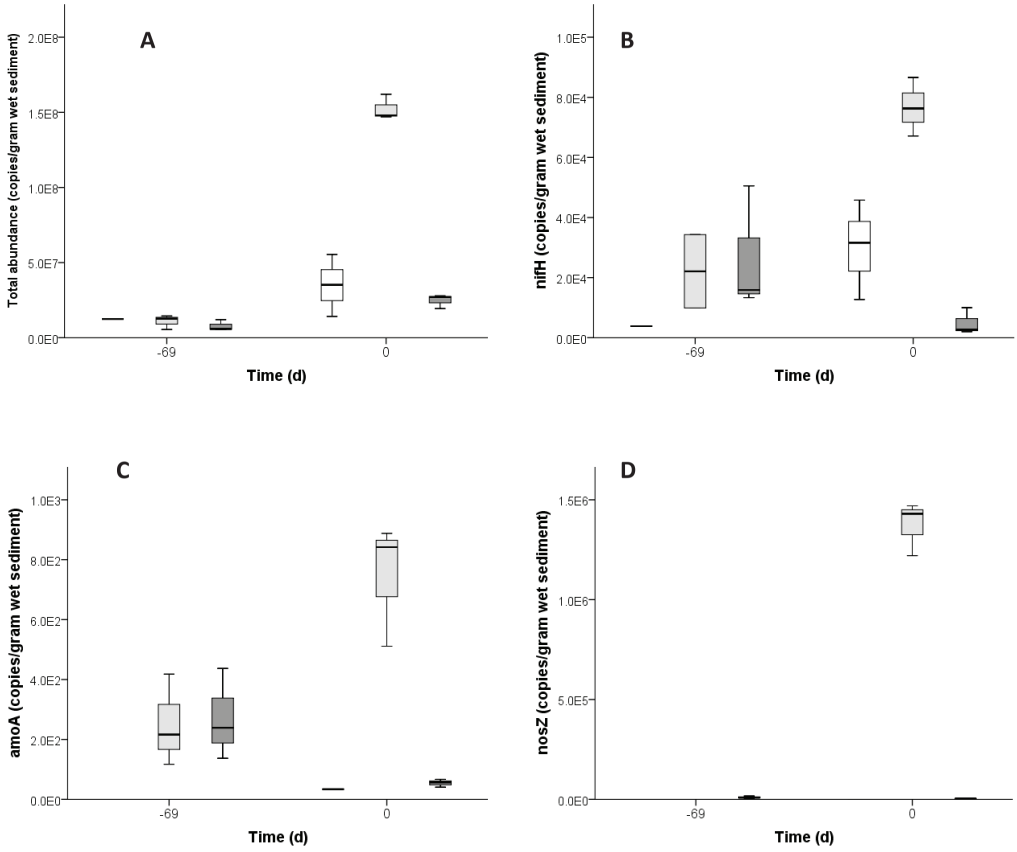

Figure S4. Total bacterial abundance (copies/gram wet sediment) (A), *nifH* abundance (copies/gram wet sediment) (B) *amoA* abundance (copies/gram wet sediment) (C) and *nosZ* abundance (copies/gram wet sediment) (D) at start of the pre-equilibration time (t=-69, n=3), at the end of the pre-equilibration time/start of experiment (t=0, n=3) for low (white), medium (light grey) and high (medium grey) organic matter content.

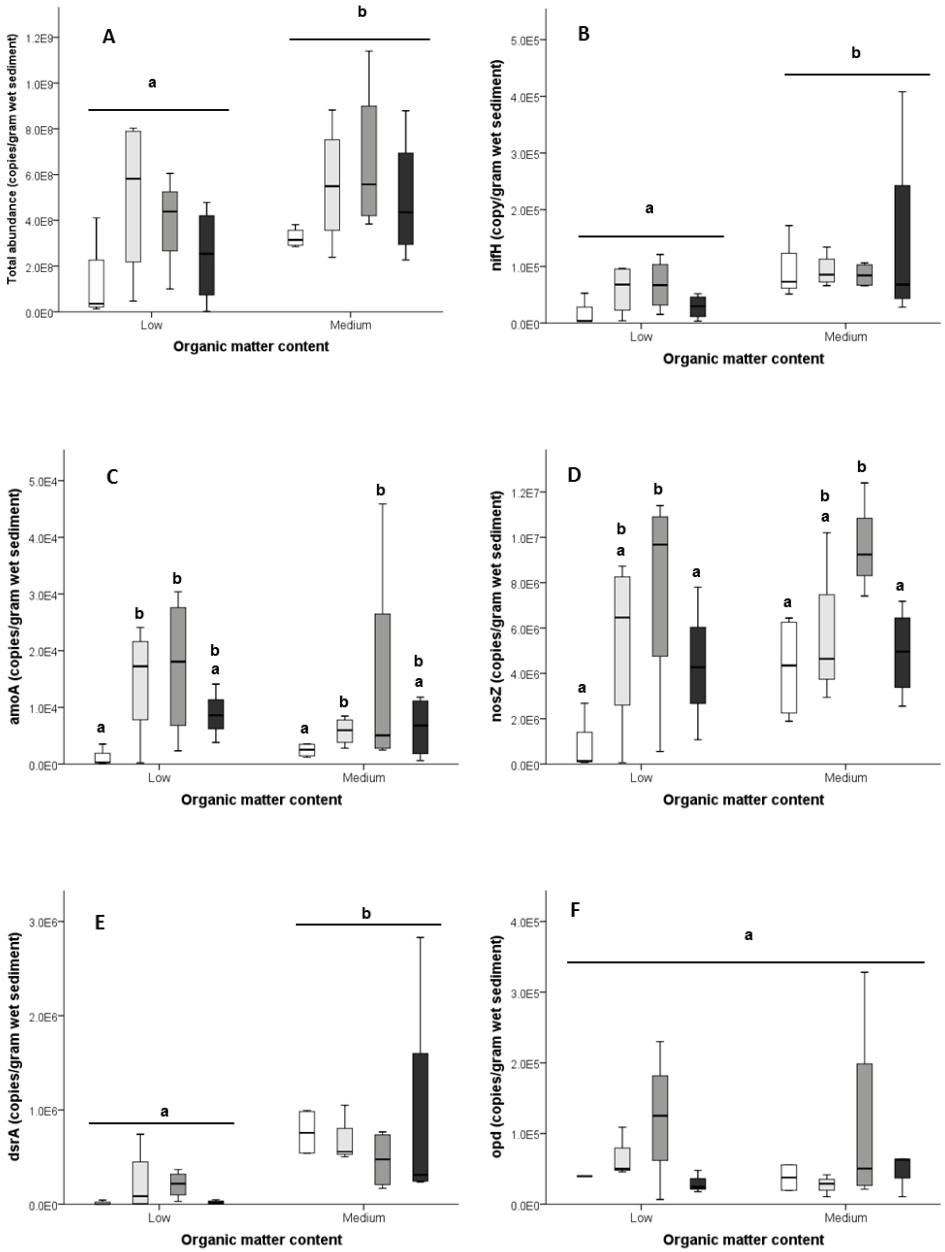

Figure S5. Total bacterial abundance (copies/gram wet sediment) (A), *nifH* abundance (copies/gram wet sediment) (B), *amoA* abundance (copies/gram wet sediment) (C), *nosZ* abundance (copies/gram wet sediment) (D), *dsrA* abundance (copies/gram wet sediment) (E) and *opd* abundance (copies/gram wet sediment (F) at the end of the bioaccumulation experiment (t=35 d, n=4) at low and medium organic matter content for *Arenicola marina* (white), *Nereis virens* (light grey), *Macoma balthica* (medium grey) and *Corophium volutator* (dark grey). Lines indicate no significant difference in abundance between species within a treatment. Small letters indicate significant differences in abundance between treatments (α=0.05).

Table S6. Overview of gene abundance in natural marine sediment.

Gene	Sediment	Abundance (min - max)	Unit	Remarks	Refs	This study (min-max)
16S	Marine	$2x10^7$ - $3x10^9$	copies/g wet sediment	Assumptions: 3.6 copies per cell. Density of sed 1.7 g/cm^3 [47]. No EMA treatment[a]	48	$7.8x10^6$ – $6.6x10^8$
16S	Marine	$4.7x10^7$ - $2.6x10^9$	copies/g wet sediment	Assumptions: 3.6 copies per cell	49	
amoA	Salt marsh	$5.6x10^4$ - $1.3x10^6$	copies/g wet sediment		53	$3.4x10^1$ – $1.7x10^4$
amoA (AOB)	Marine	$6.55×10^4$ - $3.26×10^7$	copies/g sediment		50	
nifH (group NB3)	Marine	$1.5x10^6$ - $1.5x10^8$	copies/g sediment		51	$3.9x10^3$ – $1.4x10^5$
nifH (group NB7)	Marine	$1X10^6$ - $1.5x10^8$	copies/g sediment		51	
dsrA (distribution of SRB)	Marine	$1.7x10^6$ - $2.8x10^8$	copies/g wet sediment	Assumptions: 1 copies per cell. Density of sediment 1.7 g/cm^3 [47]. No EMA treatment[a]	48	$2.3x10^3$ – $3.0x10^6$
dsrA (distribution of SRM)	Marine	$8x10^5$ (min) $5.1x10^7$ (mean)	copies/g wet sediment	Assumptions: 1 copies per cell	49	
nosZ	Estuarine wetland	$1.9x10^6$ - $2.9x10^7$	copies/g dry soil	Assumption: fraction of water 0.9 (in first 1 cm)	52	$3.8x10^3$ – $9.6x10^6$

[a] Ethidium monoazide (EMA) is a specific treatment to avoid the qPCR quantification of dead cells or free DNA.

Table S7. Difference of total bacterial, *nifH, amoA, nosZ* and *dsrA* abundance and Shannon diversity index between start (t=0) and end (t=35) of bioaccumulation test was tested with an independent t-test. *opd, nosZ* and *dsrA* for low OM could not be tested as values at t=0 were below detection limit. Values between brackets show degrees of freedom. Reported p values are two-tailed and significant values are shown in bold.

	Total abundance		*nifH*		*amoA*		*nosZ*		*dsrA*		Shannon	
	t	p	t	p	t	p	t	p	t	p	t	p
Low OM	(5) 3.642	**0.015**	(5) 1.245	0.268	(3) 1.363	0.266					-8.343	**0.000**
Medium OM	(3.018) 4.063	**0.027**	(5) 1.028	0.351	(5) 1.652	0.159	(3.060) 6.043	**0.009**	(5) -3.999	**0.01**	-1.089	0.352

Table S8. Effect of organic matter and species on total bacterial, *nifH, amoA, nosZ, dsrA* and *opd* abundance at the end of the bioaccumulation experiment (t=35 d) with a two way-ANOVA or Kruskal-Wallis. Values between brackets show degrees of freedom. p values in bold are significant.

	Total abundance[1]		*nifH*[2]		*amoA*[6]		*nosZ*[1]		*dsrA*[2]		*opd*[6]		Shannon[1]	
	F	p	X^2	p	F	p	F	p	X^2	p	F	p	F	p
OM	(1, 24) 4.424	**0.046**	(1) 7.225[3]	**0.007**	(1, 22) 0.000	0.995	(1, 22) 1.546	0.227	(1) 14.946[3]	**0.000**	(1, 15) 0.292	0.597	(1, 24) 7.607	**0.011**
Species	(3, 24) 2.605	0.075	(3) 3.588[4]	0.31	(3,22) 3.525	**0.032**	(3, 22) 4.876	**0.010**	(3) 4.367[4]	0.224	(3, 15) 0.68	0.578	(3, 24) 1.119	0.361
OM x Species	(3, 24) 0.286	0.835	(3) 0.728[5]	0.867	(3, 22) 0.894	0.460	(3, 22) 0.361	0.782	(3) 2.184[5]	0.535	(3,15) 0.368	0.777	(3, 24) 1.123	0.360

[1]Analyese were done with a two way-ANOVA.

[2]*nifH* and *dsrA* were analyzed with the Kruskal-Wallis test, tests were done for difference in OM[3], differences in species at low OM[4] and medium OM[5].

[6]*amoA* and *opd* were log transformed to meet the normality assumption.

Table S9. Effect of single species versus mixed species at medium organic matter at the end of the bioaccumulation experiment (t=35 d) on total bacterial, *nifH, amoA, nosZ, dsrA* and *opd* abundance tested with an independent t-test or Kruskal-Wallis. Values between brackets show degrees of freedom. Reported p values are two-tailed.

Total abundance[1]		*nifH*[2]		*amoA*[1]		*nosZ*[1]		*dsrA*[3]		*opd*[2]	
t	p	t	p	t	p	t	p	X^2	p	t	p
(6) 1.661	0.148	(6) -0.887	0.409	(6) -0.013	0.990	(6) 2.421	0.052	(1) 0.333	0.564	(6) 1.552	0.172

[1]Analyese were done with the independent t-test.

[2]*nifH* was log transformed to meet the normality assumption.

[3]*dsrA* was analyzed with the Kruskal-Wallis test.

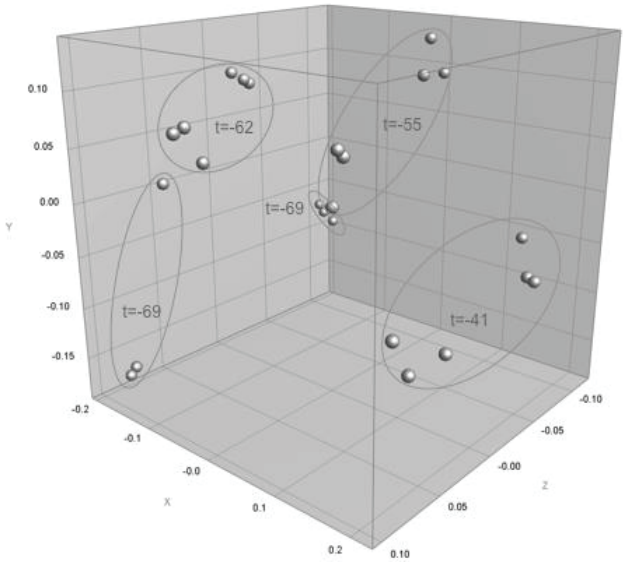

Figure S6. MDS plot of the DGGE profiles obtained from control and spiked artificial sediments (medium OM content) during the pre-equilibration period. Samples were analyzed in triplicate and all replicates are represented.

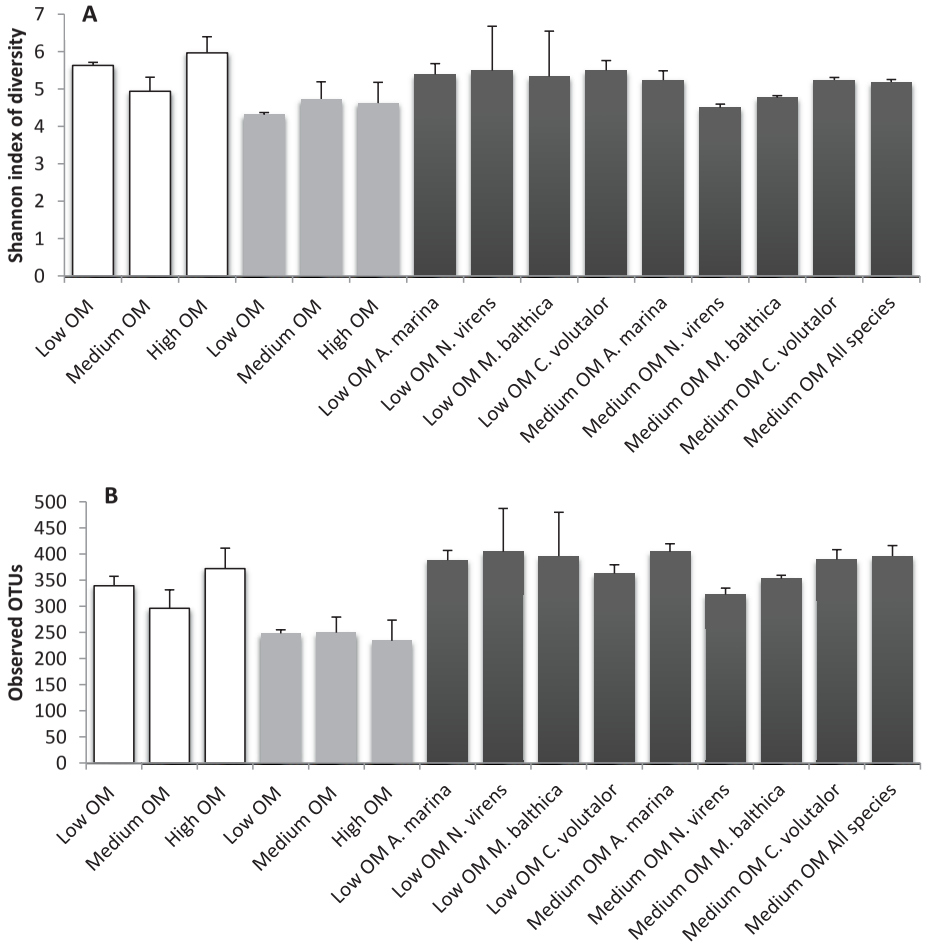

Figure S7. Shannon index of diversity (A) and observed OTUs (B) of the sediment samples collected during the experiment. White bars represent beginning of pre-equilibration period (t=-69 d), whereas light grey bars represent end of the pre-equilibration period (t=0 d). Dark grey bars represent end of the bioaccumulation test (t=35).

Table S10. Relative abundance of bacterial phyla detected in sediment samples based on 454 pyrosequencing of 16S rRNA gene fragments, during the pre-equilibration period. All phyla contributing to less than 1% of the total bacteria were grouped as 'Other'.

Bacterial phyla	Low OM t=-69	Medium OM t=-69	High OM t=-69	Low OM t=0	Medium OM t=0	High OM t=0
Proteobacteria	83.1	81.9	49.6	76.5	82.5	66.8
Acidobacteria	8.7	11.4	27.1	0.9	1	1.2
Bacteroidetes	3.9	0.3	0.6	21.5	15.2	21.8
Actinobacteria	3.3	4.8	13.3	0.3	1.1	7.7
WPS-2	0.4	0.7	1.9	0	0.1	0.2
Planctomycetes	0.3	0.3	1	0	0	0
Firmicutes	0.1	0.3	5.6	0.1	0	2.3
Other	0.2	0.3	0.9	0.6	0	0

Table S11. Relative abundance of bacterial phyla detected in sediment samples based on 454 pyrosequencing of 16S rRNA gene fragments at the end of bioaccumulation test (t=35 d). All phyla contributing to less than 1% of the total bacteria were grouped as 'Other'.

Bacterial phyla	Low OM Aronicola	Low OM Corophium	Low OM Macoma	Low OM Nereis	Medium OM Aronicola	Medium OM Corophium	Medium OM Macoma	Medium OM Nereis	Medium OM All animals
Proteobacteria	71.7	70	71.8	67.6	64.5	62.9	63.5	59.5	65.4
Bacteroidetes	25.2	24.5	23.7	26.3	32.8	31.4	31.4	37	30.4
Firmicutes	0.6	2.7	2.3	0.8	0.9	3.4	2.3	1.3	0.8
Acidobacteria	0.3	0.4	0.2	0.3	0.4	0.5	0.3	0.5	0.5
Actinobacteria	0.3	0.4	0.2	0.2	0.7	1.2	0.7	0.8	0.7
Fusobacteria	0.3	0	0	2.5	0.1	0	0	0.1	1.4
Planctomycetes	0.2	0.1	0.2	0.5	0.1	0.2	0.7	0.2	0.2
Other	1.4	1.7	1.6	1.8	0.4	0.4	0.8	0.6	0.5

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Chapter 5

Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms

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Abstract

The main objective of the present study was to assess the ecological impacts of the fluoroquinolone antibiotic enrofloxacin on the structure and functioning of tropical freshwater ecosystems. Enrofloxacin was applied at a concentration of 1, 10, 100 and 1000 μg/L for 7 consecutive days in 600-L outdoor microcosms in Thailand. The ecosystem-level effects of enrofloxacin were monitored on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoint groups for 4 weeks after the last antibiotic application. Enrofloxacin was found to dissipate relatively fast from the water column (half-dissipation time: 11.7 h), and about 11% of the applied dose was transformed into its main by-product ciprofloxacin after 24 h. Consistent treatment-related effects on the invertebrate and primary producer communities and on organic matter decomposition could not be demonstrated. Enrofloxacin significantly affected the structure of leaf-associated bacterial communities at the highest treatment level, and reduced the abundance of ammonia-oxidizing bacteria and ammonia-oxidizing archaea in the sediments, with calculated NOECs of 10 and <1 μg/L, respectively. The ammonia concentration in the microcosm water significantly increased in the highest treatment level, and nitrate production was decreased, indicating a potential impairment of the nitrification function at concentrations above 100 μg/L. The results of this study suggest that environmentally relevant concentrations of enrofloxacin are not likely to result in direct or indirect toxic effects on the invertebrate and primary producer communities, nor on important microbially mediated functions such as nitrification.

Introduction

Antibiotics used in human and veterinary medicine can enter aquatic ecosystems directly, through the discharge of waste water treatment plant effluents or aquaculture residues, or indirectly, by leaching and runoff of agricultural soils amended with manure from livestock facilities (Ternes et al., 2004, Sarmah et al., 2006 and Rico et al., 2014). Over the last few years, a considerable amount of work has been done on assessing the occurrence and environmental fate of antibiotics in the aquatic environment, indicating that measured water concentrations are, in most cases, relatively low (i.e. from 0.001 μg/L to about 10 μg/L) (Kümerer, 2009). Acute and chronic laboratory studies suggest that antibiotics are not expected to result in direct toxic effects on fish and aquatic invertebrates at environmentally relevant concentrations (Robinson et al., 2005 and Park and Choi, 2008). However, several experiments indicated that cyanobacteria and non-phototrophic microbial communities could be affected by antibiotic pollution at concentrations that are orders of magnitude lower than the threshold concentrations derived from toxicity data for standard test species (Maul et al., 2006, Ebert et al., 2011, Yergeau et al., 2012 and Wunder et al., 2013). Possibly, effects of antibiotics on cyanobacteria could affect the community structure of primary producers, which might propagate to primary and secondary consumers (Rico et al., 2014). Furthermore, the disruption of important ecosystem processes such as organic matter mineralization (Maul et al., 2006), nitrification (Klaver and Mattews, 1999), and/or degradation of organic pollutants (Näslund et al., 2008) could result in changes in water quality and might induce additional stress to aquatic organisms. To date, our knowledge on the effects of antibiotics on ecological interactions is still very limited and, therefore, further research needs to be undertaken to assess the potential side effects of antibiotics on ecological functions and on the structure of aquatic communities in multitrophic systems.

Model ecosystem studies (i.e., microcosms and mesocosms) have been used in the risk assessment of pesticides and veterinary medicines since they provide more ecological realism than laboratory bioassays and allow the identification of potential interactions between aquatic communities and ecosystem functions (Van den Brink et al., 2005). The number of studies evaluating the fate and effects of antibiotics on aquatic model ecosystems is very limited, and all of them have been performed under temperate climatic conditions (e.g. Wilson et al., 2004, Knapp et al., 2005 and Maul et al., 2006). Recent monitoring studies have detected antibiotic residues in several rivers impacted by urban and intensive animal production in (sub-)tropical regions of Asia (Yang et al., 2010, Shimizu et al., 2013 and Rico et al., 2014), suggesting that the study of the potential ecotoxicological effects of antibiotics in the tropical zone requires further attention.

