

BRIEF REPORT

Selection of aquatic microbiota exposed to the herbicides flufenacet and metazachlor

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Abstract

Herbicides are important, ubiquitous environmental contaminants, but little is known about their interaction with bacterial aquatic communities. Here, we sampled a protected natural freshwater habitat and characterised its microbiome in interaction with herbicides. We evolved the freshwater microbiomes in a microcosm assay of exposure (28 days) to flufenacet and metazachlor at environmental concentrations of 0.5, 5 and 50 µg L⁻¹. Inhibitory effects of herbicides were exemplarily assessed in cultured bacteria from the same pond (*Pseudomonas alcaligenes*, *Paenibacillus amylolyticus* and *Microbacterium hominis*). Findings were compared to long-term concentrations as provided by local authorities. Here, environmental concentrations reached up to 11 µg L⁻¹ (flufenacet) and 76 µg L⁻¹ (metazachlor). Bacteria were inhibited at minimum inhibitory concentrations far above these values; however, concentrations of 50 µg L⁻¹ of flufenacet resulted in measurable growth impairment. While most herbicide-exposed microcosm assays did not differ from controls, *Acidobacteria* were selected at high environmental concentrations of herbicides. Alpha-diversity (e.g., taxonomic richness on phylum level) was reduced when aquatic microbiomes were exposed to 50 µg metazachlor or flufenacet. One environmental strain of *P. alcaligenes* showed resistance to high concentrations of flufenacet (50 g L⁻¹). In total, this study reveals that ecologic imbalance due to herbicide use significantly impacts aquatic microbiomes.

INTRODUCTION

In natural waters, microorganisms play a key role in the vitality of ecosystems (Verrhiest et al., 2002). Changes in microbial community composition are used as biological markers of contamination (Sumampouw & Risjani, 2014) or early-warning-indicators of ecosystem disturbance (Jurado et al., 2011). Besides other contaminants, herbicides might disturb ecosystem integrity (DeLorenzo et al., 2001; Wijewardene, Wu, Hörmann, et al., 2021; Wijewardene, Wu, Qu, et al., 2021). Common herbicides used worldwide are, for example, metazachlor (Met) and flufenacet (Flu) (Carpio et al., 2020;

Maršík et al., 2021; Ulrich, Lorenz, et al., 2021; Willkommen et al., 2019). Following application, diffuse inputs of herbicides to non-target areas occur unavoidably (Chow et al., 2020; de