The main objectives of the present study were (i) to get a better understanding on the potential direct and indirect toxic effects of antibiotic pollution on tropical aquatic ecosystems, (ii) to identify sensitive structural and functional endpoints for the risk assessment of antibiotics, and (iii) to assess whether the use of threshold concentrations derived from laboratory toxicity data would result in a sufficient level of protection for tropical aquatic ecosystems. For this, we assessed the effects of the fluoroquinolone antibiotic enrofloxacin on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoint groups in outdoor freshwater microcosms in tropical Thailand. Enrofloxacin was chosen as test compound because of its broad use in livestock and aquaculture production in tropical countries (e.g. Lampang et al., 2007 and Rico et al., 2013), and because of the availability of data on its environmental fate and aquatic toxicity (Knapp et al., 2005, Robinson et al., 2005, Park and Choi, 2008, Ebert et al., 2011 and Rico et al., 2014). In our study, enrofloxacin was applied in daily pulses for a period of 7 days to eutrophic microcosms, simulating exposure patterns in tropical ecosystems receiving aquaculture effluents that contain enrofloxacin residues (Rico and Van den Brink, 2014). Enrofloxacin shows antibacterial activity against a broad spectrum of (Gram-positive and Gram-negative) bacteria and is believed to act by inhibiting bacterial DNA gyrase or topoisomerase IV, thus preventing bacterial DNA synthesis and reproduction (Hooper, 1999). Under environmental conditions, enrofloxacin is rapidly de-ethylated to form ciprofloxacin (Knapp et al., 2005), which is an antibiotic that has been listed as critically important for its use in human medicine (WHO, 2011). The occurrence of antibiotics such as enrofloxacin and ciprofloxacin in the environment has raised concerns about their selective pressure on clinically relevant bacteria and the development of antibiotic resistance (Suzuki and Hoa, 2012), and therefore the assessment of their degradation and transformation under tropical conditions adds crucial information to perform refined exposure assessments.

Material and methods

Experimental design

The present experiment was performed in ten outdoor microcosms at the Faculty of Fisheries of Kasetsart University (KU, Bangkok, Thailand). Each microcosm consisted of a PVC tank (top diameter: 122 cm; bottom diameter: 101 cm; total depth: 80 cm; water depth: 63 cm; water volume: 600 L) initially filled with approximately 3 cm of silica-based fine gravel (1–2 mm diameter) extracted from natural rivers in the north of Thailand, and tap water pre-stored for 1 week to allow dissipation of possible chlorine residues. An aeration system was installed in each microcosm in order to provide mixing of the water during the experimental period. The experiment was performed during March and April 2012 (dry season). The weather conditions during the experimental period were: air temperature 32 (24–40) °C (mean, minimum–maximum), relative humidity 63 (50–75) %, and daily precipitation 1.7 (0–37) mm (rained on 19% of days) (Don Muang Weather Station, Bangkok, Thailand). The microcosms were stocked with plankton and macroinvertebrates collected from freshwater outdoor tanks located at the Ornamental Fish Facilities of KU, from a water reservoir at KU, from the water canal located at the Asian Institute of Technology (AIT, Bangkok, Thailand) described in Daam and Van den Brink (2011), and from outdoor freshwater tanks located at the hatchery of the AIT. These sampling sites were selected because they were uncontaminated sources that showed a relatively high biodiversity of phytoplankton and invertebrates native to Thailand. The stock of the macroinvertebrates was made up by distributing the same number of animals into each microcosm, and the stock of plankton by introducing equal volumes of concentrated plankton sample into each microcosm. The planktonic and macroinvertebrate communities were allowed to establish themselves for a period of 4 weeks prior to the application of the test substance. During this period, water was exchanged between microcosms biweekly in order to homogenize the structure of the communities between the systems. Nitrogen (1.4 mg/L as urea) and phosphorus (0.18 mg/L as triple super phosphate) were added biweekly to the systems according to the recommendations provided by Daam and Van den Brink (2011) during the entire experimental period. The resulting experimental systems were plankton dominated and showed a high eutrophication level, mimicking uncontaminated aquatic systems receiving nutrient-rich effluents from aquaculture or livestock production areas which may be contaminated by antibiotic residues.

Application of the test substance

Enrofloxacin was applied to the microcosms in daily pulses (at around 4 pm) at a nominal concentration of 1, 10, 100 and 1000 μg a.i./L during a period of seven days (starting on April 3, 2012). The selected dosing scheme tried to simulate exposure regimes in aquatic ecosystems resulting from antibiotic treatments used in aquaculture or

livestock production. The enrofloxacin application was performed in eight microcosms in duplicate replicated treatments, while the remaining two microcosms were used as controls. Enrofloxacin stock solutions (667 mg/L) were prepared daily with enrofloxacin powder purchased from Sigma–Aldrich (purity ≥98%, Lot Number: 0001369030). In order to dissolve the enrofloxacin crystals, the weighted amount of the compound was introduced with distilled water in a volumetric flask and sonicated for 30 min at 45 °C. Subsequently, 200 μL of ammonia solution (25%, v/v ammonia) were introduced in the volumetric flasks. The solutions were shaken gently by hand and then sonicated for another 15–30 min under the same temperature conditions until the compound was completely dissolved. Dosing solutions of 0.60, 6.03, 60.3, and 603 mg/L were created by diluting aliquots of the stock solutions in 1 L of distilled water. Finally, the prepared dose solutions were poured over the water surface of the microcosms and mixed by stirring with a wooden stick.

Sampling and analytical verification

The concentration of enrofloxacin and ciprofloxacin (main by-product of enrofloxacin) were determined in water samples collected approximately 30 min after the first application, 24 h after the first application (prior to the second application), approximately 30 min after the last application (i.e., seventh application), 2 days after the last application, and 7 days after the last application. Depth-integrated water samples (500 mL) were collected with a Perspex tube and stored in the fridge (4 °C) for a maximum period of 24 h until analysis.

On the day of the analysis, internal standard (Norfloxcin-D5) was added to 1 mL sub-samples of the cosm water samples in order to reach a concentration of 5 μg/L. Subsequently, the sub-samples were filtered through a nylon membrane with 0.22 μm pore size and transferred into glass vials. Enrofloxacin and ciprofloxacin were analyzed by high-performance liquid chromatography (HPLC) using a Waters 2695 Alliance HPLC Separation Module. The chromatographic separation was performed by means of a Shiseido Capcell Pak C18 column (150 mm × 2 mm; 3 μm) at 30 °C. The mobile phase was formed by (A) 50 mM ammonium acetate (pH = 3.0) and (B) acetonitrile, and the flow rate was set to 0.2 mL/min. The mobile phase composition for the separation method lasted for 15 min with the following elution gradients: 90% A, to 70% A in min 3, to 40% A in min 5, to 10% A in min 6, held for 4 min, to 90% A in min 10 and held for 5 min. Sample injection volumes were 20 μL. The detection was performed by MS/MS using a Quattro Ultima (Micromass, UK, Ltd.) triple stage quadrupole mass spectrometer with the following conditions: ionization mode ESI+, capillary voltage of 3.0 kV, cone voltage of 50 V, source temperature of 120 °C, desolvation temperature of 350 °C, and nitrogen gas flow of 50 L/h in the cone and 600 L/h in the desolvation. The detection limit for both antibiotics in the water samples was 0.1 μg/L. The calculated

recoveries of the analytical method (at a concentration of 10 μg/L) were 89 ± 2% for enrofloxacin, and 104 ± 3% (mean ± SD; n = 3) for ciprofloxacin. The measured concentrations in the cosm water samples were corrected for the method recovery.

Water quality

Dissolved oxygen (DO), pH, electrical conductivity (EC) and temperature (T) were monitored on days 7 and 1 before the antibiotic treatment, 1 h after the first antibiotic application, and on days 2, 7, 9, 14, 21 and 28 after the first antibiotic application. Measurements were made in the morning (at 8 am) and at the end of the afternoon (around 6 pm) at an approximate water depth of 10 cm. DO, pH and T were measured with a HQ40d multimeter and EC with an EC-meter (Eijkelkamp 18.28).

Alkalinity levels and the concentration of ammonia, nitrite, nitrate and total phosphorus were measured in microcosm water samples collected on the same days as the other water quality parameters, except for day 9 after the first antibiotic application. A depth-integrated water sample (1 L) was collected with a Perspex tube and stored at 4 °C until analysis. Analysis of the alkalinity and nutrient concentrations was performed according to the methods described in APHA (2005).

Phytoplankton and zooplankton

Phytoplankton and zooplankton samples were taken on day 7 and 1 day before the start of the antibiotic treatment, and on days 2, 7, 9, 14, 21 and 28 after the first antibiotic application. Depth-integrated water samples of 5 L were collected using a Perspex tube and were passed through a plankton net with a mesh size of 20 μm for phytoplankton, and 55 μm for zooplankton. The 5 L water samples were concentrated to an approximate volume of 100 mL. Subsequently, the concentrated samples were fixated with Lugol's iodine solution and stored at 4 °C until further identification.

Sub-samples (200 μL) of the concentrated phytoplankton samples were analyzed with an inverted microscope (400×). Phytoplankton taxonomy was determined to the lowest practical level, and the species or genus densities were calculated as the number of individuals per litre of microcosm water. In addition, the chlorophyll-a content of the phytoplankton was used as a proxy for the phytoplankton biomass in the microcosm water. For the analysis of the chlorophyll-a, 150 mL of the microcosm water was filtered through a Whatman GF/C glass-fibre filter (mesh size: 1.2 μm). Chlorophyll-a samples were extracted according to the acetone extraction procedure described in APHA (2005).

Cladocerans, ostracods and copepods were counted in the concentrated zooplankton sample using a binocular microscope with a magnification of 15–25×. Furthermore,

a sub-sample (1–2 mL) of the zooplankton sample was taken for the identification of rotifers and copepod nauplii using an inverted microscope (magnification 100×). Rotifers and cladocerans were identified to the lowest practical taxonomic level. Copepods were identified to suborder (i.e., calanoids or cyclopoids), and a distinction was made between nauplii stages and the more mature stages. Ostracods were not further identified. The number of individuals of each species was re-calculated to numbers per liter of microcosm water. The phytoplankton and zooplankton species identification was made by using several taxonomic classification keys for tropical aquatic organisms (e.g. Wongrat, 2000 and Fernando, 2002).

Periphyton

The effects of the treatment on the periphyton community were assessed by measuring the chlorophyll-a content of the periphyton biomass on artificial substrates. Three series of 5 microscopic glass slides (7.5 cm × 2.5 cm) were introduced at a water depth of 30 cm in each microcosm 7 days before the first antibiotic application. On days 7, 14 and 28 after the first antibiotic application, a glass slide series was retrieved and the attached periphyton was collected by scraping them (in 0.5 L of water) until slides were visually clean. The chlorophyll-a in the water containing the scraped periphyton was measured according to APHA (2005). Finally, the mass of chlorophyll-a per square centimetre of glass slide was calculated by dividing the total chlorophyll-a content of the water sample by the area of the glass slide that was scraped.

Macroinvertebrates

The diversity and abundance of macroinvertebrate organisms were monitored by using pebble stone baskets that served as artificial substrates. Two pebble baskets (20 cm × 20 cm × 10 cm) were placed on the sediment's surface of each microcosm 3 weeks before the antibiotic treatment. Macroinvertebrates were sampled 1 day before the start of the antibiotic treatment, and on days 2, 9, 14, 21 and 28 after the first antibiotic application. The artificial substrates were sampled alternately. On each sampling day, one of the substrates was gently lifted from the sediment and directly enveloped by a net (51 cm × 38 cm; mesh size: 0.5 mm). The substrates were gently shaken inside of the net to collect the invertebrates inhabiting the substrates. Moreover, the net was passed through the water column next to the tank's wall covering approximately one quarter of the walls' surface in order to catch swimming macroinvertebrates. The collected invertebrates were introduced in a white plastic tray, where they were identified and counted alive. Finally, the counted invertebrates were released back into their original microcosm.

Organic matter decomposition

In order to study the effects of the antibiotic treatment on microbial organic matter decomposition, three litter bags containing approximately 2 g of *Musa* (banana) leaves

were introduced in each microcosm 1 day before the first antibiotic application. First, the banana leaves were leached in tap water for 2 days and dried in the oven at 70 °C for 48 h. A known weight (approximately 2 g) of the dried banana leaves was introduced into nylon bags (mesh size: 0.5 mm). The litter bags were suspended at an approximate water depth of 30 cm in the microcosms. One litter bag was retrieved from each microcosm on days 7, 14 and 28 after the start of the treatment. The decomposed material was dried at 70 °C for 48 h and weighted. The percentage of organic matter decomposition was calculated by comparing the initial dry weight of the banana leaves (before introduction into the microcosms) and the final dry weight after the incubation period in the microcosms.

Microorganisms

Changes in bacterial community structure present on leaf material and sediment were monitored after antibiotic application. *Musa* leaves were dried at 70 °C for 48 h and introduced into nylon bags (mesh size: 0.5 mm). Two nylon bags were hung at 30 cm depth in each microcosm seven days before the first antibiotic application. The nylon bags were retrieved from the microcosms on days 7 and 14 after the first antibiotic application. The nylon bags were opened and leaves were carefully transferred into plastic bags. Integrated sediment samples (3 cm) were collected from each microcosm on days 7, 14 and 21 after the first antibiotic application, and were introduced into plastic bags. Plastic bags containing the leaf and sediment material were frozen at −20 °C until further analysis.

Three leaf discs (1 cm diameter) were taken from every leaf sample collected, and a sub-sample of 2 g was collected from the sediment samples for microorganism analysis. Leaf discs and sediment sub-samples were subjected to total DNA extraction, using the FastDNA® Spin kit for Soil (MP Biomedicals, Santa Ana, CA) according to manufacturer's instructions (Mincer et al., 2005). The quality and quantity of the isolated DNA were checked by using a Nanodrop ND-100 spectrophotometer (Thermo Scientific, San Jose, CA). Before using the DNA samples in further experiments an equal dilution was made for all samples. The 16S rRNA gene was partially amplified (V1 to V2 region) by polymerase chain reaction (PCR). PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) according to Lin et al. (2012). Briefly, DGGE was performed on polyacrylamide gels with a denaturant gradient from 30 to 60% (100% denaturing acrylamide was defined as 7 M urea and 40% (v/v) formamide) using a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) (Muyzer et al., 1993). Aliquots of the PCR products were loaded on the gel and electrophoresis was carried out with 1× Tris–acetate–EDTA buffer (60 °C, 85 V) for 16 h. The resulting gels were silver-stained according to Sanguinetti et al. (1994) and scanned. Finally, the Bionumerics software version 4.61 (Applied Maths, Belgium) (Tzeneva et al., 2008) was

used for DGGE band detection and band intensity quantification. The results of this analysis were used to assess total operational taxonomic units (OTUs), as proxy for bacterial richness, and the relative intensity of the present bands, as a proxy for relative abundance (RA) of different OTUs (Massana and Jürgens, 2003).

Quantitative PCR (qPCR) was used to determine the abundance of total bacteria (16S rRNA gene), bacterial and archaeal ammonia oxidizers (*amoA* gene) and nitrogen-fixing bacteria (*nifH* gene) in the leaf and sediment samples. All qPCR reactions were performed in a 384-well plate (Bio-Rad) using a CFX384 Real-Time PCR Detection System (Bio-Rad). All samples were analyzed in triplicate, and reactions were carried out in a total volume of 10 µL. Single qPCR reactions were prepared using 5 µL of iQ SYBR Green super mix (Bio-Rad), 0.4 µL of forward and reverse primers (10 µM), 0.1 µL of BSA (20 mg/mL), 0.1 µL of VisiBlue™ qPCR mix colorant (TATAA Biocentre) and 4 µL of DNA (1.25 µg/mL). Primer combinations and cycle conditions are described in Table 1. At the end of each qPCR run, a melting curve analysis was performed from 60 to 99 °C with an increase of 0.5 °C every 10 s. Purity of the qPCR products was checked by the observation of a single peak on the melting curve, while correct size amplification was confirmed on a 1% (w/v) agarose gel. For each qPCR reaction a standard curve comprising 10 serial 10-fold dilutions of the target gene was created. Standards were obtained by amplifying the target genes from the following sources: *Escherichia coli* (16S rRNA gene), *Nitrososphaera viennensis* (archaeal *amoA* gene), *Nitrosospira multiformis* (bacterial *amoA* gene) and *Pseudomonas stutzeri* (bacterial *nifH* gene).

Table 1. Primers and cycle conditions used in the quantitative PCR reactions.

Target gene	Primers	Cycle conditions	References
16S rRNA	BACT1369F PROK1492R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec.	Suzuki et al. (2000)
Archaeal *amoA*	Arch-amoAF Arch-amoAR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec.	Francis et al. (2005)
Bacterial *amoA*	amoA-1F amoA-2R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 55 °C – 45 sec, 72 °C 60 sec.	Rotthauwe et al. (1997)
nifH	nifHF nifHR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 63 °C – 45 sec, 72 °C 60 sec.	Rösch et al. (2002)

Data analysis

No observed effect concentrations (NOECs) were calculated for all water quality parameters, chlorophyll-a content of the phytoplankton and periphyton community, organic matter decomposition data, and for all taxa of phytoplankton, zooplankton and macroinvertebrates. Effects were considered to be consistent when they showed statistically significant deviations pointing in the same direction for at least two consecutive sampling days or occurred on a single sampling day during or immediately after the treatment period. The NOEC calculations were performed by using the Williams test (Williams, 1972), which assumes a monotonic increasing effect with increasing exposure dose. The Williams tests were performed with the Community Analysis computer program, version 4.3.05 (Hommen et al., 1994), using a significance level of 0.05. Prior to the analysis, the species abundance data and the OTU's RA dataset were ln(Ax + 1) transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x. This was done in order to down-weigh high abundance values and approximate a normal distribution of the data (for rationale see Van den Brink et al., 2000).

The phytoplankton, zooplankton and macroinvertebrate datasets were analyzed by the principal response curve (PRC) method (Van den Brink and Ter Braak, 1999) using the CANOCO Software package, version 5 (Ter Braak and Šmilauer, 2012). The PRC method is a specific type of redundancy analysis (RDA) that is able to explain the variation in species composition between replicate microcosms from the exposure to a stressor by including the treatment regime as explanatory variable, and the interaction between the treatment regime and the sampling times as covariables. The overall significance of the antibiotic treatment regime on the variation in species composition ($p \leq 0.05$) was tested by performing 499 Monte Carlo permutations (Van den Brink and Ter Braak, 1999). The significance of the antibiotic treatment regime per sampling date was calculated by performing single RDA permutation tests for the dataset of each sampling date separately using Ln-transformed treatment concentrations as explanatory variable. Finally, the NOEC values at community level were calculated for each individual sampling date by applying Williams test to the sample scores of the first principal component of each sampling date (for rationale see Van den Brink et al., 1996).

The use of the PRC method for the analysis of microbial data requires perfect alignment of the DGGE profiles obtained from different samples, which is a laborious and difficult task, and potentially introduces an extra source of variability to the dataset (Lin et al., 2012). For this reason, the statistical significance of the antibiotic treatment on the OTU and OTU's RA datasets derived from the bacterial DGGE profiles were analyzed by RDAs performed for each sampling date separately using the Ln-transformed

treatment concentrations as explanatory variables (Monte Carlo permutation test: 499 permutations; $p \leq 0.05$). In addition, principal component analysis (PCA) bi-plots were constructed in order to graphically show the within treatment variations. The PCA and RDA analyses were performed using the CANOCO Software package version 5 (Ter Braak and Šmilauer, 2012). Bacterial community NOECs were calculated for each sampling date following the same procedure as described above. The NOECs for the total bacterial abundance, abundance of bacterial and archaeal *amoA* gene, and abundance of the *nifH* gene were calculated with the Williams test ($p \leq 0.05$; Williams, 1972).

Results

Exposure concentrations

Measured enrofloxacin concentrations after the first application were, on average, 102% of the intended concentrations (range: 88–121%) (Fig. 1). Based on the enrofloxacin concentrations measured 24 h after the first application and the equations described in Hoang et al. (2012), a first-order half dissipation time (DT50) of 11.7 ± 1.35 h and a dissipation rate constant of 1.44 ± 0.17 d−1 (mean ± standard deviation) were calculated. The concentrations of enrofloxacin measured 2 days after the last application were below the detection limit, except for the treatment with 100 and 1000 µg/L, which were 1.8 and 292 µg/L, respectively. One week after the last application, all measured enrofloxacin concentrations fell below the detection limit, except for the highest treatment level (1000 µg/L), which had a concentration of 23 µg/L (Fig. 1). The calculated 7-day average concentrations of enrofloxacin in the treated microcosms were approximately 0.7, 7, 69 and 686 µg/L, for the lowest to the highest treatment level, respectively. Enrofloxacin was rapidly transformed into ciprofloxacin. Measured ciprofloxacin concentrations 24 h after the first enrofloxacin application were, on average, 11% of the applied dose. Seven days after the last enrofloxacin application, ciprofloxacin was detected only in the 100 and 1000 µg/L treatments at concentrations of 1.1 and 40 µg/L, respectively.

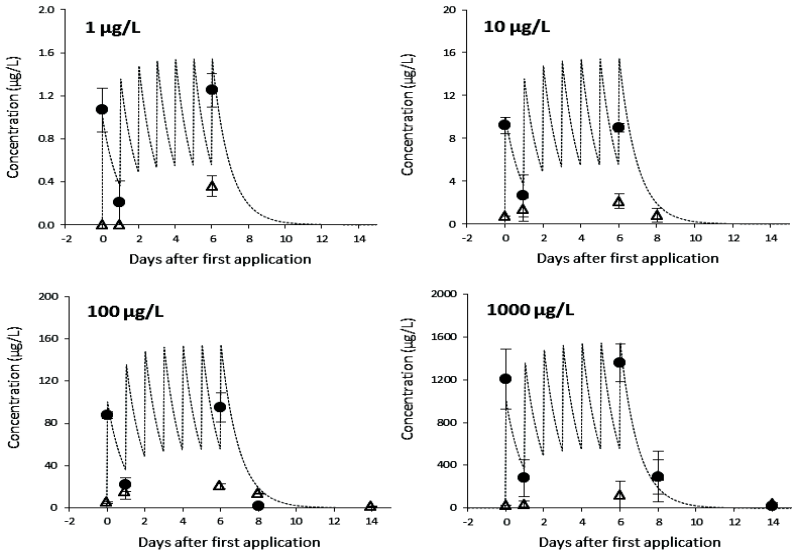

Figure 1. Measured water concentrations of enrofloxacin (dots) and ciprofloxacin (triangles) in the different treatments (mean ± standard deviation). The figure only displays those measured antibiotic concentrations that exceeded the detection limit of the analytical method (0.1 µg/L). The dashed line represents the theoretical enrofloxacin concentration in the microcosm water calculated with the first-order half dissipation time derived from the present study (DT50 = 16 h).

Water quality parameters

The daily average water temperature in the microcosms ranged between 30 and 35 °C during the experimental period. The water temperature gradually increased after the treatment period reaching a maximum water temperature of 38 °C on day 28 after the first antibiotic application (Fig. 2A and B). Average DO concentrations in the control microcosms ranged between 4.1 in the morning, to concentrations above the oxygen saturation level in the afternoon (average morning value: 5.5 mg/L; average afternoon value: 14 mg/L). On day 21 after the first antibiotic application, morning DO concentrations dropped to critical levels (below 2 mg/L) in some microcosms. The average daily oxygen production in the control microcosms (i.e., difference between morning and afternoon concentration) was 8.4 mg/L, denoting a very high primary productivity. A trend was observed towards lower DO concentrations and lower daily oxygen production in the highest treatment level (1000 µg/L), however, significant differences with the control treatment were only calculated for the oxygen production values after the second enrofloxacin pulse (Table 2 and Fig. 2C and D). The pH in the microcosms ranged between 8.0 and 10.7. Although a decrease in the pH was observed in the highest treatment level (1000 µg/L) during the treatment period, deviations to the controls were lower than 0.8 pH units and differences were not statistically significant

(Table 2 and Fig. 2). The measured EC and alkalinity levels during the whole experimental period were 259 (213–349) µs/cm and 90 (57–140) mg $CaCO_3$/L (mean, minimum–maximum), respectively. No treatment-related effects could be demonstrated for these two parameters during the experimental period (Table 2 and Fig. 2F).

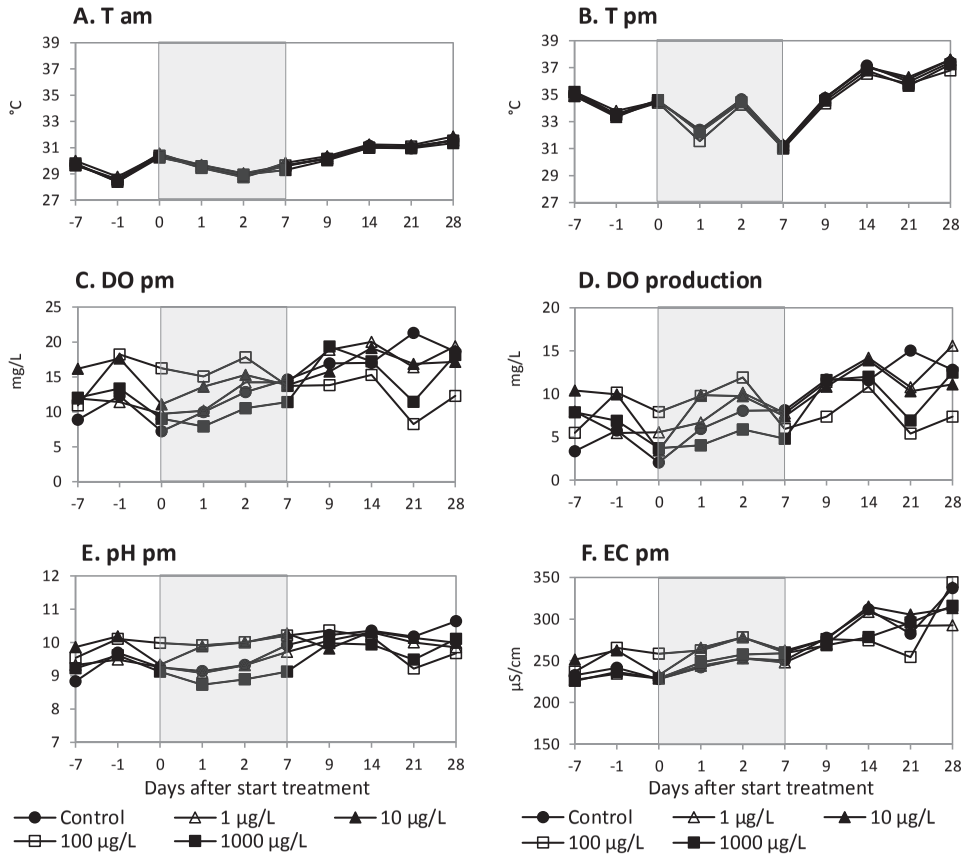

Figure 2. Water quality parameter dynamics measured during the experimental period. The figures show temperature (T) measured early in the morning (8 am) (A) and late in the afternoon (6 pm) (B), afternoon dissolved oxygen (DO) measurements (C) and dissolved oxygen production (difference between morning and afternoon levels) (D), and afternoon pH (E), and electric conductivity (EC) measurements (F). The shaded area indicates the treatment period.

Table 2. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) in µg/L (expressed in terms of nominal single-dose enrofloxacin concentration) for water quality parameters measured on each sampling date. The shaded area indicates the treatment period.

	Days after first application									
Endpoint	**-7**	**-1**	**0**	**1**	**2**	**7**	**9**	**14**	**21**	**28**
DO a.m.	>	>	>	>	>	>	>	>	>	>
DO p.m.	>	>	>	>	>	>	>	>	>	>
DO production	>	>	>	>	10 (↓)	>	>	>	10 (↓)	>
pH a.m.	>	>	>	>	>	>	>	>	>	>
pH p.m.	>	>	>	>	>	>	>	>	>	>
EC a.m.	>	>	>	>	>	>	>	>	>	>
EC p.m.	>	>	>	>	>	>	>	>	>	>
Alkalinity	>	>	NM	NM	>	>	NM	>	>	>
Ammonia	>	>	NM	NM	100 (↑)	100 (↑)	NM	100 (↑)	>	>
Nitrite	< 1 (↓)	>	NM	NM	>	>	NM	>	>	>
Nitrate	>	>	NM	NM	>	>	NM	>	>	1 (↓)
Total phosphorus	>	>	NM	NM	>	>	NM	>	>	>
Chlorophyll-a	>	>	NM	NM	>	>	NM	>	>	>

↑ = increase, ↓ = decrease, > = no significant effect (NOEC > 1000 µg/L), NM = not measured

Ammonia concentrations showed a significant increase at the highest treatment level (1000 µg/L) during the treatment period, and one week after the treatment period (Table 2 and Fig. 3A). The average ammonia concentrations on days 2, 7 and 14 after the first antibiotic application were 2.6, 3.2, and 1.0 mg/L in the highest treatment level (1000 µg/L), and 1.3, 0.7 and 0.4 in the controls, respectively. Nitrite concentrations were considerably higher in the control treatment samples than in the rest of the treatments during the pre-treatment and treatment period (Fig. 3B), and were found to decrease in the highest treatment level on days 2, 7, and 14 after the first antibiotic application, although the data did not show significant differences. Nitrate concentrations during the pre-treatment and treatment periods showed a high variability, with considerably higher values in the control (0.4–0.5 mg/L) and in the lowest antibiotic treatment (0.7–0.9 mg/L), compared to the other treatments (Fig. 3C). This variability could be visually associated to different periphyton or phytoplankton dominating states in the microcosms. Microcosms with high quantities of filamentous algae adhered to the walls of the tanks generally showed lower dissolved nitrate concentrations. A trend was observed towards lower nitrate concentrations in the highest treatment level during the antibiotic application period although, due to the high variability observed in the other treatment levels, significant differences could not be demonstrated (Table 2 and Fig. 3C). Total phosphorus concentrations ranged between 0.07 and 0.69 mg/L during the whole experimental period (average: 0.24 mg/L), and did not show any treatment-related significant variation (Table 2).

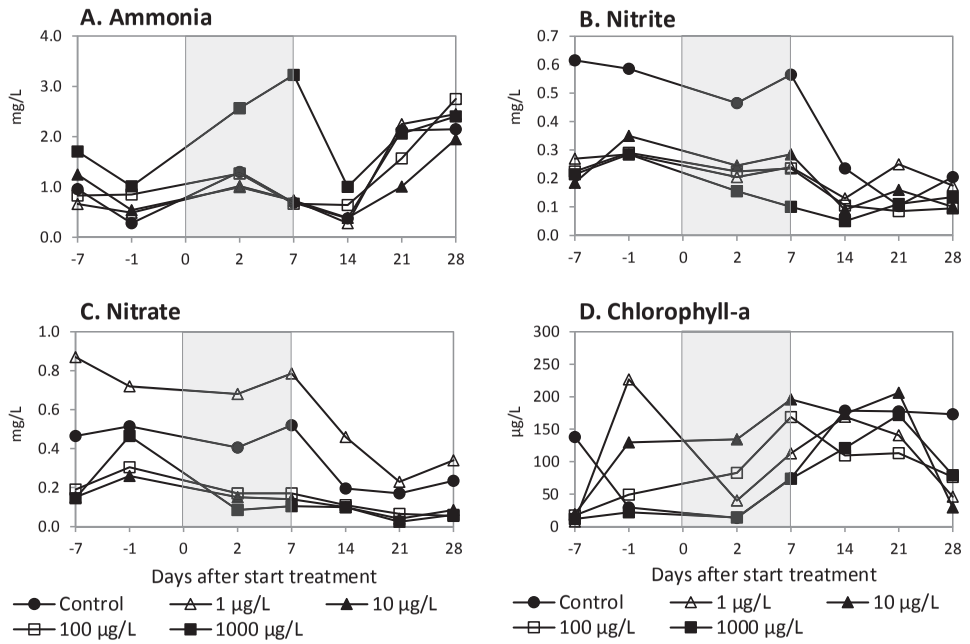

Figure 3. Ammonia (A), nitrite (B), nitrate (C), and chlorophyll-a (D) dynamics measured during the experimental period. The shaded area indicates the treatment period.

Phytoplankton community

Thirty-nine phytoplankton taxa were identified in the current study, belonging to 5 different taxonomic groups Chlorophyceae (20 taxa), Cyanobacteria (8), Bacillariophyceae (5), Desmidiaceae (3), Dinophyceae (2), and Euglenophyceae (1). The phytoplankton community was dominated by a limited number of taxa, and many occurred in low densities (<1 individual/mL) and/or were only observed on a limited number of sampling days (Table S1). The most abundant phytoplankton taxa in decreasing order were: *Chlorella sp.* (Chlorophyceae) and *Coelastrum sp.* (Chlorophyceae). The total phytoplankton abundance in the controls considerably decreased after the pre-treatment period, however, the relative abundance of species remained relatively constant (Fig. S1).

The total taxa richness observed on day 14 was slightly higher in all treated microcosms compared to the controls (Table 3 and Fig. S2A). These differences, however, occurred in one isolated sampling day and were very small (i.e., from 11 taxa in controls to 16 taxa in the 1000 µg/L treatment level) and, hence, a clear dose–response effect relationship could not be identified. The water concentration of chlorophyll-a in the microcosms was relatively high, indicating a high primary productivity in the systems, and increased during the treatment period. However, significant effects of the antibiotic

could not be demonstrated (Table 2 and Fig. 3D). The results of the PRC analysis did not show significant effects of the enrofloxacin treatment on the composition of the phytoplankton community ($p = 0.53$). Consistent statistically significant treatment-related effects were calculated for only 1 out of the 39 phytoplankton taxa. A *Scenedesmus* species showed a higher abundance at the three highest treatment levels compared to the controls (Table 3 and Fig. S2B,C).

Table 3. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) expressed in terms of nominal single-dose of enrofloxacin concentration (µg/L) for the phytoplankton, zooplankton, macroinvertebrate and microorganism endpoints evaluated. Only individual taxa that showed a treatment-related effect on at least one sampling date are included. The shaded area indicates the treatment period.

Endpoint	Day after first application								Note
	-7	-1	2	7	9	14	21	28	
Phytoplankton									
Community	>	>	>	>	>	>	>	>	
Total taxa richness	>	>	>	>	>	<1(↑)	>	>	Fig. S2A
Chlorophyta	>	>	>	>	>	>	>	>	
Scenedesmus sp. II	NP	>	>	>	10(↑)	<1(↑)	<1(↑)	>	Low density [a], Fig. S2B
Cyanophyta	>	>	>	>	>	>	>	>	
Desmidiaceae	>	>	>	>	>	>	>	>	
Diatomeae	>	>	>	>	>	>	>	>	
Diatom sp. IV	>	>	>	>	>	>	10(↑)	>	Low density [a], Fig. S2C
Dinoflagellata	>	>	NP	NP	NP	NP	NP	NP	
Euglenophyceae	>	NP	NP	NP	NP	NP	NP	NP	
Zooplankton									
Community	>	>	>	>	>	>	>	>	
Total taxa richness	>	>	>	>	>	>	>	>	Fig. S3A
Cladocera	>	>	>	>	>	>	1(↑)	>	Fig. S3B
Alonella sp.	>	>	>	100(↑)	>	>	>	>	Low density [b], Fig. S3C
Ceriodaphnia reticulata	>	>	<1(↓)	<1(↓)	100(↓)	>	>	>	Low density [b], Fig. S3D
Diaphanosoma senegal	>	>	>	>	100(↑)	>	>	>	Low density [b], Fig. S3E
Copepoda	>	>	>	>	>	>	>	<1(↑)	Fig. S3F
Nauplii	>	>	>	>	>	>	>	<1(↑)	Fig. S3G
Ostracoda	>	>	>	>	>	>	100(↑)	>	Fig. S3H
Rotifera	10(↑)	>	>	>	>	>	>	>	Fig. S3I
Brachionus angularis	>	>	>	>	>	>	>	100(↓)	Fig. S3J
Brachionus caudatus	>	>	>	>	>	100(↑)	>	>	Fig. S3K
Brachionus forficula	NP	>	NP	NP	>	>	>	<1(↓)	Low density [b], Fig. S3L
Filinia longiseta	NP	NP	NP	NP	>	>	100(↓)	>	Fig. S3M
Hexarthra sp.	>	>	>	>	>	100(↑)	10(↑)	>	Fig. S3O
Macroinvertebrates									
Community	NM	>	>	NM	>	>	>	>	
Total taxa richness	NM	<1(↑)	>	NM	>	>	>	>	Fig. S4A
Insecta	NM	>	>	NM	>	>	>	>	
Mollusca	NM	>	>	NM	>	>	>	>	
Melanoides tuberculata	NM	>	100(↑)	NM	>	NP	NP	NP	Low density [c], Fig. S4B
Physella acuta	NM	10(↑)	>	NM	>	100(↑)	>	>	Low density [c], Fig. S4C
Annelida	NM	NP	NP	NM	>	NP	NP	NP	
Microorganisms									
Leaf samples									
Bacterial OTUs	NM	NM	NM	100	NM	100	NM	NM	
Bacterial RA OTUs	NM	NM	NM	100	NM	100	NM	NM	Fig. 4A
Total bacteria	NM	NM	NM	100(↓)	NM	>	NM	NM	Fig. S6A
Bacterial *amoA* gene	NM	NM	NM	>	NM	>	NM	NM	Fig. S6C
Archaeal *amoA* gene	NM	NM	NM	>	NM	>	NM	NM	Fig. S6D
nifH gene	NM	NM	NM	100(↓)	NM	>	NM	NM	Fig. S6E
Sediment samples									
Bacterial OTUs	NM	NM	NM	>	NM	>	>	NM	
Bacterial RA OTUs	NM	NM	NM	>	NM	>*	>	NM	Fig. 4B
Total bacteria	NM	NM	NM	>	NM	>	>	NM	Fig. S6B
Bacterial *amoA* gene (sediment)	NM	NM	NM	10(↓)	NM	10(↓)	>	NM	Fig. 5A
Archaeal *amoA* gene	NM	NM	NM	<1(↓)	NM	<1(↓)	<1(↓)	NM	Fig. 5B
nifH gene in sediment	NM	NM	NM	>	NM	100(↓)	>	NM	Fig. S6F

> = no significant effect (NOEC > 1000 µg/L), NM = not measured, NP = not present (taxa not present in the analyzed samples).

[a] The number of individuals per sample was, on average, lower than 1 individual/mL when the statistically significant effect was observed.

[b] The number of individuals per sample was, on average, lower than 10 individuals/L when the statistically significant effect was observed.

[c] The number of individuals was, on average, lower than 5 per sample when the statistically significant difference was observed.

* Significant effects (Monte Carlo permutation test $p = 0.05$), but calculated NOEC was higher than 1000 µg/L.

Periphyton biomass

The periphytonic chlorophyll-a density in the control microcosms ranged between 2 and 7 μg/dm^2 of glass slide (Fig. S5B). On average, chlorophyll-a contents increased on day 14 after the first antibiotic application of the four treatments. However, the results of the univariate analysis did not show a significant effect of the antibiotic treatment on the chlorophyll-a content at any of the sampling dates.

Zooplankton community

The sampled zooplankton community consisted of 20 Rotifera taxa, 6 Cladocera taxa, 2 Copepoda taxa and 1 Ostracoda taxon. The most abundant taxa belonged to Rotifera (i.e., *Brachionus angularis*, *Filinia longiseta*, *Brachionus caudatus*, *Hexathra sp.*, *Ploesoma sp.*, *Brachionus calyciflorus*, *Polyarthra vulgaris*, and *Trichocerca sp.*) and Copepoda (i.e., nauplii stages and cyclopoids) (Table S2). The control microcosms were dominated by cyclopoid copepods, the rotifers *Ploesoma sp.*, *P. vulgaris*, *B. angularis*, and the cladoceran *Ceriodaphnia reticulata*. During the experimental period, the relative abundance of Copepoda, Cladocera, and *Ploesoma sp.*, decreased. The numbers of the *Brachionus* rotifers increased sharply during the last 2 weeks of the experimental period, probably due to the increased water temperatures, and resulted in a notable increase of the total zooplankton abundance (Fig. S1).

The results of the PRC analysis did not show significant effects of the enrofloxacin treatment on the zooplankton community ($p = 0.62$). Significant univariate responses were calculated for 8 taxa, but only one species (*C. reticulata*) showed a consistent response (Table 3). *C. reticulata* abundance was significantly lower in the treated microcosms than in the controls after the start of the treatment. However, such differences were already appreciable in the pre-treatment period (Fig. S3D).

Macroinvertebrate community

During the experimental period, 17 different macroinvertebrate taxa were identified, the majority of which belonged to Insecta (11 taxa), followed by Mollusca (5) and Annelida (1) (Table S3). The most abundant genera in decreasing order were Chironomidae, *Micronecta* sp., and Notonectidae. The relative abundance of these three taxa in the control microcosms remained relatively constant during the experimental period. The total macroinvertebrate abundance was generally low in the pre-treatment period and in the last 2 weeks of the experimental period (Fig. S1).

The total macroinvertebrate taxa in all the treatment levels slightly decreased during the experimental period. The results of the PRC analysis did not show a significant effect of the enrofloxacin treatment on the macroinvertebrate community ($p = 0.30$).

The results of the univariate analysis indicated a significant increase in the abundance of two snail species (*Melanoides tuberculata* and *Physella acuta*) in the highest enrofloxacin treatment level (1000 µg/L) (Table 3). However, these significant effects were observed on isolated sampling dates and the abundance of these species in the microcosm samples was very low (Fig. S4B,C).

Organic matter decomposition

The decomposition of the *Musa* leaves in the control microcosms were 24%, 43% and 76%, after an incubation period of 1, 2 and 4 weeks, respectively (Fig. S5A). The results of the univariate analysis did not show treatment-related effects on the percentage of decomposition in any of the sampling dates. It must be noted, however, that in some instances macroinvertebrates (e.g. Chironomidae) were found to be feeding on the leaves, which could have influenced the leaf breakdown.

Microorganism community

The RDA analysis indicated significant effects of the antibiotic treatment on the bacterial OTUs and the RA of OTUs in the *Musa* leaf samples at the end of the treatment period (day 7) and one week after the antibiotic treatment (day 14), with calculated NOECs of 100 µg/L for both datasets and both sampling dates (Table 3 and Fig. 4A). The total bacteria and the *nifH* gene abundance in the leaf samples of the highest treatment level decreased on day 7 after the first antibiotic application (Fig. S6A,E), however, the abundance of the bacterial and archaeal *amoA* gene did not show significant treatment-related effects (Fig. S6C,D and Table 3).

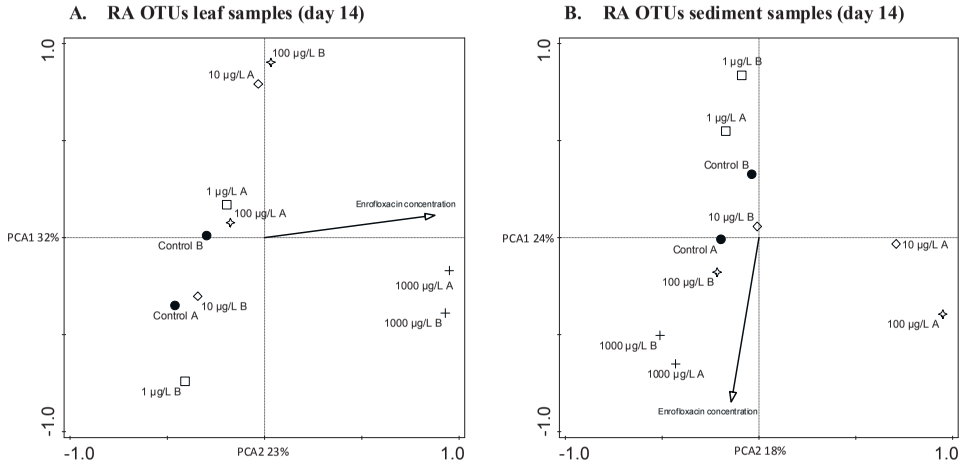

Figure 4. Principal component ordination diagrams of the Relative Abundance (RA) of the Operational Taxonomic Units (OTUs) datasets derived from the DGGE profiles for the leaf (A) and sediment (B) samples collected on day 14. The calculated NOECs are presented in Table 3. The letters A and B in the graphs refer to the two replicates in each treatment level.

The RDA analysis on the bacterial OTUs in the sediment samples did not show any treatment-related effects. The sediment bacterial OTUs' RA dataset only showed significant antibiotic-related effects for the samples collected 1 week after the antibiotic treatment using the Monte Carlo permutation test, but the calculated NOEC was higher than 1000 µg/L (Table 3 and Fig. 4B). The total abundance of bacteria in the sediment samples did not show significant treatment related effects (Fig. S6B). A significant decrease was observed in the *amoA* gene abundance during and after the antibiotic treatment, with calculated NOECs of 10 µg/L and below 1 µg/L for the sediment bacteria and archaea communities, respectively (Table 3 and Fig. 5A and B). A significant decrease of the bacterial *nifH* gene abundance was only observed at the highest treatment level on the sediment samples collected 1 week after the antibiotic treatment (Fig. S6F and Table 3).

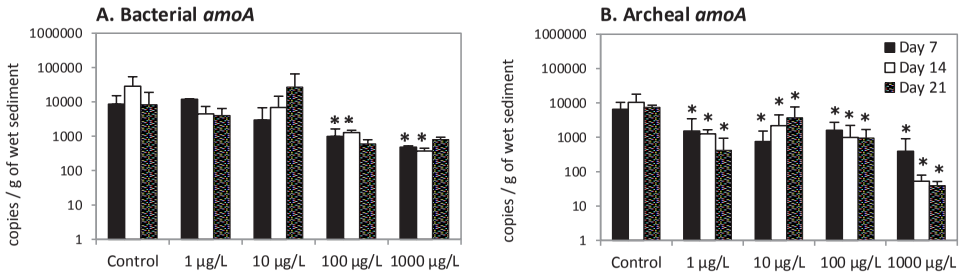

Figure 5. Bacterial (A) and archaeal (B) amoA gene abundance in the sediment samples collected on day 7, 14 and 21 after the first antibiotic application (mean ± standard deviation). The asterisk indicates significant differences with controls (Williams test; $p < 0.05$).

Discussion

Dissipation of enrofloxacin

The results of our experiment showed that the dissipation of enrofloxacin from the water column and the formation of its by-product ciprofloxacin were quick processes. Several semi-field studies have demonstrated that photodegradation and sorption to organic matter are the main processes influencing the dissipation of fluoroquinolone antibiotics from surface waters (Cardoza et al., 2005 and Knapp et al., 2005). Knapp et al. (2005) evaluated the dissipation and transformation of enrofloxacin under different light conditions (i.e., full-light exposure, partial shading, and almost complete shading) in a mesocosm experiment performed during autumn in Kansas (USA). The enrofloxacin DT50 calculated by Knapp et al. (2005) (approximately 19 h) in the mesocosms with full-light exposure was slightly higher than the value calculated in our experiment (DT50 = 11.7 h), suggesting that tropical environmental conditions favour the dissipation of enrofloxacin from the aquatic environment, probably due to higher photodegradation.

Enrofloxacin effects on primary producers

In our experiment, the phytoplankton community and the biomass of the established periphyton community did not show a significant response to the antibiotic treatment. Laboratory toxicity studies have reported short-term growth inhibition EC50 values for enrofloxacin and ciprofloxacin in the range of 10–173 µg/L for cyanobacteria (*Microcystis aeruginosa* and *Anabaena flos-aquae*) and 3100–18,700 µg/L for green algae (*Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*) (Robinson et al., 2005 and Ebert et al., 2011). Wilson et al. (2004) found a concentration-dependent reduction in the abundance and species richness of phytoplankton, with cyanobacteria and cryptophyta/dinophyta being the most affected populations, in microcosms that were chronically exposed to a mixture of four tetracycline antibiotics, which have a similar toxicity to primary producers than fluoroquinolone antibiotics (Park and Choi, 2008). The significant effects observed by Wilson et al. (2004) 7 days after the start of the treatment period occurred in the microcosms that were exposed to an antibiotic concentration that was 2–3 times lower than the highest enrofloxacin concentration tested in our study. On the basis of this data, we expected to find a decline of the cyanobacterial population in the microcosms with the highest enrofloxacin concentration and a potential shift in the overall phytoplanktonic community structure, however, such trend could not be identified. A potential explanation for the absence of effects on the phytoplankton community in our experiment could be related to the high water pH measured in the microcosms. Enrofloxacin is a weak acid and the pH range measured in the test microcosms was rather alkaline (8.0–10.7). Based on the ionic component distributions shown in Kim et al. (2010), about 80–100% of the compound could have remained in

its anionic form during the experimental period. Several studies have demonstrated that the bioaccumulation and toxicity of ionizable organic substances decreases when the molecule is in its ionized form (Rendal et al., 2011 and references therein). For example, Fahl et al. (1995) found that the toxicity of chlorsulfuron, an ionizable herbicide, on *Chlorella fusca* growth was enhanced 25-fold by lowering the pH of the growth medium from 6.5 to 5.0, and Kim et al. (2010) found that the toxicity of enrofloxacin and ciprofloxacin to *Daphnia magna* increased in waters with lower pH. Thus, studies aimed at assessing the effects of pH on the toxicity of enrofloxacin and other ionizable antibiotics on primary producers, especially cyanobacteria, are recommended in order to confirm this hypothesis and to quantify the variability of the sensitivity to antibiotic exposure under different pH ranges. Another potential explanation for the lack of effects on primary producers resides in the dominance of Chlorophyceae species and the variability observed in the occurrence and abundance of potentially sensitive taxa (cyanobacteria) in the studied microcosms. Daam and Van den Brink (2011) argued that the phytoplankton community structure of tropical ecosystems largely depends on seasonally related weather conditions. And cyanobacterial taxa, typically *Microcystis*, tends to dominate during situations of nutrient scarcity and/or light limitations, the latter most commonly occurring during the rainy season. Therefore, in order to better observe potential phytoplankton structure damages by antibiotic exposure under tropical conditions, further semi-field tests with cyanobacteria-dominated systems during rainy season are recommended.

Enrofloxacin effects on invertebrates

The analysis of the zooplankton and macroinvertebrate communities did not show a significant response to the enrofloxacin application. Sporadic significant responses of certain taxa were observed, but were isolated and did not show a concentration response relationship. Acute toxicity studies with freshwater cladocerans and macroinvertebrates show acute EC50 values higher than 50 mg/L (Park and Choi, 2008 and Rico et al., 2014). Long-term studies assessing the effects of enrofloxacin and ciprofloxacin on reproduction (Park and Choi, 2008) and life-history traits (Martins et al., 2012) of *Daphnia magna* found NOEC values higher than the highest antibiotic concentration tested in our study. Furthermore, previous microcosm experiments performed in temperate regions have not been able to identify negative responses of invertebrate communities to environmentally relevant antibiotic exposure concentrations (Wilson et al., 2004 and Maul et al., 2006). Therefore, based on the available literature and the results of this study we can conclude that (tropical and temperate) aquatic invertebrate communities are highly tolerant to enrofloxacin under realistic exposure conditions (i.e., several micrograms per litre).

Enrofloxacin effects on microorganisms and ecosystem metabolism

Enrofloxacin clearly affected the structure of leaf-associated bacterial communities and reduced bacterial abundance at concentrations higher than 100 μg/L, however, little or no effects were identified for the sediment bacterial community (Table 3). Observed differences between the sensitivity of both bacterial communities could be related to differences in exposure patterns and characteristics of these bacterial communities. The bacterial community of our (3 cm) depth integrated sediment samples might have been exposed to a gradient of antibiotic exposure concentrations (from higher concentrations in the top layer, to lower concentrations in the bottom layers) and environmental conditions (from aerobic conditions in the top layer, to less aerobic or anaerobic conditions in the deeper layers). The higher richness of the sediment bacterial community compared to the leaf one (as shown by the number of OTUs in the DGGE profiles; Table S4 and Fig. S7), might make them more resilient to antibiotic exposure (Girvan et al., 2005) and is likely to hamper the identification of effects on less dominant species due to the fact that DGGE in general only allows to analyze populations of at least 1% in relative abundance (Muyzer et al., 1993). Knapp et al. (2005) did not find significant effects of enrofloxacin on water-living bacterial communities in microcosms exposed to a single dose of 25 μg/L, but suggested that effects could be more prominent on organic matter surfaces, where prolonged exposures are more likely, as shown in our study. Maul et al. (2006) demonstrated a shift in carbon source utilization of leaf-associated microbial communities repeatedly exposed for 12 days to 100 μg/L of ciprofloxacin. In our study, the alteration of the bacterial community structure and decrease in total bacterial abundance observed at the highest enrofloxacin concentration did not influence the organic matter decomposition rates in the leaf samples, however, such trend could have been masked by the influence of invertebrates on the leaf breakdown. Therefore, we recommend to include such endpoint in further microcosm experiments with antimicrobial substances, but to lower the mesh size of the litter bags containing the decomposing material to 300 μm or less to prevent any interaction with invertebrates.

Our study demonstrated that sediment bacterial and archaeal ammonia oxidizers are highly sensitive to enrofloxacin (NOEC = 10 and 1 μg/L, respectively), and a causal link with their nitrification function could be demonstrated, showing an increase in the ammonia concentrations and a trend towards inhibition of the nitrate formation during the antibiotic exposure period at 1000 μg/L. Several studies have demonstrated that nitrification is largely inhibited in aquatic systems exposed to therapeutic doses of antibiotics used in aquaculture (several mg/L), suggesting potential toxic effects for aquatic organisms due to the accumulation of ammonia (Klaver and Matthews, 1994 and Nymenya et al., 1999). Nymenya et al. (1999) estimated that ammonia oxidation and nitrate production will be reduced by about 1% and 2.3%, respectively,

within 24 h by enrofloxacin concentrations of 1 mg/L. This might explain the changes in nutrient concentrations observed at the highest treatment level of our experiment. Despite the reduction in sediment-born ammonia oxidizing microorganisms that was observed at almost all treatment levels, a significant increase in the microcosm ammonia concentrations was only demonstrated for the microcosms exposed to 1000 μg/L, returning to levels similar to controls within 2 weeks after the treatment. This suggests that water-living microorganisms (which were not evaluated) could have recovered faster than sediment microorganisms (potentially due to a lower exposure and damage), denoting a high resilience of the whole water–sediment microbial community and a fast recovery from antibiotic exposure. In conclusion, our study confirms that microbial functions such as nitrification might be affected in aquatic systems exposed to therapeutic concentrations of enrofloxacin such as those used in aquaculture bath treatments, but are not likely to be affected in natural aquatic ecosystems that are exposed to antibiotic residual concentrations, which typically are 2–3 orders of magnitude lower than therapeutic concentrations (Rico and Van den Brink, 2014).

Study limitations

To our knowledge, this is the first study that evaluated the fate and ecological effects of an antibiotic in tropical freshwater model ecosystems. The experimental set-up and methodological approach followed the recommendations provided for the ecotoxicological assessment of pesticides in tropical microcosms (see Daam and Van den Brink, 2011). However, we found some limitations that are worth to discuss in order to improve the methodological approach for testing the ecological effects of antibiotics. For example, nutrient additions have been recommended in order to sustain the plankton-dominated status of tropical model ecosystems (Daam and Van den Brink, 2011). In our experiment, biweekly pulsed nutrient (nitrogen and phosphorus) applications were performed, which could have masked the antibiotic effects on nitrogen transformation rates. In addition, aeration was constantly supplied to prevent temperature stratification in the microcosm water under such hot conditions and to avoid critical oxygen drops at night. We believe that such nutrient applications and aeration system were crucial to maintain the planktonic communities in such eutrophic systems, but could have hampered the observation of effects on microbial functional endpoints and ecosystem metabolism (e.g. nitrogen transformation, microbial respiration and aerobic organic matter mineralization). This suggests that worst-case effects of antibiotics in ecosystem functional endpoints should be better evaluated in less eutrophic systems and during the rainy season in which, as discussed previously, solar radiation and water temperatures are lower and the dominance of sensitive cyanobacteria is more likely. In addition, the metabolism of bacteria is known to be generally higher in tropical aquatic ecosystems with high temperatures (Amado et al., 2013), and the recovery potential of microorganisms exposed to non-selective bacteriostatic compounds is also expected

to be higher, supporting the use of lower temperature systems to observe microbial-related effects.

During the first three weeks of the pre-treatment period, about 20% of the microcosm water was exchanged biweekly in order to homogenize the microcosms, however, this turned out to be not enough to prevent differences in dominating periphyton and suspended algae taxa that competed for light and nutrients. We tried to avoid that by exchanging more than 50% of the microcosm water during the week before the antibiotic treatment. However, after a few days the microcosms often returned to their original states. This probably influenced the diversity of the planktonic and microbial communities in the microcosms (data not shown) and increased the variability between replicates, lowering the power of the statistical test. Therefore, future experiments should try to provide intensive mixing during the whole pre-treatment period (more than 50%, if possible every 2–3 days) and increase replication.

Threshold concentrations and implications for risk assessment

Of all endpoints investigated in the current study, the abundance of bacterial and archaeal ammonia oxidizers was found to be the most sensitive endpoint (NOECs of 10 and <1 μg/L, respectively). Therefore, according to the results of this study, the cut-off value used in the first-tier risk assessment of veterinary medicinal products (1 μg/L; VICH, 2000) provides a sufficient protection level for plant and invertebrate aquatic communities, and microbial-associated function (i.e. nitrification), but fails to protect the relative abundance of important microbial groups in sediments. Most of the second-tier threshold concentrations derived from toxicity data for standard test species and assessment factors appear to ensure a sufficient protection level for aquatic primary producers, invertebrate and microorganism communities, and for nitrification, whereas the threshold concentration derived from toxicity data for *Microcystis aeruginosa* (0.49 μg/L) ensures the most conservative protection for key sediment microorganisms (i.e. nitrifiers) (Table 4). Table 4 also shows that the threshold concentration derived from the luminescence inhibition test performed with the marine bacterium *Vibrio fischeri*, which is often used as surrogate for aquatic bacterial communities in risk assessments, does not result in a sufficient level of protection for all aquatic bacterial taxa, and probably neither for microbial-associated functions. According to the results of this study, an assessment factor of at least 10 is recommended when safe concentrations are calculated from median HC5 values (hazardous concentration for the 5th sensitivity percentile of species) derived with species sensitivity distributions (SSDs) for primary producers, including species of green algae and cyanobacteria (Table 4).

Table 4. Threshold concentrations for enrofloxacin derived from laboratory toxicity data for bacteria, primary producers, invertebrates and fish. The last column indicates whether these threshold concentrations are protective or not for the abundance of ammonia oxidizing microbes (calculated NOEC < 1 µg/L) and their associated ecological function (calculated NOEC = 100 µg/L).

Taxonomic group	Species	Endpoint	Toxicity value (µg/L)	Assessment factor [a]	Threshold (µg/L)	Protective for ammonia oxidizing microbes/function?
Bacteria	*Vibrio fischeri*	IC50-15min (luminescence inhibition)	326,800 [b]	100	327	No/No
Primary producers	*Pseudokirchneriella subcapitata*	EC50-3d (growth inhibition)	3,100 [c]	100	31	No/Yes
	Microcystis aeruginosa	EC50-5d (growth inhibition)	49 [c]	100	0.49	Yes/Yes
	Assemblage	Median HC5 from SSD	8.80[d]	10	0.88	Yes/Yes
Invertebrates	*Daphnia magna*	EC50-2d (immobility)	56,700 [b]	1,000	57	No/Yes
	Daphnia magna	NOEC-21d (reproduction)	5,000 [b]	10	500	No/No
	Assemblage	Median HC5 from SSD	28,190[d]	10	2,819	No/No
Fish	*Oryzias latipes*	EC50-4d (mortality)	> 100,000 [b]	1,000	100	No/Yes

[a] Assessment factors for standard test species of primary producers, invertebrates and fish based on the international guidelines for the environmental risk assessment of veterinary medicinal products (VICH 2004). The assessment factors for the bacteria IC50 and for the HC5 for species assemblages were based on authors' judgement.

[b] Park and Choi (2008).

[c] Robinson et al. (2005).

[d] Rico et al. (2014).

The majority of the fluoroquinolone antibiotic concentrations monitored in tropical surface waters are below the µg/L range. Some studies, however, have measured concentrations up to several µg/L in rivers impacted by aquaculture pollution (Rico et al., 2014), and in effluents of animal farms and hospitals (see Suzuki and Hoa, 2012 for a review). At such environmental concentrations, enrofloxacin is likely to impact, at least temporarily, the structure and function of bacterial and archaeal communities, particularly in sediments, but not to directly affect algal primary producers or invertebrates. One of the aims of our experiment was to assess whether the effects of antibiotic pollution on microbial communities and important ecosystem functions could result in side-effects on primary producers and invertebrates. This experiment did not suggest indirect effects at higher trophic levels, however, the exposure period used in this study was relatively short, and the recovery of the ecosystem function impairment was relatively quick. Therefore, more studies are required with prolonged exposure periods and using other antibiotics (if possible, with higher environmental persistence) and under different environmental and biological conditions (with lower temperatures and with higher dominance of cyanobacteria). Such experiments should also include fish, as they have been demonstrated to show a lower tolerance to ammonia accumulation in surface waters than invertebrates (Arthur et al., 1987).

Acknowledgments

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Supplementary information

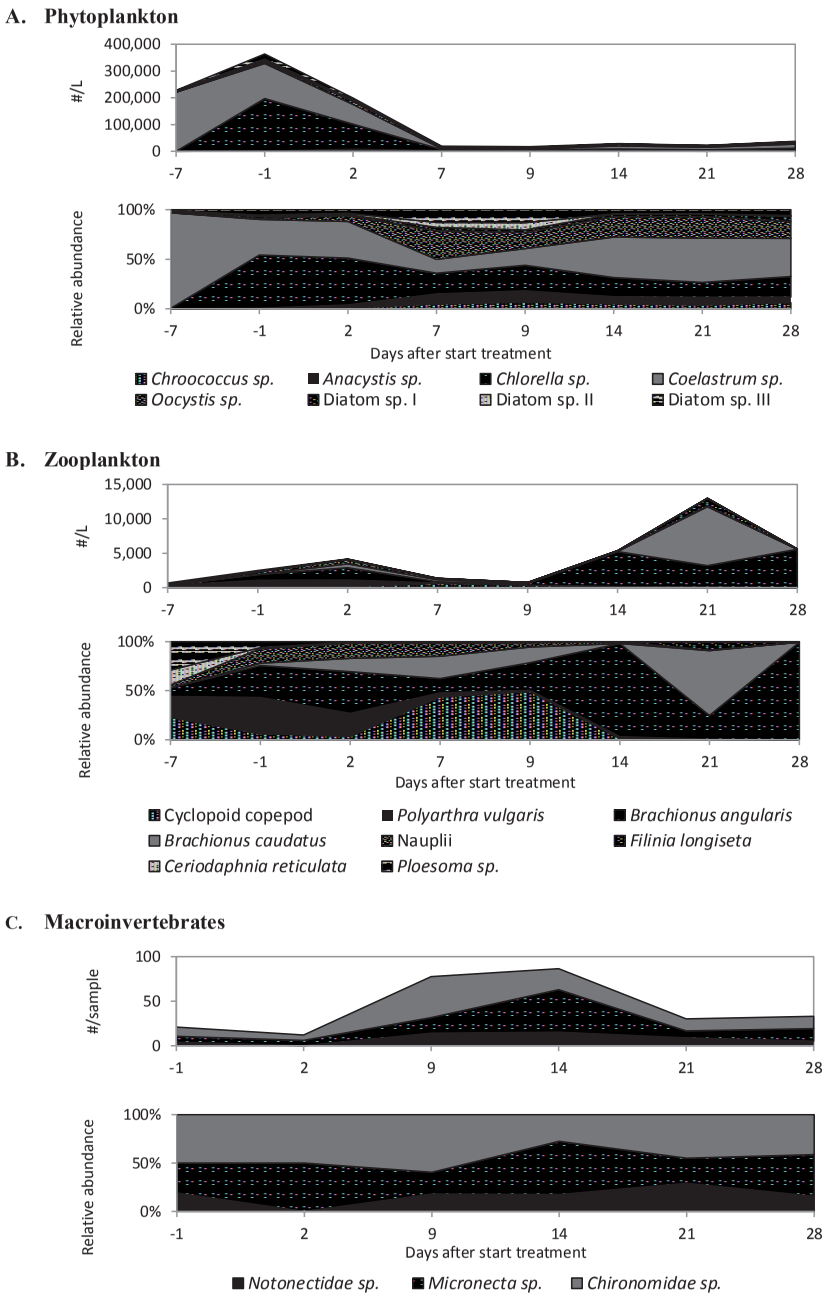

Figure S1. Total and relative abundance of the most dominant taxa of phytoplankton (A), zooplankton (B) and macroinvertebrates (C) in the control microcosms during the experimental period.

Table S1. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) for phytoplankton taxa. Concentrations expressed as nominal single-dose of enrofloxacin, in µg/L.

	Day after first application								
Phytoplankton	**-7**	**-1**	**2**	**7**	**9**	**14**	**21**	**28**	**Density [a]**
Chlorophyta									
Ankistrodesmus sp.	>	>	>	>	>	>	>	>	Medium, Fig. S2H
Chlorella sp	>	>	>	>	>	>	>	>	High, Fig. S2D
Coelastrum sp	>	>	>	>	>	>	>	>	High, Fig. S2E
Crucigenia sp.	NC	>	>	>	NC	NC	NC	NC	Low
Golenkinia sp.	>	>	>	>	NC	NC	NC	NC	Low
Micractinium sp.	>	NC	NC	NC	NC	NC	NC	NC	Low
Oocystis sp.	>	>	>	>	>	>	>	>	Medium, Fig. S2F
Pediastrum sp.	>	NC	NC	NC	NC	NC	NC	>	Low
Scenedesmus sp. I	>	>	>	>	>	>	>	>	Low
Scenedesmus sp. II	NC	>	>	>	10 (↑)	<1 (↑)	<1 (↑)	>	Low, Fig. S2B
Scenedesmus sp. III	NC	>	>	>	NC	NC	>	NC	Low
Scenedesmus sp. IV	NC	>	>	NC	NC	NC	NC	>	Low
Scenedesmus sp. V	NC	>	>	>	>	>	>	>	Low
Scenedesmus sp. VI	NC	>	>	>	>	>	>	>	Low
Scenedesmus sp. VII	NC	NC	>	>	NC	NC	NC	NC	Low
Scenedesmus sp. VIII	NC	NC	>	NC	NC	NC	NC	NC	Low
Selenastrum sp.	NC	NC	>	NC	NC	NC	NC	NC	Low
Sphaerocystis sp	>	>	>	>	>	>	>	>	Medium
Tetrahedron sp.	>	>	>	>	>	>	>	>	Low
Unknown (green algae)	>	>	>	>	NC	NC	NC	NC	Low
Cyanophyta									
Chroococcus sp.	>	>	>	>	>	>	>	>	Medium
Phormidium sp.	>	>	>	>	>	>	>	>	Low
Anacystis sp.	NC	NC	>	>	>	>	>	>	Medium, Fig. S2G
Scytonema sp.	NC	NC	NC	NC	NC	NC	>	>	Low
Microcystis sp.	>	>	>	>	>	>	>	>	Low
Merismopedia sp.	NC	>	>	>	>	>	>	>	Low
Oscillatoria sp.	NC	>	>	NC	>	>	>	NC	Low
Spilurina sp.	NC	NC	>	NC	>	NC	NC	NC	Low
Desmidiaceae									
Closterium sp.	>	NC	NC	>	>	>	>	>	Low
Cosmarium sp.	>	NC	NC	>	NC	NC	NC	NC	Low
Unknow (desmid)	NC	>	>	>	NC	NC	NC	NC	Low
Diatomeae									
Diatom sp. I	>	>	>	>	>	>	>	>	Medium
Diatom sp.II	>	>	>	>	>	>	>	>	Medium
Diatom sp. III	>	>	>	>	>	>	>	>	Medium
Diatom sp. IV	>	>	>	>	>	>	10 (↑)	>	Low, Fig. S2C
Diatom sp. V	>	>	>	>	>	>	>	>	Low
Dinoflagellata									
Ceratium sp.	>	NC	NC	NC	NC	NC	NC	NC	Low
Peridiniopsis sp.	>	>	NC	NC	NC	NC	NC	NC	Low
Euglenophyceae									
Phacus sp.	>	NC	NC	NC	NC	NC	NC	NC	Low

↑ = increase in abundance, ↓ = decrease in abundance, > = no significant effect (NOEC > 1000 µg/L), NC = not calculated (species not present).

[a] High: more than 10 individuals/mL; Medium: between 1 and 10 individuals/mL; Low: lower than 1 individual/mL.

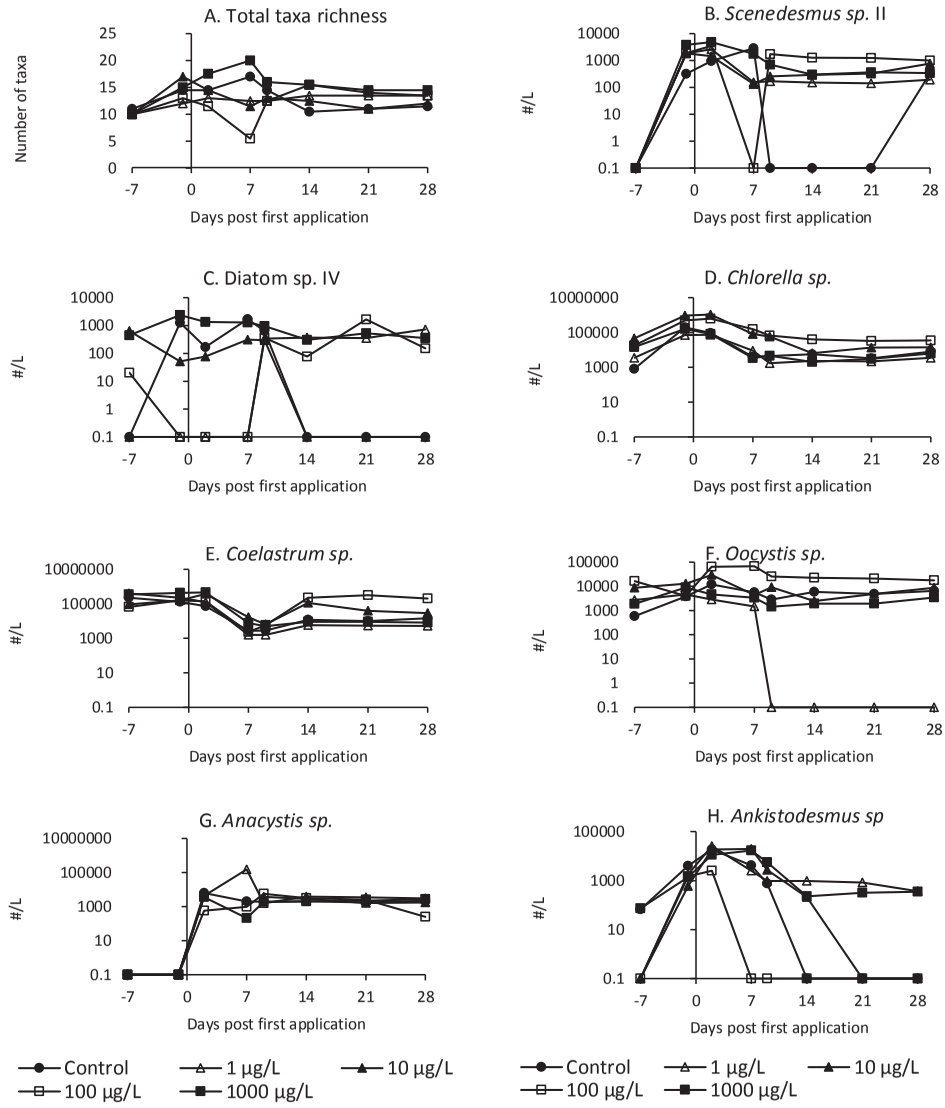

Figure S2. Total taxa richness and population dynamics of phytoplankton taxa in the different enrofloxacin treatments. Only the taxa that showed a significant response in the univariate analysis (*Scenedesmus sp.* II and *Diatom sp.* IV) and the most abundant taxa are included. The NOECs for treatment-related responses are shown in Table S1. The figures show the mean values per treatment. A value of 0.1 denotes absence of the taxon in the analyzed samples.

Table S2. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) for zooplankton taxa. Concentrations expressed as nominal single-dose of enrofloxacin, in µg/L.

	Day after first application								
Zooplankton	**-7**	**-1**	**2**	**7**	**9**	**14**	**21**	**28**	**Density [a]**
Cladocera									
Alona sp.	NC	NC	NC	NC	NC	>	NC	NC	Low
Alonella sp.	>	>	>	100 (↑)	>	>	>	>	Low, Fig. S3C
Ceriodaphnia reticulata	>	>	<1 (↓)	<1 (↓)	100 (↓)	>	>	>	Medium, Fig. S3D
Dadaya macrops	NC	NC	NC	NC	NC	>	NC	NC	Low
Diaphanosoma senegal	>	>	>	>	100 (↑)	>	>	>	Low, Fig. S3E
Grimaldina brazzai	NC	NC	NC	NC	NC	NC	NC	>	Low
Copepoda									
Calanoid copepod	>	NC	NC	NC	NC	NC	NC	NC	Low
Cyclopoid copepod	>	>	>	>	>	>	>	>	High
Nauplii	>	>	>	>	>	>	>	<1 (↑)	High, Fig. S3G
Ostracoda	>	>	>	>	>	>	100 (↑)	>	Low, Fig. S3H
Rotifera									
Anuraeopsis fissa	>	>	>	>	>	>	>	NC	Medium
Ascomorpha sp.	NC	NC	NC	NC	NC	NC	>	>	Low
Asplachna sp.	>	NC	NC	NC	NC	NC	NC	NC	Low
Brachionus angularis	>	>	>	>	>	>	>	100 (↓)	High, Fig. S3J
Brachionus calyciflorus	>	>	>	NC	NC	>	>	>	High
Brachionus caudatus	>	>	>	>	>	100 (↑)	>	>	High, Fig. S3K
Brachionus falcatus	>	>	>	>	>	NC	NC	NC	Low
Brachionus forficula	NC	>	NC	NC	>	>	>	<1 (↓)	Low, Fig. S3L
Colurella sp.	>	>	NC	NC	NC	>	NC	NC	Low
Filinia longiseta	NC	NC	NC	NC	>	>	100 (↓)	>	High, Fig. S3M
Hexarthra sp.	>	>	>	>	>	100 (↑)	10 (↑)	>	High, Fig. S3O
Keratella tropica	>	>	>	>	>	>	>	>	Medium
Lecane gr luna	>	>	>	>	>	>	>	>	Medium
Lecane gr lunares	>	>	NC	>	>	>	NC	>	Low
Mytilina sp.	>	NC	NC	NC	NC	>	>	NC	Low
Ploesoma sp.	>	>	>	>	>	>	>	>	High
Polyarthra vulgaris	>	>	>	>	>	>	>	>	High
Rotatoria sp.	NC	NC	>	>	>	>	>	>	Medium
Trichocerca cf capucina	>	>	>	>	>	>	>	>	High
Unkown sp. I	NC	NC	NC	NC	NC	NC	>	>	Low

↑ = increase in abundance, ↓ = decrease in abundance, > = no significant effect (NOEC > 1000 µg/L), NC = not calculated (species not present).

[a] High: more than 100 individuals/L; Medium: between 10 and 100 individuals/L; Low: lower than 10 individuals/L.

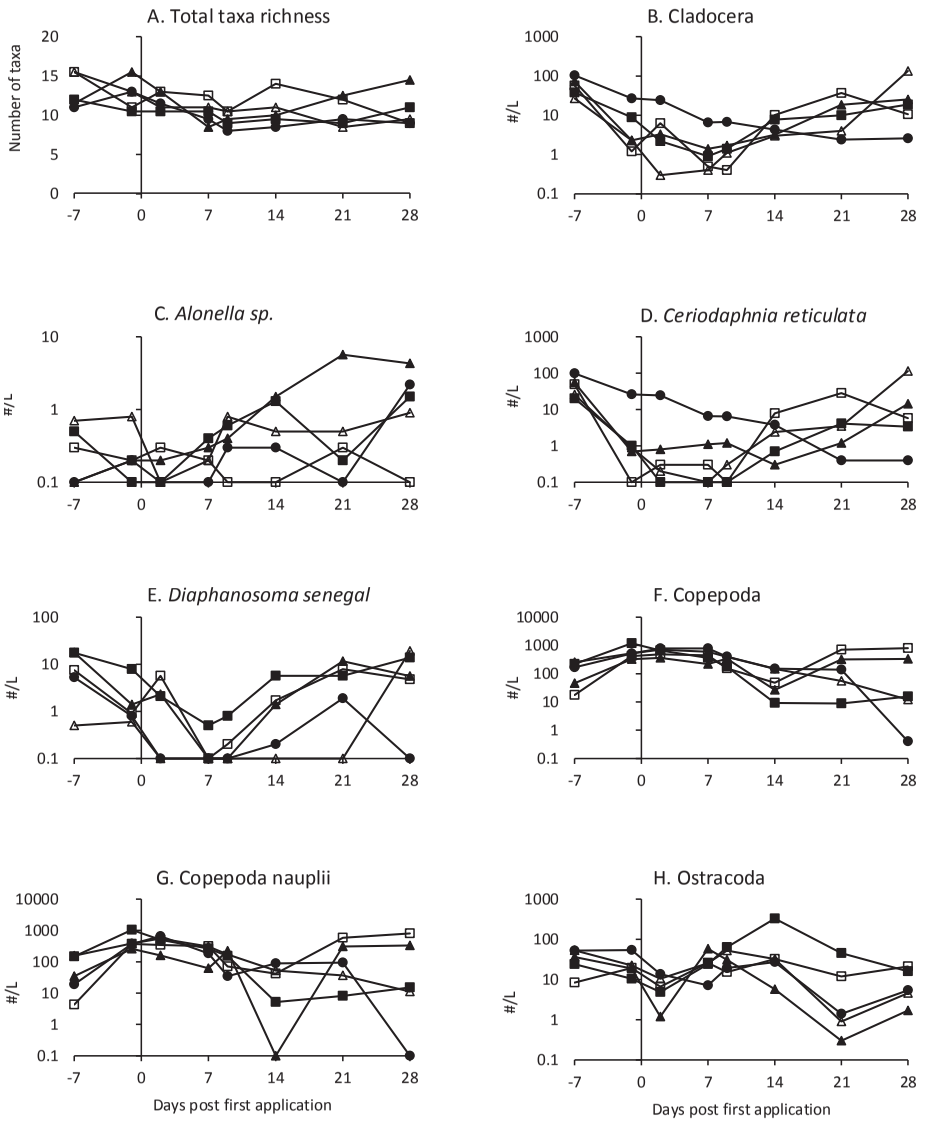
A. Total taxa richness
Number of taxa
B. Cladocera
#/L
C. Alonella sp.
D. Ceriodaphnia reticulata
E. Diaphanosoma senegal
F. Copepoda
G. Copepoda nauplii
H. Ostracoda
Days post first application
Control
1 µg/L
10 µg/L
100 µg/L
1000 µg/L

Figure S3. Continued.

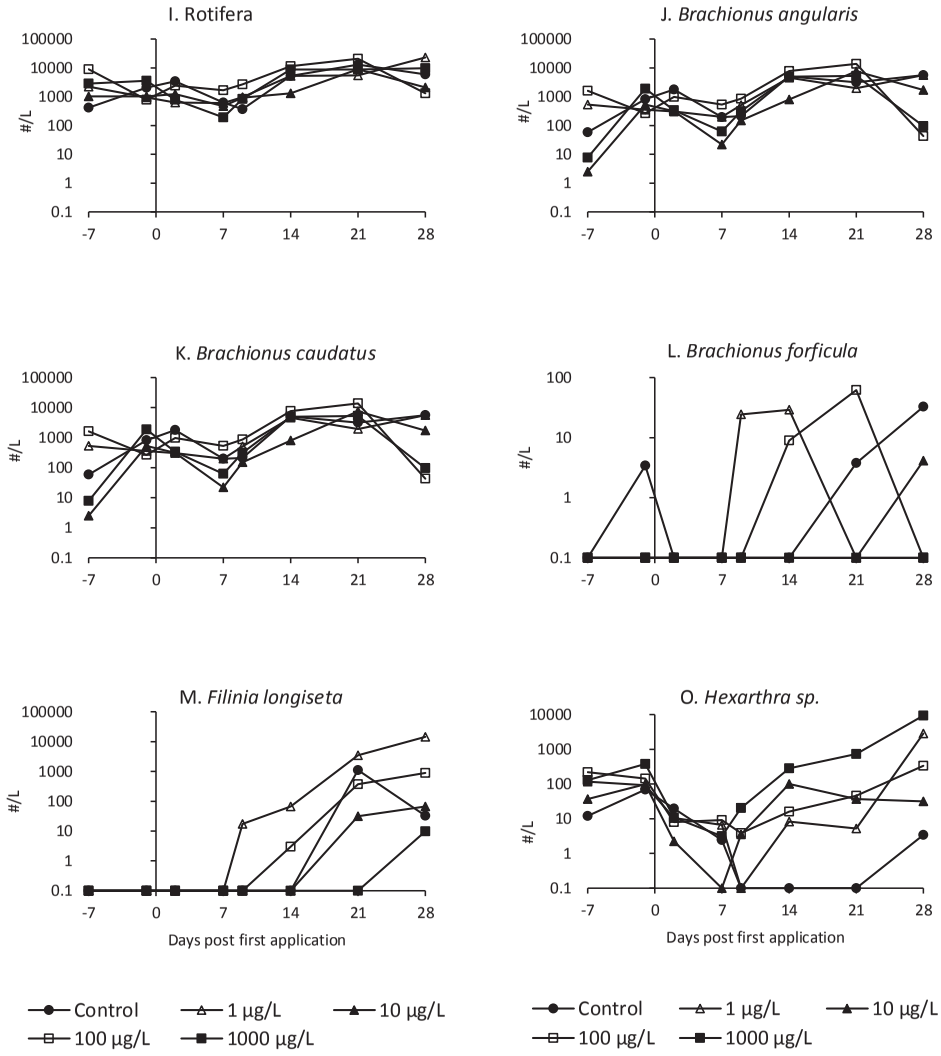

Figure S3. Total taxa richness and population dynamics zooplankton taxa in the different enrofloxacin treatments. Only the taxa that showed a significant response in the univariate analysis are included. The NOECs for treatment-related responses are shown in Table S2. The figures show the mean values per treatment. A value of 0.1 denotes absence of the taxon in the analyzed samples.

Table S3. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) for macroinvertebrate taxa. Concentrations expressed as nominal single-dose of enrofloxacin, in µg/L.

	Day after first application						
Macroinvertebrates	**-1**	**2**	**9**	**14**	**21**	**28**	**Density [a]**
Insecta							
Anisoptera (larvae)	>	>	>	>	NC	NC	Low
Chironomidae sp.	>	>	>	>	>	>	High, Fig. S4D
Cloeon sp.	>	>	>	>	>	NC	Low
Corixa/sigara	NC	NC	NC	>	>	>	Low
Dytiscidae (larvae)	>	NC	NC	NC	NC	NC	Low
Ilyocoris sp.	>	NC	NC	NC	NC	NC	Low
Micronecta sp.	>	>	>	>	>	>	High, Fig. S4E
Nepa sp.	>	>	>	>	>	>	Low
Notonectidae sp.	>	>	>	>	>	>	High, Fig. S4F
Plea sp.	NC	>	>	>	>	>	Low
Zygoptera (larvae)	>	>	>	NC	NC	NC	Low
Mollusca							
Melanoides	>	100 (↑)	>	NC	NC	NC	Low, Fig. S4B
tuberculata Physella	10 (↑)	>	>	100 (↑)	>	>	Low, Fig. S4C
acuta Physidae sp.	>	>	>	>	>	NC	Low
Planorbis sp.	>	>	>	NC	NC	NC	Low
Pomacea sp.	>	>	>	NC	>	NC	Low
Annelida							
Naididae sp.	NC	NC	>	NC	NC	NC	Low

↑ = increase in abundance, ↓ = decrease in abundance, > = no significant effect (NOEC > 1000 µg/L), NC = not calculated (species not present).

[a] High: more than 10 individuals/sample; Medium: between 5 and 10 individuals/sample; Low: lower than 5 individuals/sample.

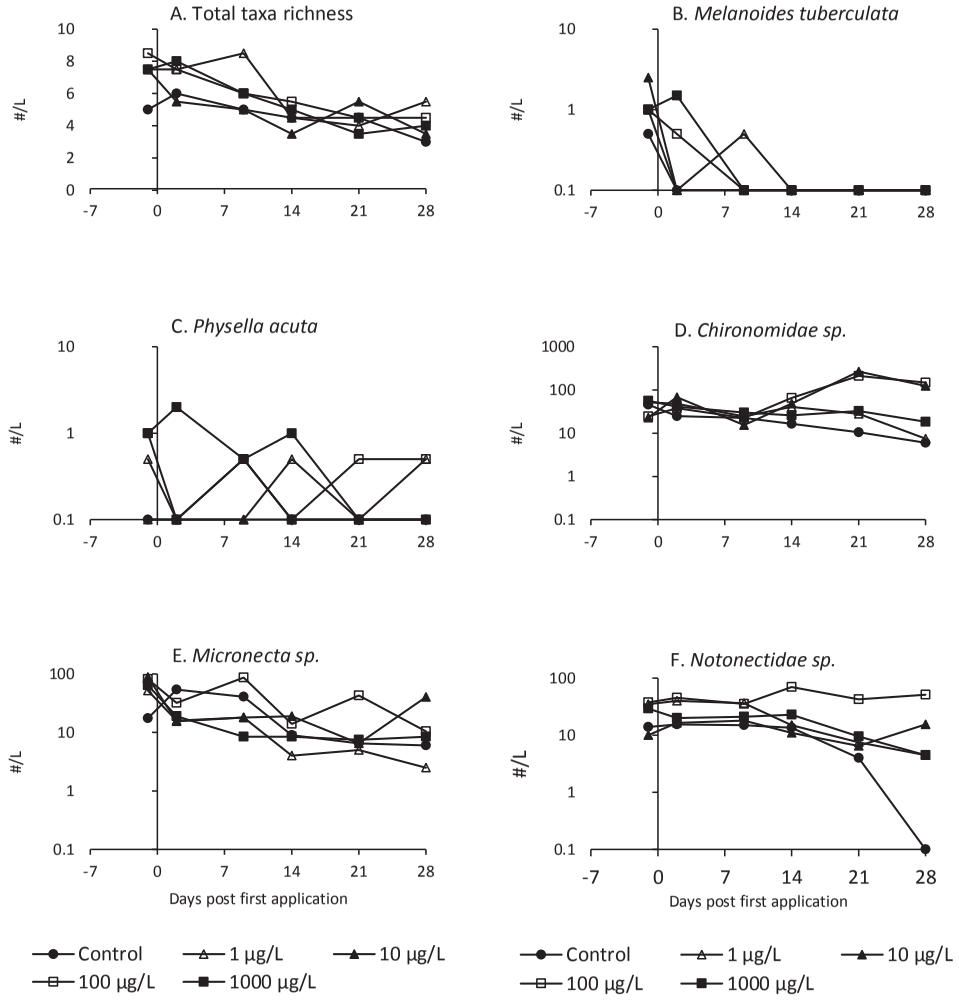

Figure S4. Total taxa richness and population dynamics of macroinvertebrates in the different enrofloxacin treatments. Only the taxa that showed a significant response in the univariate analysis (i.e., *Melanoides tuberculata, Physella acuta*) and the three most abundant taxa are included. The NOECs for treatment-related responses are shown in Table S3. The figures show the mean values per treatment. A value of 0.1 denotes absence of the taxon in the analyzed samples.

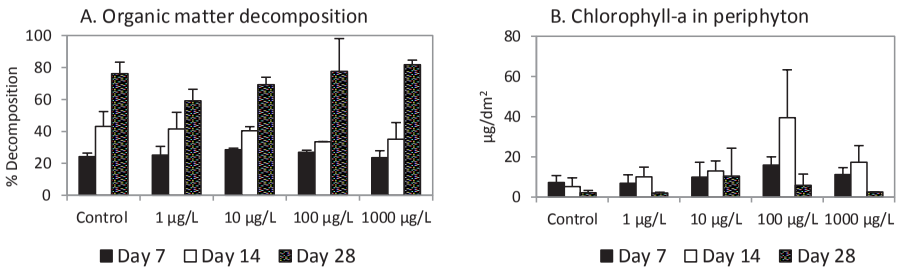

Figure S5. Decomposition of *Musa* leaves (A) and concentration of chlorophyll-a in periphyton (B) on day 7, 14, and 28 after the first antibiotic application (mean ± standard deviation).

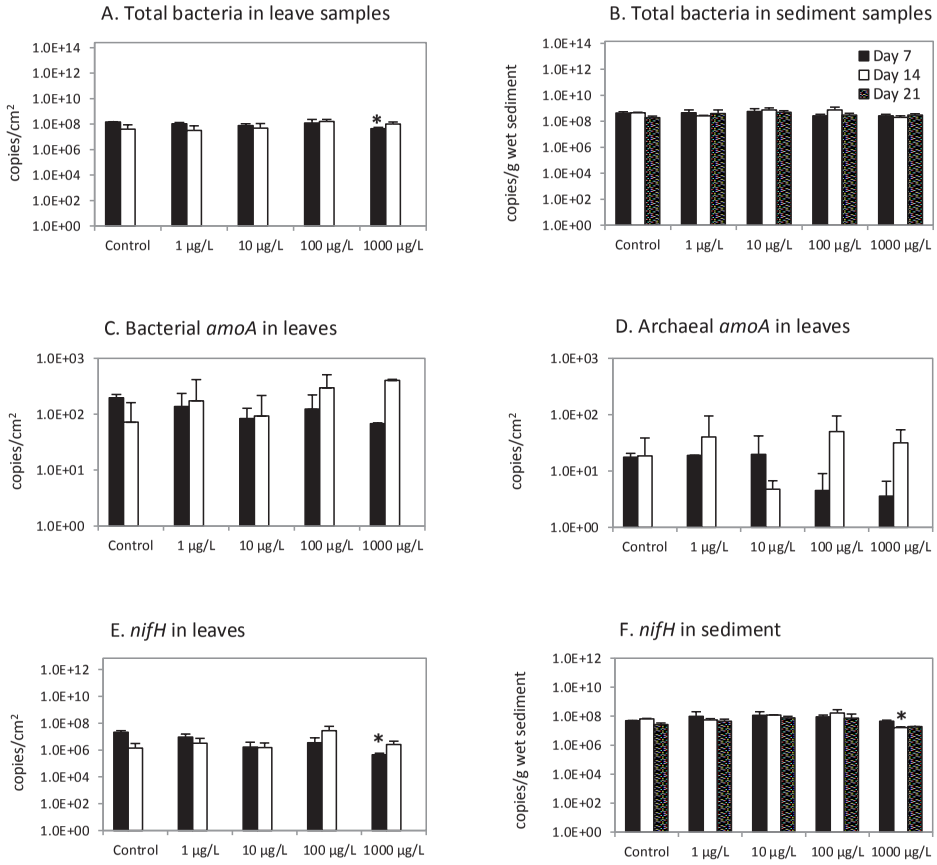

Figure S6. Total bacteria abundance in the leave (A) and sediment (B) samples, bacterial (C) and archaeal (D) *amoA* gene abundance in the leaf samples, and *nifH* gene abundance in the leaf (E) and sediment (F) samples. The asterisk indicates significant differences with controls (Williams test; $p < 0.05$).

Table S4. Number of operational taxonomic units (OTUs) in the leaf and sediment samples (mean±SD).

	Leaves		Sediment		
	Day 7	Day 14	Day 7	Day 14	Day 21
Control	26.0±1.4	28.5±2.1	44.5±2.1	36.5±3.5	34.0±7.1
1 µg/L	23.0±2.8	25.0±4.2	40.0±0.0	30.0±9.9	30.0±1.4
10 µg/L	26.5±4.9	28.5±2.1	49.0±8.5	35.0±1.4	43.0±2.8
100 µg/L	19.0±1.4	25.0±5.7	40.0±2.8	41.0±0.0	36.0±2.8
1000 µg/L	23.5±9.2	26.0±1.4	47.5±0.7	36.0±4.2	34.5±0.7

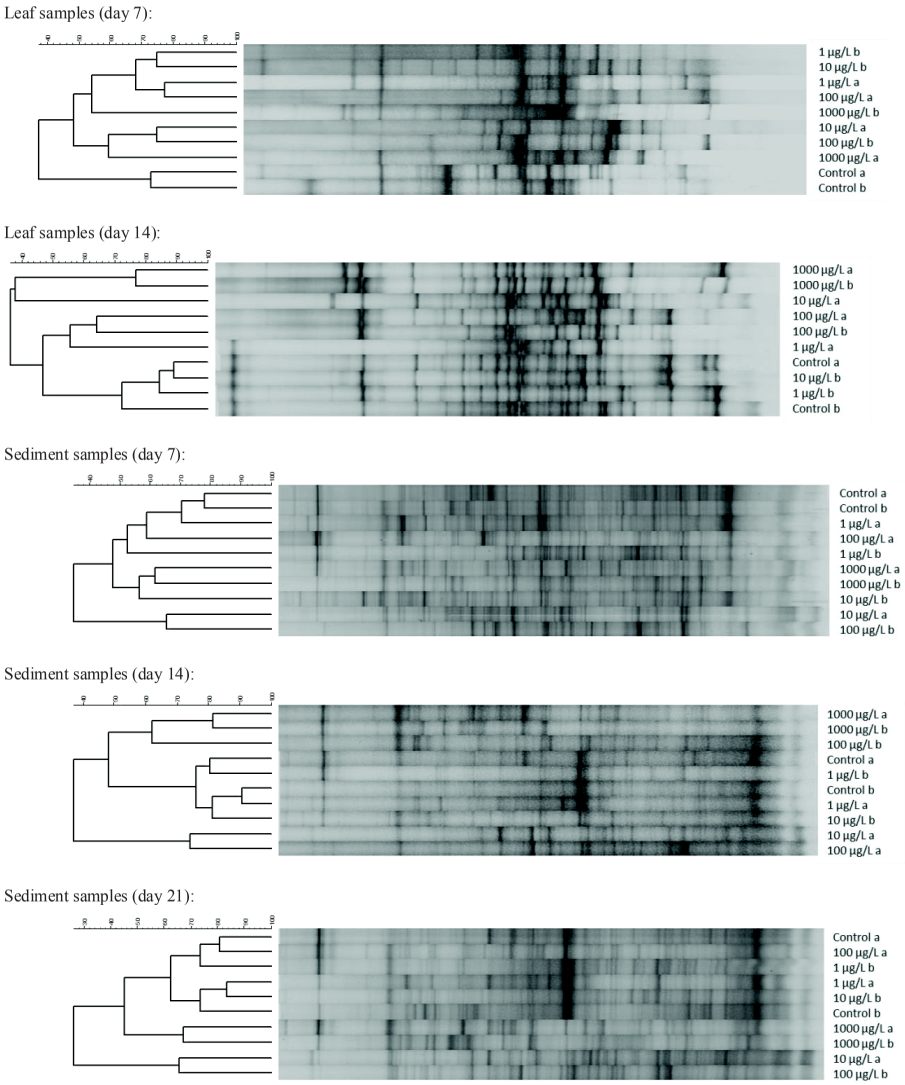

Figure S7. DGGE profiles of 16S rRNA gene pools obtained from the leaf and sediment samples, and clustering of the profiles based on the Pearson correlation coefficient (0-100%). Each band is one operational taxonomic unit (OTU).

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Chapter 6

Effects of the antibiotic enrofloxacin on sediment bacterial community composition and antibiotic resistance genes in tropical freshwater microcosms

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Manuscript in preparation

Abstract

The extensive use of antibiotics either to treat bacterial infections or as prophylactic measures in human and veterinary medicine may lead to serious environmental problems, including the development of bacterial resistance and the alteration of ecosystem functioning. Enrofloxacin is a fluoroquinolone antibiotic that is widely used in aquaculture and livestock production in tropical countries. In this study, we assessed the effects of enrofloxacin on sediment bacterial community composition and the relative abundance of multiple antibiotic resistance genes in tropical freshwater microcosms. Enrofloxacin was applied to the microcosm water for seven consecutive days at a concentration of 10 and 1000 μg/L. Bacterial community composition as well as relative abundance of multiple antibiotic resistance genes were assessed on the last application day (day 7) and seven days after the last enrofloxacin application (day 14). Decrease in the relative abundance of relevant bacterial taxa, such as Acidobacteria, Cyanobacteria and Nitrospira were observed. However, overall community structure was not significantly affected by the antibiotic treatment neither on day 7 nor on day 14. A treatment-related effect was observed on the relative abundance of antibiotic resistance genes, with a significant increase on day 14. Abundance of resistance genes associated to quinolone resistance was relatively low in this study as compared to the abundance levels monitored for genes conferring resistance to other antibiotics. The most notable increase in the abundance of resistance genes was observed for those related to aminoglycoside antibiotics, which can be co-selected by the toxic pressure exerted by quinolone antibiotics.

Introduction

Antibiotics are compounds that can inhibit growth or kill bacteria, and their discovery and use have revolutionized human medicine. Antibiotics are also frequently applied in order to treat and prevent various diseases in animals and plants (Cabello, 2006; Patricia et al., 2002). However, the extensive use of antibiotics to improve human, animal and plant health comes with a cost. Large quantities of antibiotics have been detected in different terrestrial and aquatic ecosystems across the globe (Monteiro and Boxall, 2010; Rico et al., 2014b; Zhu et al., 2013). In some cases, reported environmental concentrations are as high as therapeutic levels (Brandt et al., 2015; Larsson et al., 2007), fueling concerns regarding the potential threats to environmental health and the selection of antibiotic resistant bacteria that may pose a risk to human health (Brandt et al., 2015).

In the last few years, several studies have evaluated the environmental occurrence and fate of antibiotics in the aquatic environment (Rico et al., 2014a; Rico et al., 2014b). Studies have suggested that environmentally relevant concentrations of antibiotics may pose a higher risk to cyanobacteria than to higher aquatic organisms like invertebrates and fish (Halling-Sorensen et al., 2000; Robinson et al., 2005). Moreover, effects of antibiotics on aquatic bacterial community composition and function have been demonstrated (Maul et al., 2006; Rico et al., 2014a). However, fewer studies are available on how the exposure to antibiotics may increase the prevalence and abundance of antibiotic resistance genes in aquatic environments. Model ecosystems (i.e., microcosms and mesocosms) are often used to study fate and effects of chemical in aquatic environments. Such systems provide a more ecological perspective as compared to single-species laboratory assays (Van den Brink et al., 2005). Therefore, they are ideal to study the possible direct and indirect effects of antibiotic contamination on ecosystem structural and functional parameters and to monitor the development of bacterial resistance.

Enrofloxacin is a fluoroquinolone antibiotic that is widely used in Asian aquaculture and also in livestock production in tropical countries (Rico et al., 2014b; Rico et al., 2013). Furthermore, its environmental fate and aquatic toxicity is well documented in literature (Knapp et al., 2005; Rico et al., 2014a; Robinson et al., 2005). Therefore, enrofloxacin was chosen as test compound for this study. The main goals of this study were i) to assess the effects of enrofloxacin application on bacterial community composition in freshwater sediments, and ii) to evaluate its impacts on the resistome using tropical freshwater microcosms. For this, we used high-throughput molecular techniques that allow a screening resolution not often seen in the aquatic risk assessment of antibiotics.

Material and methods

Experimental procedure

Samples used in the present study originated from an outdoor microcosm experiment performed at the Faculty of Fisheries of Kasetsart University (KU, Bangkok, Thailand). Here, only a brief description is provided, whereas a full description of the experiment is given by Rico et al. (2014a). Please note that the original experiment consisted of five treatment levels, while only three of them were sampled for this paper.

The experiment was performed in March and April 2012 (dry season) in six outdoor freshwater microcosms. Microcosms consisted of PVC tanks (volume capacity 600 L) containing approximately 3 cm of silica-based fine gravel (1–2 mm diameter) filled with dechlorinated tap water. Silica-based fine gravel (sediment bed) was obtained from natural rivers in the north of Thailand. Microcosms received an equal volume of concentrated plankton and an even number of macro-invertebrates, both collected from uncontaminated sources in Thailand. Before application of the test substance, a period of four weeks was used for stabilization of the microcosms. To homogenize the experimental units, water was exchanged two times per week between microcosms during the stabilization period. In addition, nitrogen (1.4 mg/L as urea) and phosphorus (0.18 mg/L as triple super phosphate) were supplied to the systems twice per week during the entire experimental period.

Enrofloxacin (Sigma–Aldrich, St. Louis, MO, USA) was daily applied to the microcosms at a nominal concentration of 0, 10 and 1000 μg/L for a period of seven consecutive days. Concentrations of enrofloxacin were chosen to mimic realistic exposure regimes in aquatic ecosystems. Such exposures may result from the environmental release of wastewater from aquaculture and livestock production facilities. Treatments were performed in duplicate, with four microcosms receiving antibiotic treatment and the remaining two microcosms being used as control treatments. The experiment had a duration of 28 days after the first application of the test substance. On days 7 and 14 after the first antibiotic application, sediment was sampled (3 cm) in order to monitor treatment effects on sediment bacterial community composition and abundance of selected antibiotic resistance genes. Sediment samples were kept at -20 °C until further analysis.

Sediment bacterial community composition

Sediment samples were subjected to total DNA extraction, which was performed using the FastDNA® Spin kit for Soil (MP Biomedicals, Solon, OH, USA) according to manufacturer's instructions. Quality and quantity of the isolated DNA were checked by using a Nanodrop ND-100 spectrophotometer (Thermo Scientific, Wilmington, DE,

USA). Before being used in further experiments, DNA samples were diluted to an equal concentration. Sediment bacterial community composition was assessed based on next generation technology sequencing of partial 16S rRNA gene (V1 and V2 regions) fragments, which were amplified by Polymerase Chain Reaction (PCR). Primers and cycle conditions used for PCR reactions are described in Table 1. Full description of library preparation and sequencing is provided by Dimitrov et al. (2014). The sequencing platform used was 454-pyrosequencing (454 Life Sciences, Roche).

Table 1. Primers and cycling conditions used for targeting bacterial communities present in the sediment samples.

Primers	Sequence 5'– 3'	Cycle condition	Reference
27F-DegS	GTTYGATYMTGGCTCAG	95 °C – 2min; 30 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C – 60 sec	van den Bogert et al. (2011)
338R-I	GCWGCCTCCCGTAGGAGT		Daims et al. (1999)
338R-II	GCWGCCACCCGTAGGTGT		Daims et al. (1999)

Quantification of antibiotic resistance genes

Ninety-five selected antibiotic resistance genes and mobile genetic elements, associated to antibiotic resistance (Table S1), were targeted by high-throughput qPCR analysis using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, USA). High-throughput qPCR was performed by ServiceXS B.V. (Leiden, the Netherlands), according to manufacturer's instructions, with the exception that primer-annealing temperature during PCR reaction was lowered to 56 °C. In order to normalize the abundance of antibiotic resistance genes present in a sample, their relative abundances were calculated based on the total bacterial abundance (abundance of the 16S rRNA gene) present in a given sample. However, total bacterial abundance is expected to be higher than abundance of antibiotic resistance genes. Therefore, prior to perform the qPCR reaction on the 96.96 BioMark™ Dynamic Array, sediment DNA was subjected to a specific target amplification with the same primer sets used for qPCR, excluding the 16S rRNA primer set. Conditions of the Specific Target Amplification, as well as further information on the high-throughput qPCR reaction can be found in Bülow (2015).

Four replicates of each sample were used in the high-throughput qPCR assay. A reference sample, consisting of an equimolar mixture of metagenomic DNA isolated from various environments under selective pressure of several antibiotics, was used to determine primer efficiency. Only primers for which efficiency was determined to be between 80% and 120% were used to calculate abundance of the target genes. As recommended by the manufacturer, only CT values lower than 20 were taken into consideration for abundance calculations. Melting curves were used to assess primer specificity. Out of the four replicates used, at least three had to show CT values below 20 to be included in further analysis.

Data analysis

Sequencing analyses were performed using a Snakemake workflow (Koster and Rahmann, 2012), which follows a standard operating procedure for 454 data analysis in mothur version 1.33.2 (Schloss et al., 2009). An OTU (Operational Taxonomic Unit) table was generated following the procedure described in Chapter 02. Such table contains information about the total number of sequences each OTU comprises and in which samples those OTUs were found. Taxonomic information of each OTU is also provided. The OTU table was filtered before to be used for further analyses. OTUs that were not assigned to any kingdom, as well as OTUs classified as Chloroplast were removed from the original dataset. In addition, singletons and doubletons were also removed.

Calculation of normalized abundance of antibiotic resistance genes was performed according to (Bülow, 2015). Briefly, normalized gene abundances were calculated relative to the abundance of the 16S rRNA gene (total bacteria). Delta-delta CT values were calculated as $2^{-(CT\text{antibiotic resistance genes} - CT16S\ rRNA)}$.

Redundancy analysis (RDA) was used to assess whether there was a significant effect of the antibiotic application on the sediment bacterial community composition and on the relative abundance of antibiotic resistance genes found in the studied sediment communities. RDA was also used to determine whether specific OTU(s) affected the relative abundance of antibiotic resistance genes. When that was the case, the relationship of these OTUs with antibiotic resistance genes was established by principal component analysis (PCA) bi-plots. Prior to analyses, OTU raw abundances were transformed to relative abundance, treatment concentrations (explanatory variables) were ln-transformed and normalized abundance of antibiotic resistance genes were ln(Ax+1) transformed. In the transformation ln(Ax+1), x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero as x (for rationale see Van den Brink et al. (2000). The significance level ($p < 0.05$) was determined by a Monte Carlo permutation test (499 permutations). Analyses were performed using

the CANOCO software package, version 5 (ter Braak and Šmilauer, 2012). A heatmap depicting the normalized relative abundances of all antibiotic resistance genes and mobile genetic elements found was created using the software MeV, version 4.9 (Saeed et al., 2003). Normalized abundance values were log2-transformed before the heatmap was created.

Results

Sediment bacterial community composition

A total of 64,683 16S rRNA gene sequences with an acceptable quality were obtained with an average of 5,390 reads per sample, being 4,480 and 6,757 the lowest and highest number, respectively. Based on 97% sequence similarity as threshold, and after filtering, a total of 960 OTUs were found.

RDA analysis did not show significant treatment-related effects on the sediment bacterial community on day 7 ($p = 0.34$) and day 14 ($p = 0.58$). However, for both days RDA plots showed a clear separation between samples originating from the highest treatment level (1000 μg/L) and the lowest treatment level (10 μg/L) (data not shown). Control samples (0 μg/L) grouped close to the lowest treatment level (data not shown). Therefore, despite not being statistically significant as a whole, the antibiotic treatment seemingly induced some changes in the sediment bacterial community structure. The relative abundance of various bacterial phyla was affected by the enrofloxacin application. For instance, Acidobacteria appeared to be very sensitive to the treatment, since its relative abundance in the sediment dropped considerably on both days 7 and 14, compared to control treatments (Figure 1, Table S2). Another phylum that had its relative abundance lowered due to the treatment was Cyanobacteria, with its abundance being almost zero at the highest treatment level on day 14. Despite being present at a very low relative abundance, Nitrospira was the single observed phylum that completely disappeared after the antibiotic treatment (Table S2). In contrast, an increase in relative abundance was observed for Bacteroidetes, Firmicutes, Proteobacteria, Chloroflexi and Verrucomicrobia on at least one sampling date (Table S2). An increase in relative abundance of unclassified bacteria (phylum level) could be seen in both sampling days.

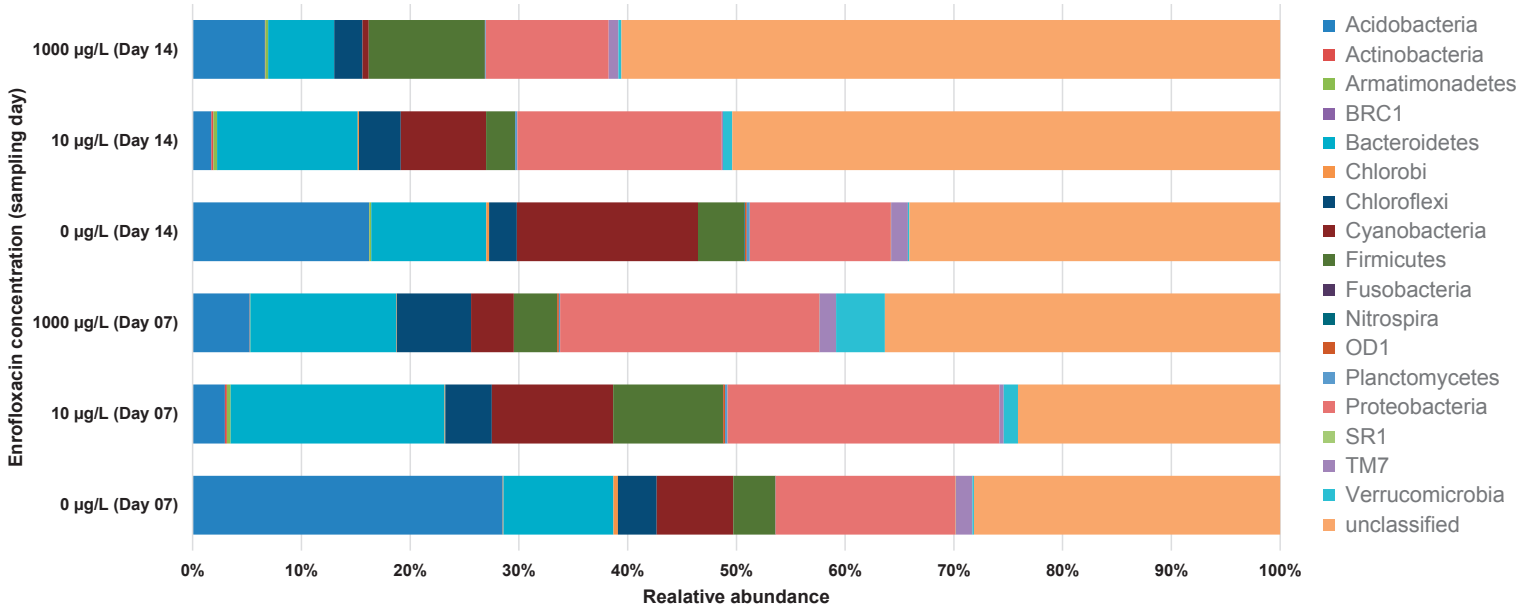

Figure 1. Relative abundance of all bacterial phyla detected in the sediment. Average values are shown for each treatment and sampling day.

Abundance of antibiotic resistance genes

A total of twenty-seven antibiotic resistance genes and mobile genetic elements, conferring resistance to nine different antibiotic classes, were detected in the sediment samples collected during the experiment (Figure 2, Table S3). Several antibiotic resistance genes and mobile genetic elements were detected in all samples, including control samples. Seven resistance genes were detected in sediment samples from the treated microcosms only (Figure 2), and one of these genes is known to promote resistance to quinolone antibiotics (*qnr*S). However, only one out of four targeted genes conferring resistance to quinolone antibiotics was detected (Figure 2, Table S1). A Monte Carlo permutation test performed under the RDA option showed that there was a significant effect (p = 0.038) of the antibiotic treatment on the number and relative abundance of antibiotic resistance genes on day 14. We observed a clear separation of the highest treatment level (1000 µg/L) from the other treatments in the resulting RDA biplot, and a higher number of antibiotic resistance genes were associated with this treatment as compared to the lowest treatment level and control microcosms (Figure 3). Application of enrofloxacin appeared to have promoted resistance to aminoglycosides, and beta-lactam, since almost all detected resistance genes, which are associated to these classes of antibiotics were mainly present and more abundant in the treated samples. On day 14, the abundance of a single gene related to quinolone resistance (*qnr*S) was higher in samples that had received the highest concentration of antibiotic (1000 µg/L), compared to control samples. Quinolone resistance genes were not detected in samples that had received the lowest enrofloxacin concentration (10 µg/L) neither on day 7, nor on day 14.

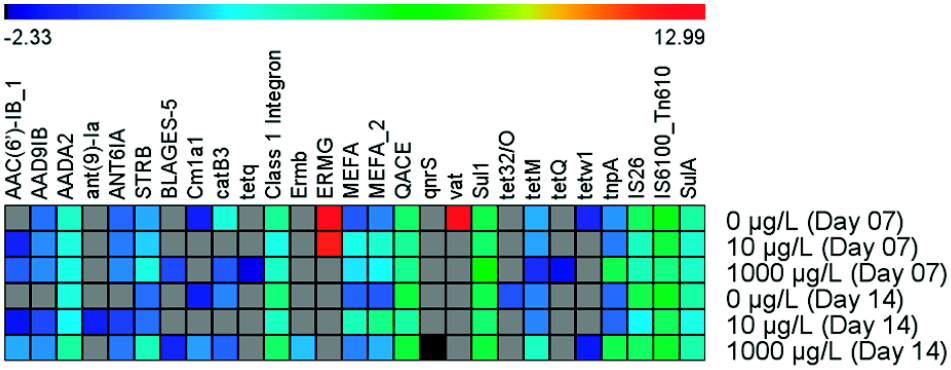

Figure 2. Heatmap depicting the normalized abundances of all antibiotic resistance genes and mobile genetic elements detected in the sediment. Colour scale ranges from dark blue (least abundant) to red (most abundant), whereas grey squares indicate that the respective antibiotic resistance gene was not detected.

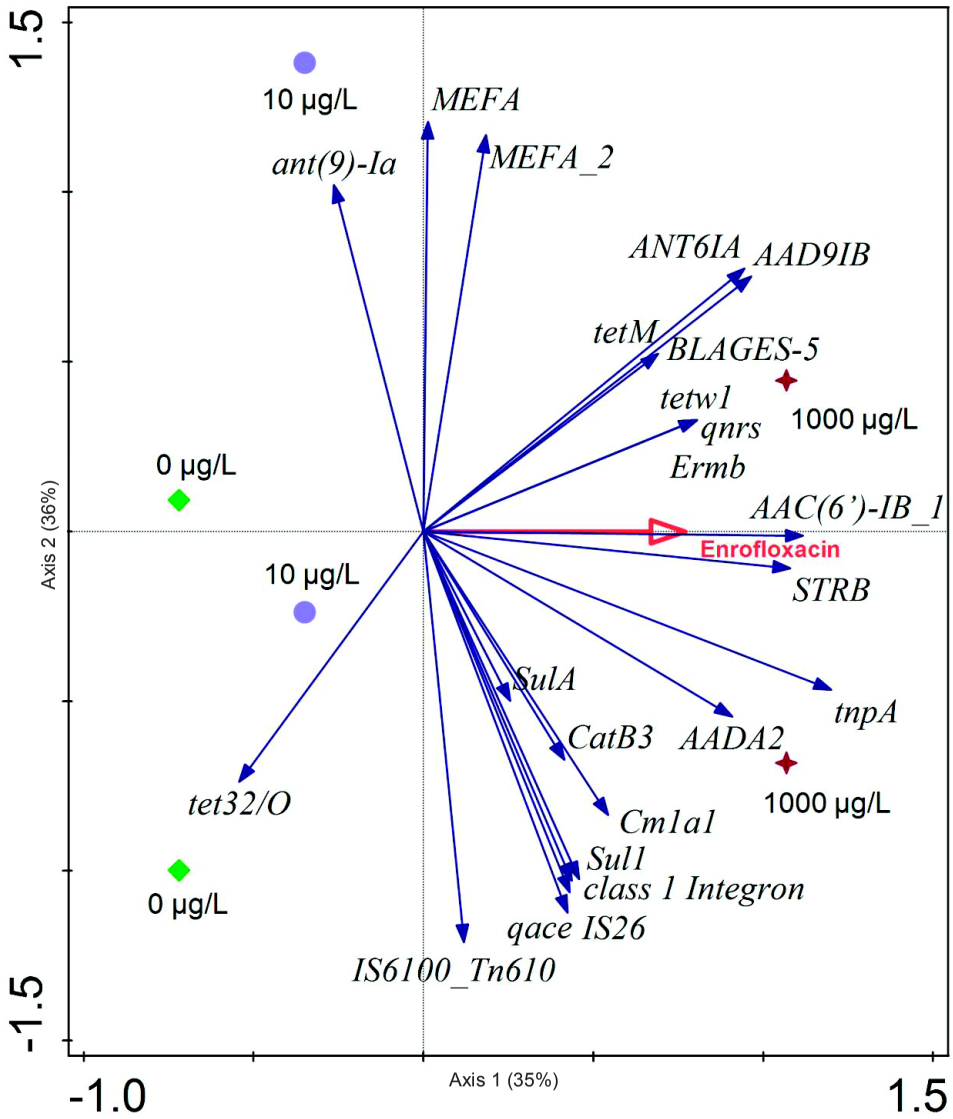

Figure 3. Redundancy analysis diagram of the relative abundance of antibiotic resistance genes detected on the sediment samples at day 14. Duplicates of treatments are shown. Of all variance observed in the relative abundance of antibiotic resistance genes, 71% could be explained by the treatment. Of this explained variance, 35% is displayed in the first (canonical) axis, whereas 36% is attribute to the second axis. The result of the Monte Carlo permutation test indicates that a significant part of the variance on the relative abundance of antibiotic resistance genes is explained by the treatment (p = 0.038).

Sediment bacterial community and antibiotic resistance

Overall, RDA analyses did not show a significant effect of the treatment on the sediment bacterial community at any sampling day. However, there was a significant effect of the treatment on the relative abundance of antibiotic resistance genes on day 14. RDA analysis indicated that a total of twenty-two OTUs significantly affected the relative abundance of antibiotic resistance genes, on day 14 (data not shown). In order to visualize the relationship of the OTUs that significantly affected the abundance of antibiotic resistance genes, a PCA analysis was performed. On day 14, seven days after the last antibiotic application, sixteen out of the twenty-two OTUs had increased relative abundance at the highest treatment level. Figure 4 depicts the correlation of the twelve most significant OTUs (lowest significance values) with the detected antibiotic resistance genes on day 14. From the twelve most significant OTUs, eleven showed a significant increase in relative abundance at the highest treatment level. Most OTUs were classified as belonging to the following phyla: Bacteroidetes, Firmicutes and Proteobacteria. However, some of the OTUs were classified only up to Kingdom level, which means that they were not assigned to any of the known existing phyla. According to the PCA analysis of the samples from day 14, OTUs that increased in abundance in the highest treatment level, showed a correlation with antibiotic resistance genes conferring resistance to the following classes of antibiotics: aminoglycoside, β-lactam, macrolides, quinolones, tetracycline and sulphonamide (Figure 4).

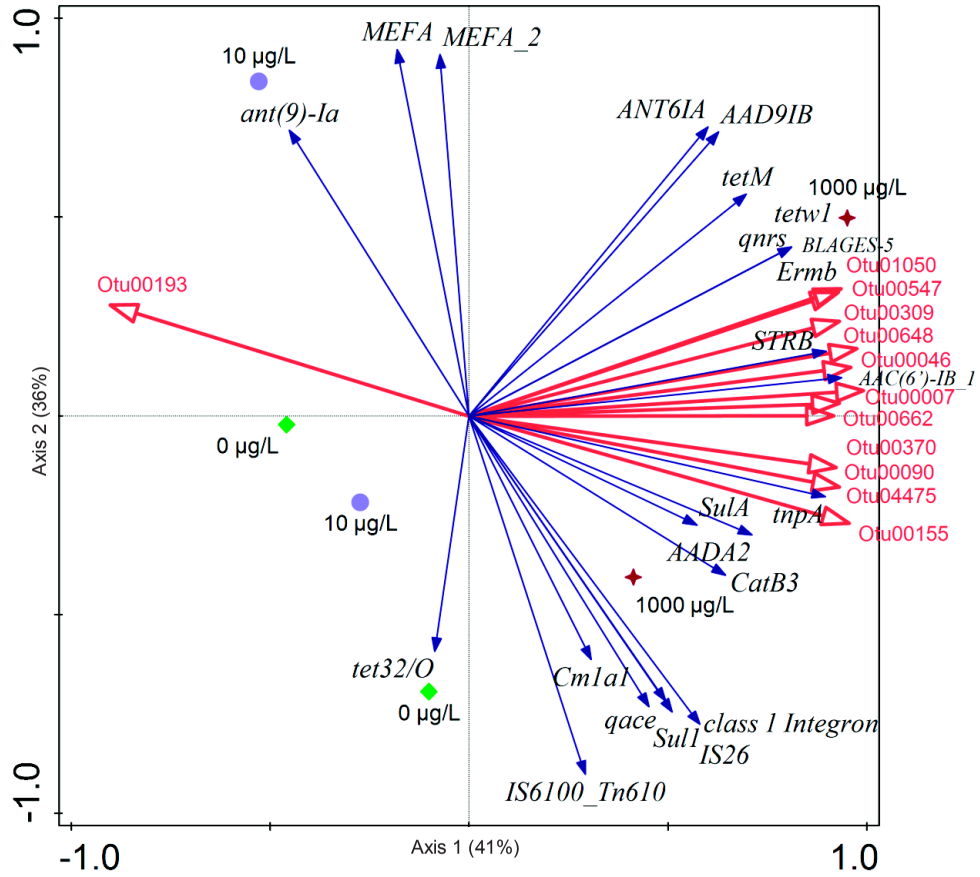

Figure 4. Principal component analysis diagram showing the correlation of detected antibiotic resistance genes and the twelve most significant OTUs on day 14. From all OTUs shown here, OTU00193 (Cyanobacteria) was the only one that had its relative abundance decreased on day 14. The remaining OTUs, OTU00007, OTU00155, OTU00547, OTU01050 (unclassified Bacteria), OTU00046, OTU00090, OTU00309, OTU00370 (Proteobacteria), OTU00622, OTU 04475 (Bacteroidetes) and OTU00648 (Firmicutes) showed an increase relative abundance compared to control samples.

Discussion

Overall, no significant treatment-related effects on sediment bacterial community composition could be observed in any of the samples analyzed in this study. A similar observation was described by Rico et al. (2014a), when the same samples used in this study were analyzed by denaturing gradient gel electrophoresis (DGGE). Rico et al. (2014) showed a significant effect of the treatment on the bacterial sediment community only on day 14. However, a NOEC (No Observed Effect Concentration) of 1000 μg/L was calculated, indicating that concentrations higher than the highest treatment level would be needed to affect sediment bacterial communities. Similarly, Knapp et al. (2005) did not detect a significant effect of enrofloxacin on water bacterial communities, which had been exposed to a single application (25 μg/L) of the antibiotic. However, effects of ciprofloxacin (main by-product of enrofloxacin) on bacterial community composition have been shown in cosm experiments with salt marsh sediments (Cordova-Kreylos and Scow, 2007) and wetlands (Weber et al., 2011). Since photo-degradation and sorption to organic matter are the two major enrofloxacin dissipation processes (Cardoza et al., 2005; Knapp et al., 2005), a non-significant overall effect on the bacterial community could be a result of the high rate of dissipation of this compound from the water phase found during the experiment (Rico et al., 2014a). Additionally, decreased toxicity potential due to high water pH in the experimental systems may have contributed to the non-significant overall effect on bacterial community. To this end it should be noted that the experiment was performed in a tropical environment, which is characterized by high solar irradiance and high algae growth, resulting in elevated pH values that increased the ionized fraction of the evaluated substance (Rico et al., 2014a). Nevertheless, relative abundance of various bacterial phyla showed to be affected by the treatment. Quinolone antibiotics are effective against Gram-positive and Gram-negative bacteria (Cordova-Kreylos and Scow, 2007), however, all phyla that showed a notable decline in their relative abundance in the treated samples were Gram-negative bacterial phyla. Ciprofloxacin was found to be highly toxic to *Microcystis aeruginosa* (Cyanobacteria), with EC_{50} in the range of 1-60 μg/L (Halling-Sorensen et al., 2000). The high sensitivity of Cyanobacteria to ciprofloxacin could explain the drastic decrease in relative abundance of this phylum in the treated microcosms, especially on day 14. Rico et al. (2014a) detected a concentration of 40 μg/L of ciprofloxacin in the highest treatment level after seven days of the last antibiotic application (day 14). It is interesting to note that Rico et al. (2014) also observed a significant effect on the total abundance of the *nifH* gene in the highest treatment level on day 14. *nifH* encodes for nitrogenase, the key enzyme in bacterial nitrogen fixation, which is a trait that is, among others, associated with Cyanobacteria. The drastic decrease in the relative abundance of Cyanobacteria at the highest treatment level on day 14 might have contributed to the decrease in *nifH* abundance as observed by Rico et al. (2014a).

Bacterial phyla containing important human pathogens, which are known to be resistant to quinolone antibiotics, such as Proteobacteria and Firmicutes (Drudy et al., 2007; Neuhauser et al., 2003), were higher in relative abundance in most of the treated microcosms.

As expected, antibiotic resistance genes and mobile genetic elements were found in all microcosms, including the control treatment. However, the highest enrofloxacin concentration used here likely promoted the significant effect on the relative abundance and quantity of antibiotic resistance genes detected on day 14. Unexpectedly, targeted antibiotic resistance genes known to confer resistance to quinolone antibiotics were not amongst the most abundant genes detected. Nevertheless, a quinolone resistance gene (*qnr*S) became detectable in the samples exposed to the highest concentration of enrofloxacin at day 14, seven days after antibiotic application was terminated. Quinolone antibiotics act on DNA replication, hampering the process and impeding DNA duplication (Strahilevitz et al., 2009). Mechanisms of bacterial resistance to antibiotics are various, ranging from point mutations on the enzymes targeted by the antibiotics to production of proteins that protect the targeted enzymes (Strahilevitz et al., 2009; Wright, 2010). Such mechanisms may arise from mutations in the bacterial chromosomal DNA or by the presence of resistance genes, which may be located on mobile genetic elements. Bacteria possess a variety of complex mobile genetic elements (MGEs) such as bacterial plasmids, transposons or cassettes of integrons that enable horizontal transfer of antibiotic resistance genes (Partridge, 2011). Several integrases and tranposases were highly abundant in the sediment samples in both sampling days. Antibiotic resistance integrons, such as the class 1 integron, are widely distributed among Gram-negative bacteria and are considered as a paradigm of genetic transfer between the environmental resistome and both commensal and pathogenic bacteria (Stalder et al., 2012). The class 1 resistance integron frequently carries aminoglycoside, tetracycline, sulfonamide and/or fluoroquinolone resistance genes (Canton and Coque, 2006; Strahilevitz et al., 2009). The selection for, and/or the spread of mobile genetic elements is likely to contribute to simultaneous emergence and spread of multiple antibiotic resistance genes, by a process called co-selection (Gnanadhas et al., 2013). Co-selection or co-resistance, refers to the spread of multiple antibiotic resistance genes present in a single genetic mobile element. Co-selection may have occurred in the treated microcosms, explaining the emergence of quinolone resistance and the simultaneous significant increase in the abundance of various aminoglycoside resistance genes.

The present work indicates that the use of enrofloxacin may lead to shifts in sediment bacterial community composition and increase in the relative abundance of antibiotic resistance genes. Despite effects had only been observed in the highest level treatment, which exceeds reported concentrations found in freshwater ecosystems, intensive use

of enrofloxacin in aquaculture may lead to long-term exposure that is likely to impact the resistome of microbial communities present in aquatic environments. Such impact may lead to the emergence of multi-resistant bacteria, which can pose a serious threat to human health, since aquaculture products as well as other components of aquatic ecosystems (e.g. water, sediments) may work as vectors in the transmission of resistant pathogens to humans.

Supplementary information

Table S1. Antibiotic resistance genes targeted in the present study. For a description of primer sequences please refer to chapter 4 of Bülow (2015).

Antibiotic resistance genes	Antibiotic class
AAC(6')-II	Aminoglycoside
AAC(6')-IB_1	Aminoglycoside
AACC2	Aminoglycoside
AAD9IB	Aminoglycoside
AADA	Aminoglycoside
AADE-like gene	Aminoglycoside
ANT(9)-IA	Aminoglycoside
ANT6IA	Aminoglycoside
APH(2')-IB	Aminoglycoside
APH(2)-ID	Aminoglycoside
APH3IIIA	Aminoglycoside
APH-6AII	Aminoglycoside
KAMR	Aminoglycoside
STRB	Aminoglycoside
BACA	Bacitracin
BACA_1	Bacitracin
BACA_2	Bacitracin
CBIA	β-lactam
CEPA	β-lactam
cfxA4	β-lactam
KPC-2	β-lactam
CEPA_2	β-lactam
ACC-1	β-lactam
BIC-1	β-lactam
BL1_EC	β-lactam
BLACTX-M-1	β-lactam
BLAGES-5	β-lactam
BLAIMP-1	β-lactam
BLANDM-1	β-lactam
BLAOXA-48	β-lactam
BLA-TEM1	β-lactam
BLAVIM-1	β-lactam
CMY-2	β-lactam
DHA-1	β-lactam
IMI-1	β-lactam
BLA-CMY-10	β-lactam
SHV-12	β-lactam
CATA13	Chloramphenicol
MDTL	Chloramphenicol

Table S1 continued.

Antibiotic resistance genes	Antibiotic class
Cm1a1	Chloramphenicol
ACRF	Efflux systems
ARCA	Efflux systems
CATB3	Tetracycline
Macrolide-Efflux-Protein	Efflux systems
TOLC	Efflux systems
TET(A)Q	Efflux systems/QAC
FOSB	Fosfomycin
CLASS 1 Integrase	Integrase
CFR_E.FEACALIS	Linezolid R
CFR_STAPH	Linezolid R
MDTF	Macrolide
ERMA	Macrolide
ERMB	Macrolide
ERMC	Macrolide
ERMF	Macrolide
ERMG(C)	Macrolide
MEFA	Macrolide
MEFA_2	Macrolide
MACB	MRSA/methillin
MECA	MRSA/methillin
ARNA (polymixin)	Polymixin
MDTO	Puromycin
QACA	QACs
QACC	QACs
QACE	QACs
qnrA	Quinolones
qnrB	Quinolones
qnrC	Quinolones
qnrS	Quinolones
VAT	Streptogramin
VATB	Streptogramin
SUL1	Sulfamethoxazole
TET32/O	Tetracycline
TETB(A)	Tetracycline
TETM	Tetracycline
TETQ	Tetracycline
TETW1	Tetracycline
TETX	Tetracycline
DRAFA27	Thrimethroprim
DFRF	Thrimethroprim

Table S1 continued.

Antibiotic resistance genes	Antibiotic class
tnpA	Transposase
IS26	Transposase
IS4group	Transposase
IS6 group	Transposase
IS6_ISS1N	Transposase
IS6100_Tn610	Transposase
IS613	Transposase
IS942	Transposase
ISEcp1B	Transposase
VanA	Vancomycin
VANB	Vancomycin
VanR	Vancomycin
VANUG	Vancomycin
Vanx	Vancomycin
SulA	Sulfonamide

Table S2. Relative abundance of all bacteria phyla detected in the sediment. Average values are shown for each treatment and sampling day.

	Day 07			Day 14		
Phyla	0 µg/L	10 µg/L	1000 µg/L	0 µg/L	10 µg/L	1000 µg/L
Acidobacteria	28.50	2.95	5.24	16.21	1.72	6.65
Actinobacteria	0.02	0.20	0.01	0.02	0.18	0.05
Armatimonadetes	0.05	0.33	0.04	0.17	0.34	0.25
BRC1	0.02	0.00	0.00	0.00	0.01	0.00
Bacteroidetes	10.11	19.67	13.42	10.60	12.92	6.07
Chlorobi	0.42	0.08	0.04	0.25	0.11	0.00
Chloroflexi	3.55	4.28	6.83	2.53	3.84	2.60
Cyanobacteria	7.05	11.15	3.95	16.66	7.84	0.56
Firmicutes	3.83	10.12	3.97	4.27	2.68	10.69
Fusobacteria	0.00	0.01	0.00	0.00	0.00	0.02
Nitrospira	0.02	0.00	0.00	0.06	0.00	0.00
OD1	0.04	0.15	0.16	0.16	0.01	0.00
Planctomycetes	0.03	0.23	0.13	0.28	0.20	0.06
Proteobacteria	16.54	25.01	23.85	12.98	18.80	11.30
SR1	0.02	0.00	0.01	0.01	0.00	0.00
TM7	1.53	0.39	1.50	1.54	0.12	0.90
Verrucomicrobia	0.12	1.31	4.49	0.15	0.84	0.28
Unclassified	28.16	24.11	36.35	34.08	50.39	60.58

Table S3. Antibiotic resistance genes detected in this study and the represented antibiotic classes.

Antibiotic resistance gene	Antibiotic class
AAC(6')-IB_1	Aminoglycosides
AAD9IB	Aminoglycosides
AADA2	Aminoglycosides
ant(9)-Ia	Aminoglycosides
ANT6IA	Aminoglycosides
STRB	Aminoglycosides
BLAGES-5	β-lactam
Ermb	Macrolides
ERMG	Macrolides
MEFA	Macrolides
MEFA_2	Macrolides
QACE	QACs[1]
Cm1a1	Chloramphenicol
qnrS	Quinolones
vat	Streptogramin
Sul1	Sulfonamides
SulA	Sulfonamides
catB3	Tetracyclines
tet32/O	Tetracyclines
tetM	Tetracyclines
tetQ	Tetracyclines
tetw1	Tetracyclines
tetq	Tetracyclines
Class 1 Integron	Integrase (MGE[2])
tnpA	Transposase (MGE[2])
IS26	Transposase (MGE[2])
IS6100_Tn610	Transposase (MGE[2])

[1] quaternary ammonium compounds.

[2] mobile genetic element.

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Chapter 7

General discussion

The importance of microorganisms

Microorganisms make up an unseen world. However, one cannot underestimate the central role these tiny organisms play supporting life on Earth. Microorganisms are ubiquitous, inhabiting a great variety of ecosystems, from deep ocean vents to the skin and gastrointestinal tract of humans and animals. Microbes are responsible for a vast range of processes that not only support, but also allowed life to evolve and exist, as we know it. For instance, cyanobacteria were the first oxygen-producing phototrophic organisms to inhabit our planet and, therefore, directly responsible for oxygenating the atmosphere (Crowe et al., 2013), which allowed the conditions for aerobic life forms to evolve. Many other essential processes that maintain life are mediated by microorganisms, such as degradation of organic matter and recycling of nutrients. Microorganisms can also improve plant nutrition (Mendes et al., 2013), suppress diseases (Mendes et al., 2011), mitigate climate change (Bender et al., 2014) and remediate contaminated ecosystems (Maphosa et al., 2012). Moreover, microorganisms serve as food source to higher organisms, fuelling food webs in terrestrial and aquatic ecosystems.

Aquatic ecosystems provide a habitat to many microorganisms, including bacteria, fungi, protozoans and plankton. As discussed above, microorganisms drive a broad range of biogeochemical cycles and food webs that secure life on Earth. Consequently, detrimental effects on aquatic microbial communities from exposure to synthetic chemicals may seriously affect ecosystem services. Therefore, for the protection of aquatic ecosystems and the services they provide, it is critical to understand how and to which extent chemical pollution may affect aquatic microbial community composition and function.

Effects of synthetic chemicals on aquatic microbial communities

A vast range of synthetic chemicals, such as pesticides, pharmaceuticals and chemicals from personal and home care products, end up in aquatic ecosystems, where they may pose a risk to microbial communities (Kummerer, 2009; Steen et al., 2001). However, studies assessing the effects that synthetic chemicals (e.g. pesticides) might have on aquatic microbial community composition and function are limited, especially with respect to heterotrophic microorganisms (e.g. bacteria and fungi). Many freshwater ecosystems are fuelled by allochthonous organic matter, and heterotrophic microbes are crucial for the conversion of organic matter into animal biomass (Bärlocher, 2005; Webster and Meyer, 1997). Consequently, pollutant-induced changes in the function and composition of aquatic microbial communities could have far-reaching ecological consequences. The general aim of this thesis was to contribute to the understanding of the effects synthetic chemicals, which are often, but not exclusively, used in agriculture and aquaculture, may have on aquatic microbial communities. To this end, we used

a range of complementary cultivation-independent molecular techniques that are not normally applied to studies assessing environmental effects of chemicals.

Previous studies have reported effects of a variety of pesticides (fungicides, herbicides and insecticides) on freshwater sediment bacterial community composition and function (Widenfalk et al., 2008b; Widenfalk et al., 2004). Fungicides are widely used in modern agriculture, however, relatively little is known about their effects on aquatic microbial communities, especially on non-target fungi. A reason for this knowledge gap might be that regulatory risk assessment of fungicides in Europe does not require toxicity data for heterotrophic microbes (Commission, 2013). Instead, in order to protect species that are not required to be tested, assessment factors are applied to data that are obtained by standard toxicity tests. However, it is unclear to which extent such assessment factors account for uncertainties associated with extrapolating from standard toxicity data, which are normally performed with single species and under laboratory circumstances, to microbial communities in the natural environment. A way to reduce this uncertainty is to characterize variation in toxicity sensitivity by constructing species sensitivity distributions (SSDs) (Posthuma et al., 2002). Maltby et al. (2009) created SSDs based on acute single species toxicity data for 39 fungicides and derived threshold concentrations that were hazardous to either 5% (HC5) or 1% (HC1) of the species. For 12 fungicides, derived values were compared to semi-field studies in order to evaluate whether they were protective of adverse ecological effects. A limitation of this study was that no toxicity data for heterotrophic microbes were available. In order to address the limitation faced by Maltby et al. (2009) we performed a study to assess the effects of a widely used fungicide (tebuconazole) on heterotrophic microbes in aquatic microcosms, when applied at its HC5 concentration previously derived without toxicity data for heterotrophic microbes (**Chapter 3**). Therefore, the study reported in **Chapter 3** contributes to the understanding of the potential effects fungicides might have on aquatic microbial communities, including non-target fungi. The main finding of the study described in **Chapter 3** is that tebuconazole, when applied at a single pulse at its HC5 concentration (as derived by Maltby et al. (2009), may affect aquatic fungal diversity and fungi-mediated processes such as feeding behaviour of leaf-shredding organisms. Tebuconazole had a significant effect on conidia production and fungal community composition associated to leaf material. Our study corroborates findings of a previous study that showed a significant decrease in fungal sporulation due to tebuconazole exposure (Zubrod et al., 2011). Change in the structure of fungal communities associated with leaf material, due to tebuconazole exposure, had been demonstrated earlier (Artigas et al., 2012), however, to our knowledge our study was the first to use next-generation sequencing technology to demonstrate that leaf-associated fungal communities might be affected by tebuconazole exposure, providing information at much higher resolution as compared to previous studies. Moreover,

leaf-shredding organisms showed a significant decrease in feeding rate when fed with leaf material that had been exposed to tebuconazole. Such finding is in line with a study of Bundschuh et al. (2011) who reported that leaf-shredding organisms preferred leaf material that had not been exposed to tebuconazole over leaf material exposed to concentrations of either 50 or 500 μg/L tebuconazole. Our study was also the first to demonstrate, by using next-generation sequencing technologies, that tebuconazole, at its HC5 concentration, did not cause a significant effect on freshwater sediment bacterial and fungal community composition. Therefore, in **Chapter 3** we argue that despite limited ecological impact of tebuconazole on the microbial communities of the aquatic microcosms studied, such impact should not be ignored; instead should alarm for the necessity of further research, especially because potential effects on higher organisms were observed, which could lead to more severe ecological effects.

As for fungicides, the use of antibiotics to treat or prevent bacterial infections in human and veterinary medicine may lead to serious environmental problems, including the development of antibiotic resistance and alteration of ecosystem functioning. The reason that antibiotics may lead to environmental pollution and eventually affect microbially-mediated ecosystem services, is that antibiotics are normally poorly adsorbed by the organisms receiving them (Sarmah et al., 2006) and, therefore, are likely to end up in the environment. For example, high percentages (30 - 90%) of administered antibiotics may be excreted via urine as active substances (Alcock et al., 1999). Indeed, several antibiotics that are normally used in human and veterinary medicine have been detected in aquatic environments (Monteiro and Boxall, 2010; Rico et al., 2014), and in some cases at reported concentrations that exceed therapeutic levels (Larsson et al., 2007). Effects of antibiotic pollution on aquatic microorganisms or microbial community functioning have been demonstrated. Halling-Sorensen et al. (2000) reported that both mecillinam and ciprofloxacin antibiotics were highly toxic to the cyanobacterium *Mycrocists aeruginosa*, whereas Maul et al. (2006) demonstrated an effect of ciprofloxacin on the carbon substrate utilization of leaf associated microbial communities. However, effects are often seen at concentrations that are above what is normally detected in aquatic environments (Wunder et al., 2013). Nevertheless, even at non-lethal concentrations, long-term, chronic exposures to antibiotics are likely to affect microorganisms (Andersson and Hughes, 2012). In **Chapter 5** and **Chapter 6**, we assessed the effects of the antibiotic enrofloxacin on the ecology of tropical freshwater microcosms. Effects on organisms belonging to different trophic levels, such as phytoplankton, zooplankton, periphyton and bacteria, were assessed. Enrofloxacin is a fluoroquinolone antibiotic that is widely used in aquaculture in tropical countries (Rico et al., 2014; Rico et al., 2013). The main findings described in **Chapter 5** indicate that environmentally relevant concentrations (μg/L range) of enrofloxacin are not likely to cause direct or indirect toxic effects neither on primary producer

communities nor on bacterial community composition and relevant microbially-mediated functions (e.g. nitrification). However, effects on cyanobacteria and plankton communities, after a long-term exposure to an antibiotic mixture, have been demonstrated (Wilson et al., 2004). We reasoned that the lack of a clear treatment related effect of enrofloxacin on plankton and bacterial communities in our study could be a result of the high dissipation rate of this compound as well as a high water pH observed during the experiment. In our study reported in **Chapter 5**, at enrofloxacin levels higher than environmentally relevant concentrations, transient effects on microbial community composition and function were observed. Effects on bacterial community composition associated with leaf material and nitrifying sediment archaea and bacteria were detected for at least one sampling day. However, processes linked to those communities, such as organic matter degradation and nitrification were either not significantly affected or quickly recovered once the treatment had ceased. Such results indicate a high degree of functional redundancy as well as high recovery capacity of aquatic microbial communities. Results discussed in **Chapter 5** were partially obtained by using molecular techniques, which allowed characterization of aquatic microbial community structure in sediment and leaf material (DGGE) as well as absolute quantification of genes (qPCR) related to nitrogen cycling (nitrification, nitrogen fixation). In **Chapter 6**, we selected three treatment levels of the study described in **Chapter 5** to be analyzed with state of the art molecular techniques in order to provide a higher taxonomic resolution and sensitivity of the potential effects of enrofloxacin exposure on aquatic bacterial communities, when compared to techniques used in **Chapter 5**. In **Chapter 6**, we confirmed observations reported on **Chapter 5**. Overall sediment bacterial community composition, as assessed by next-generation sequencing of PCR-amplified 16S rRNA gene fragments, was not affected by the enrofloxacin treatment. Nevertheless, despite this lack of an overall effect, changes in relative abundance of a number of bacterial phyla were observed as an effect of the treatment. It should be noted that DGGE fingerprinting, the method chosen for Chapter 5, does not directly provide any information regarding the identity of populations affected by a given treatment, and information at higher taxonomic ranks is difficult, if not impossible to obtain unless it is combined with extensive sequence analysis of excised bands. Some bacterial phyla, such as Cyanobacteria, showed a steep decline in relative abundance due to exposure to enrofloxacin. However, since only two sampling dates were analyzed, it was not possible to detect whether relative abundances of affected bacterial phyla, returned to levels similar to control treatments. Nevertheless, a clear example of how next generation sequencing technologies may add to the identification of chemical effects on aquatic microbial communities is given in **Chapter 6**. Sediment samples were also screened for the presence and abundance of antibiotic resistance genes. Several genes known to cause resistance to a vast number of antibiotics, as well as mobile genetic elements that are known to be associated to resistance genes, were quantified by

a high throughput qPRC assay. This approach allowed us to comprehensively determine gene abundances in multiple samples in a single run. Antibiotic resistance genes were abundant in all samples, including control treatment. However, enrofloxacin (highest treatment level) was found to induce a significant increase in relative abundances of multiple antibiotic resistance genes. Surprisingly, the majority of the detected antibiotic resistance genes were responsible for conferring resistance to aminoglycoside antibiotics, and only a single quinolone resistance gene was detected in the treated sediment samples (highest treatment level). Multiple genetic mobile elements were also detected. Class 1 integron is a genetic mobile element known to carry numerous antibiotic resistance genes, including aminoglycoside and quinolone resistance genes (Canton and Coque, 2006; Strahilevitz et al., 2009). Class 1 integron was found to be more abundant in treated samples (highest treatment level), which might also explain the increase in abundance of aminoglycoside resistance genes by a phenomenon known as co-selection or co-resistance (Gnanadhas et al., 2013). Therefore, the main finding of **Chapter 6** is that enrofloxacin exposure might not only affect selected bacterial phyla but also promote bacterial resistance against multiple classes of antibiotics, besides quinolone antibiotics. Multi-resistant bacteria pose a serious threat to human health, and therefore, should not be ignored, especially when the emergence and spread of resistance may happen in an environment that all life forms depend on. As for **Chapters 3 and 5**, **Chapter 6** gives another example of how high-throughput molecular techniques may be applied to the understanding of how chemicals affect aquatic microbial communities. Results obtained in **Chapter 5** and **Chapter 6** indicate that antibiotic pollution is likely to exert chronic exposure of non-lethal concentrations on aquatic microbial communities. Therefore, we argue that further research should be conducted and focus on the assessment of potential effects of these long-term antibiotic exposures on aquatic microbial community composition, function as well as development and persistence of antibiotic resistance. Moreover, it is important to address to which extent functional redundancy in aquatic microbial communities is able to cope with long-term exposure to antibiotic pollution.

Sediment toxicity testing

Microorganisms interact with chemicals in multiple ways. Chemicals that enter aquatic environments might affect microbial community composition and function (**Chapter 3, 5 and 6**) (Widenfalk et al., 2004). In turn, biodegradation and bioavailability of chemicals that accumulate in aquatic sediments may be affected by microorganisms, which can accumulate and degrade a vast range of chemicals (Karpouzas and Singh, 2006; Singh and Walker, 2006; Widenfalk et al., 2008b). Degradation of chemicals by the microbial community may change chemical exposure patterns to higher sediment organisms. Therefore, the understanding of fate of chemicals in aquatic environments is pivotal to determine whether a chemical poses a risk to aquatic organisms or not. To that end, fate

and effects of chemical contaminants in aquatic ecosystems may be assessed by sediment toxicity testing, however, the importance of microorganisms is often overlooked in such tests (Diepens, 2013). In **Chapter 4**, we studied the development of bacterial communities in artificial sediments during pre-equilibration and exposure phases of a standard whole-sediment test. Next-generation sequencing and qPCR targeting 16S rRNA genes were used in order to characterize bacterial communities associated with different test stages and conditions. Furthermore, qPCR was used to quantify abundance of selected functional genes, which are involved in important ecosystem functions mediated by microbes. The choice of targeted functional genes was based, when possible, on whether or not those genes were involved in processes that could affect water quality (e.g. nitrogen cycling) and chemical fate (e.g. biodegradation), as these may influence the outcome of sediment toxicity testing. Sediment toxicity testing is often standardized by using artificial sediments rather than natural sediments. Natural sediments are highly complex and heterogeneous in space and time. Therefore, they are less suitable to be used for sediment toxicity testing, since test outcomes would most likely not be reproducible. Studies have demonstrated that microbial communities are poorly developed in artificial sediments. Even a poorly developed microbial community may directly or indirectly influence water and sediment quality (Goedkoop et al., 2005; Verrhiest et al., 2002). By targeting selected functional genes using qPCR, we demonstrated in **Chapter 4**, that chemical fate and water quality variables, which might affect performance of test species, may be influenced by microbial communities present in the artificial sediment. This observation gives rise to the question whether sediment toxicity testing should be performed with single (eukaryotic) test species only, in order to avoid interactions with other organisms that could influence test outcome. It may be preferred when targeting scientific question, but not on a sediment toxicity testing set up. Despite the fact that a single species test would pose major experimental challenges in order to be achieved (germ-free animals/plants, sterile material and environment, etc.), one might ask how ecologically relevant such test would be. A scenario where higher organisms would not be under influence of microorganisms is unreal. Therefore, on the one hand, tests conducted in the absence of microbial communities would be ecologically less relevant. However, on the other hand, how to account for direct and indirect effects microbes may exert on chemicals and/or on organism(s) being tested? A solution for such dilemma could be the use of a standard inoculum, consisting of a synthetic microbial community, when preparing artificial sediments. However, how to assure that this community behaves and develops consistently in different tests carried out at different times and/or different laboratories? It is clear that further studies are necessary to address the questions raised here, and the data presented in **Chapter 4** reinforced that molecular techniques, in combination with innovative culturing methods for the establishment of representative defined microbial consortia, may be of assistance to address such questions.

Molecular techniques and risk assessment of chemicals – future perspectives

As previously discussed, microbes are essential for the sustainability of life as we know it. Therefore, one could assume that, since microorganisms are so important, their protection from chemical pollution would be a priority. However, the assessment of contaminant effects on microbial communities is precarious, especially in sediments (Widenfalk et al., 2008a). Traditionally, chemical toxicity effects to microorganisms are often evaluated in laboratory tests with single species or by assessing microbial community level endpoints, such as density of selected microorganisms, respiration inhibition and nitrogen transformation tests (Brandt et al., 2015; Kahru et al., 1996; Schafer et al., 2011). However, single species tests and community level endpoint analyses may fail to detect effects on microbial community composition, which could lead to deviations in important ecosystem functions (Widenfalk et al., 2008a). When looking at community level endpoint analyses, effects on community composition may be overseen due to the presence of functional redundancy, which means that loss of microbial species could be compensated by others that perform the same function. However, resistance or resilience to disturbance might depend on the existing level of functional redundancy in microbial communities (Griffiths and Philippot, 2013). Therefore, even when no major effects on selected functional endpoint are seen, chemical contamination may affect community composition, which could lead to loss of diversity, and thus impaired resilience, after prolonged and/or repeated exposure.

Molecular techniques, more precisely 'omics' techniques, are not routinely applied in the risk assessment of chemicals. However, the increasing use of such techniques in a vast range of applications has shown great potential to improve ecological studies (Brandt et al., 2015). The so called 'omics' techniques (genomics, transcriptomics, proteomics and metabolomics) have allowed researchers to have a deeper understanding of how pollutants cause toxicity (Garcia-Reyero and Perkins, 2011). For instance, transcriptomics, which entails the genome-wide study of transcripts, allow researchers to compare gene expression profiles of organisms that were subjected to contrasting situations, such as chemical and no chemical exposure. Comparison of gene expression profiles, associated to genomic information of the organisms being studied, may shed light on how the organisms under study react and protect themselves against chemical exposure. The same principle of transcriptomics holds for other 'omics' techniques. What changes are the biological molecules being studied, for instance, proteomics consists of the study of proteins rather than transcripts. The same techniques can be applied to the study of complex microbial communities in the environment, with the difference that in this case no single species are being studied, but rather communities. In such cases 'omics' techniques are denominated meta-omics, which means a collection of all available biological material of interest, collected from the environment being studied. When the

biological material of interest is DNA the technique is called metagenomics, however, when transcripts are of interest, metatranscriptomics. Metagenomics, as opposed to the 16S rRNA gene targeted approach that was used to characterize bacterial community composition in **Chapters 2, 3, 4 and 6**, allow researchers to gather taxonomic and functional information about the microbial communities under study. Combined taxonomical and functional information may lead to a deeper comprehension of the interaction microorganisms might have with synthetic chemical. For example, Fang et al. (2014) used metagenomic analysis to assess the abundance and diversity of biodegradation genes as well as potential degradation pathways of persistent pesticides such as DDT in marine and freshwater ecosystems. By using metagenomics Fang et al. (2014) were able to identify nearly complete biodegradation pathways for two persistent pesticides (DDT and atrazine). Such finding may help to develop more efficient ways to remediate contaminated sites. However, molecular techniques, as all techniques, bring its fair share of bias. When using molecular techniques to study environmental microbial communities from complex matrices, such as soil and sediment, bias will be present at least in one step of the study; the extraction of the targeted biomolecules (DNA, RNA, proteins). Bias related to soil and sediment DNA extraction is widely acknowledged in the literature (Frostegard et al., 1999; Guo and Zhang, 2013). However, a few studies have attempted to reduce bias related to soil DNA extraction by extracting DNA multiple times from the same sample (Burgmann et al., 2001; Feinstein et al., 2009). In **Chapter 2**, we elaborated on the study from Feinstein et al. (2009) by investigating how bias, due to incomplete DNA extraction, may affect soil microbial characterization. The main findings reported in **Chapter 2** are that considerable amounts of soil DNA are not extracted when only a single DNA extraction is performed. Furthermore, successive DNA extractions of the same soil sample may yield significantly different microbial communities. Therefore, we argue that to improve microbial characterization of environmental samples, successive DNA extractions of the same sample should be performed. DNA obtained in successive extractions should be then pooled prior use in further experiments, as indicated by Feinstein et al. (2009).

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Appendices

Summary

SUMMARY

Nowadays, an increasing number of synthetic chemicals are used in a vast range of applications. When reflecting about our daily activities, it is not difficult to realize how much of our life style is dependent on synthetic chemicals. From the food we eat, medicines we take to the health care products we use, all have their fair share of synthetic chemicals. However, many of the synthetic chemicals we depend on are released into the environment, where they could become a threat to non-target organisms, ecosystems, as well as us, human beings. Due to their use and application, synthetic chemicals may be found in terrestrial, aquatic and atmospheric environments. Aquatic environments receive direct and indirect inputs of chemicals such as pesticides and antibiotics. Pesticides, as well as antibiotics may have a direct or indirect effect on aquatic organisms, including microorganisms, and may affect different trophic levels.

Microorganisms are an essential part of aquatic ecosystems, carrying out crucial ecosystem functions such as primary production, nutrient cycling and decomposition. Moreover, microbial communities have a large influence on abundance and diversity of higher organisms (i.e. benthic invertebrates) by controlling carbon dynamics and providing a food source. On the one hand, microorganisms may influence the degradation and bioavailability of synthetic chemicals. In turn, synthetic chemicals may affect microbial community composition and function in aquatic ecosystems. Surprisingly, not many studies assessing the toxicity of synthetic chemicals on microbial community composition and function have been performed, especially in aquatic environments. Most studies have focused on the microbial degradation of pesticides, for instance, rather than on the effects on microbial communities. Therefore, little attention is given to the potential effects of chemicals on aquatic microbes, especially effects on heterotrophic microbes. Since microorganisms play a central role in many important ecosystem processes, understanding pollutant-induced effects on microbial communities in aquatic ecosystems is pivotal for the protection of such ecosystems.

Sediment toxicity testing is used to assess effects of chemical contaminants in aquatic ecosystems. Such tests are pivotal for the understanding of how chemicals may affect sediment biota, since chemical exposure in the sediment may be different from exposure in the aquatic phase. As previously stated, microorganisms may influence the degradation and bioavailability of chemicals in aquatic sediment, which consequently influences chemical exposure to higher organisms. Therefore, studies evaluating the effects microorganisms might have on the fate of chemicals in the sediment are necessary, since microbes eventually may affect the outcome of sediment tests with higher organisms.

The objectives of this thesis were to implement currently available molecular techniques for the assessment of potential effects of a variety of synthetic chemicals on aquatic

microbial communities, and how in turn microbial communities might affect the fate of such chemicals in aquatic ecosystems.

In **Chapter 3,** we explored the validity of the assumption that regulatory acceptable fungicide concentrations based on ecotoxicological data obtained from studies with fish, invertebrates and primary producers are protective to all other aquatic organisms. For that, the effects of a widely used fungicide, tebuconazole, on non-target aquatic bacterial and fungal communities were assessed in a semi-field study, using currently available molecular techniques. Moreover, indirect effects at a higher trophic level, resulting from the effects on the microbial communities, were studied when tebuconazole exposed leaf material was fed to leaf-shredding organisms. Tebuconazole was applied at its HC5 concentration (concentration that is hazardous to 5% of the tested data) that was derived from acute single species toxicity tests on fish, invertebrates and primary producers. A treatment-related effect of tebuconazole (238 μg/L) on either fungal biomass associated with leaf material or leaf decomposition or the composition and biomass of the fungal community associated with sediment could not be demonstrated. Moreover, treatment-related effects on bacterial communities associated with sediment and leaf material were not detected. However, tebuconazole exposure did significantly reduce conidia production and altered fungal community composition associated with leaf material. An effect at a higher trophic level was observed when *Gammarus pulex* were fed tebuconazole-exposed leaves, which caused a significant decrease in their feeding rate. Therefore, tebuconazole may affect aquatic fungi and fungi-mediated processes even when applied at its "non-microbial derived" HC5 concentration.

In **Chapter 4**, we assessed the development of bacterial communities in artificial sediment during a 28 day bioaccumulation test with polychlorinated biphenyls, chlorpyrifos and four marine benthic invertebrates. DGGE and 454-pyrosequencing of PCR-amplified 16S rRNA genes were used to characterise bacterial community composition. Abundance of total bacteria and selected genes encoding enzymes involved in important microbially-mediated ecosystem functions were measured by qPCR. Community composition and diversity responded most to the time course of the experiment, whereas organic matter (OM) content showed a low but significant effect on community composition, biodiversity and two functional genes tested. Moreover, OM content had a higher influence on bacterial community composition than invertebrate species. Medium OM content led to the highest gene abundance and is preferred for standard testing. Our results also indicated that a pre-equilibration period is essential for growth and stabilization of the bacterial community. The observed changes in microbial community composition and functional gene abundance may imply actual changes in such functions during the test duration, with consequences for exposure and toxicity assessment.

Chapters 5 and 6 assessed the ecological impact of the fluoroquinolone antibiotic enrofloxacin on the structure and function of tropical freshwater ecosystems, as well as on the development of antibiotic resistance. In **Chapter 5**, enrofloxacin was applied at a concentration of 1, 10, 100 and 1000 μg/L for seven consecutive days in outdoor microcosms in Thailand. The ecosystem-level effects of enrofloxacin were monitored on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoint groups for 4 weeks after the last antibiotic application. Consistent treatment-related effects on the invertebrate and primary producer communities and on organic matter decomposition could not be demonstrated. Enrofloxacin significantly affected the structure of leaf-associated bacterial communities at the highest treatment level, and reduced the abundance of ammonia-oxidizing bacteria and ammonia-oxidizing archaea in the sediments. The ammonia concentration in the microcosm water significantly increased at the highest treatment level, and nitrate production was decreased, indicating a potential impairment of the nitrification function at concentrations above 100 μg/L. The results reported in **Chapter 5** suggest that environmentally relevant concentrations of enrofloxacin are not likely to result in direct or indirect toxic effects on the invertebrate and primary producer communities, nor on important microbially mediated functions such as nitrification. In **Chapter 6**, we expand on the findings described on **Chapter 5** by using three treatment levels (0, 10 and 1000 μg/L) of the outdoor microcosm experiment to assess the effects of enrofloxacin on sediment bacterial community composition and the relative abundance of multiple antibiotic resistance genes on the last application day (day 7) and seven days after the last enrofloxacin application (day 14). Shifts in the relative abundance of relevant bacterial taxa (Acidobacteria, Bacteriodetes, Chloroflexi, Cyanobacteria, Firmicutes and Proteobacteria) were observed, however, overall community structure was not significantly affected by the antibiotic treatment neither on day 7 nor on day 14. A treatment-related effect was observed on the relative abundance of antibiotic resistance genes, with a significant increase on day 14. Abundance of resistance genes associated to quinolone resistance was relatively low in this study as compared to levels observed for genes conferring resistance to other antibiotics. The most notable increase in the abundance of resistance genes was observed for those related to aminoglycoside antibiotics, which can be co-selected by the toxic pressure exerted by quinolone antibiotics.

In **Chapter 2**, we performed an assessment of how much incomplete DNA extractions from a complex environmental matrix, such as soil, may affect microbial community characterization when using molecular approaches. Independently of the molecular approach used, soil DNA extraction is a crucial step. Success of downstream procedures used for microbial characterization will depend on how well DNA extraction was performed. Often, studies describing and comparing soil microbial communities are

based on a single DNA extraction, which may not lead to a representative recovery of DNA from all organisms present in the soil. To determine whether successive DNA extractions, performed on the same soil sample, would lead to different observations in terms of microbial abundance and community composition, we performed three successive extractions, with two widely used commercial kits, on six different soil samples. Successive extractions increased considerably DNA yield, as well as total bacterial and fungal abundances in most of the soil samples. 454-pyrosequencing analyses of the 16S and 18S rRNA genes revealed that microbial community composition (taxonomic groups) observed in the successive DNA extractions were similar. Successive DNA extractions revealed a few additional microbial groups, which were not observed with a single extraction. However, relative abundance of these additional groups was very low. Nevertheless, for some soil samples shifts in microbial community composition were observed, mainly due to shifts in relative abundance of a number of microbial groups.

In **Chapter 7**, a general discussion of the main findings of this thesis is provided, and future perspectives for the use of molecular tools for the ecological risk assessment of chemicals are given.

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About the author

ABOUT THE AUTHOR

Mauricio Rocha Dimitrov was born on 28th of April, 1983 in São Paulo, Brazil. In 2002 he started the study of Biology at the São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil. During his studies he worked at the Laboratory of Biochemistry of Microorganisms and Plants (LBMP) on the construction of soil metagenomic libraries for the screening of bacterial genes related to the production of polyhydroxyalkanoates (PHAs). He obtained his B.Sc. degree in 2006 and moved to the University of São Paulo (USP) to start his M.Sc. studies in Biotechnology. During his M.Sc. studies he continued to work on soil metagenomic libraries and bacterial genes related to the production of PHAs. Towards the end of his M.Sc. studies, he spent four months as a guest researcher at the Netherlands Institute of Ecology (NIOO-KNAW), Heteren, the Netherlands. In 2009 he obtained his M.Sc. degree and moved to the Netherlands to start his Ph.D. research. He was appointed as a Ph.D student in the Molecular Ecology group at the Laboratory of Microbiology, Wageningen University, Wageningen, the Netherlands. His Ph.D. research focused on the assessment of the effects of chemicals on aquatic microbial ecosystems.

List of publications

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Veraart, A.J., Audet, J., Dimitrov, M.R., Hoffmann, C.C., Gillissen, F., de Klein, J.J.M., 2014. Denitrification in restored and unrestored Danish streams. Ecological Engineering 66, 129-140.

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Lin, R., Buijse, L., Dimitrov, M.R., Dohmen, P., Kosol, S., Maltby, L., Roessink, I., Sinkeldam, J.A., Smidt, H., Van Wijngaarden, R.P., Brock, T.C., 2012. Effects of the

fungicide metiram in outdoor freshwater microcosms: responses of invertebrates, primary producers and microbes. Ecotoxicology 21, 1550-1569.

Paixao, D.A.A., Dimitrov, M.R., Pereira, R.M., Accorsini, F.R., Vidotti, M.B., Lemos, E.G.D., 2010. Molecular Analysis of the Bacterial Diversity in a Specialized Consortium for Diesel Oil Degradation. Rev Bras Cienc Solo 34, 773-781.

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* Contributed equally

In preparation

Dimitrov, M.R. et al., Successive DNA extractions improve characterization of soil microbial communities.

Dimitrov, M.R. et al., Effects of the antibiotic enrofloxacin on sediment bacterial community composition and antibiotic resistance genes in tropical freshwater microcosms.

Paulo, A.M.S. et al., Identification and characterization of aerobic and anaerobic bacteria that degrade sodium lauryl ether sulfate (SLES).

Veraart, A.J. et al., Abundance, richness, and activity of denitrifiers in drainage ditches in relation to sediment characteristics, vegetation and land-use.

Khatikarn, J. et al., Ecological impacts of the antimicrobial triclosan on freshwater communities in tropical microcosms.

Overview of completed training activities

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

For specialised PhD training

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE) declares that

Mauricio Rocha Dimitrov

born on 28 April 1983 in São Paulo, Brazil

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 17 March 2016

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Huub Rijnaarts	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

K O N I N K L I J K E N E D E R L A N D S E
A K A D E M I E V A N W E T E N S C H A P P E N

The SENSE Research School declares that **Mr Mauricio Rocha Dimitrov** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 46.1 EC, including the following activities:

SENSE PhD Courses

- Environmental Research in Context (2010)
- New Frontiers in Microbial Ecology (2010)
- Basic statistics (2011)
- Principles of Ecological Genomics (2011)
- Research in Context Activity: 'Co-organising PhD study trip of the Laboratory of Microbiology to China and Japan', Wageningen University (2011)
- Environmental Risk Assessment of Chemicals (2012)

Other PhD and Advanced MSc Courses

- PhD competence assessment, Wageningen University (2010)
- Project and time management, Wageningen University (2010)
- Scientific writing, Wageningen University (2011)
- De novo Assembly of NGS data, Wageningen University, The Netherlands Bioinformatics Centre, and Leiden University Medical Center (2013)
- ARB training - phylogenetic software for microbial genomics, Wageningen University (2014)
- Metagenomics approaches and data analysis, Radboud University Nijmegen (2014)

Management and Didactic Skills Training

- Supervising MSc student with thesis entitled 'Effects of Aquiculture antibiotics on non-target bacterial communities in freshwater ecosystems' (2013)
- Supervising BSc student with thesis entitled 'Bacterial abundance and community structure in OECD sediments before and during a sediment toxicity test' (2013)
- Supervising internship student with thesis entitled 'Detection of antibiotic resistant bacteria in sediment samples from aquaculture farms in the Tha Chin River, Thailand' (2013)
- Teaching practical courses in the MSc course 'Research Methods Microbiology' (2010) and in the BSc course 'Microbial physiology' (2010-2012)

Oral Presentations

- *Effects of the fungicide tebuconazole on fungal and bacterial communities - insights from outdoor freshwater microcosms*. 3rd Young Environmental Scientist Meeting , 11-13 February 2013, Krakow, Poland
- *Importance of diversity and functional redundancy for the structure and functioning of freshwater microbial communities*. SENSE Summer Symposium - Ecosystem under stress: assessing the impact of chemical and physical disturbances on ecological processes and ecosystem structure, 14 December 2012, Wageningen, The Netherlands
- *Population and community level effects of fungicide exposure in outdoor freshwater microcosms*. PhD study trip – Laboratory of Microbiology, 15 April-1 May, Beijing, China

SENSE Coordinator PhD Education

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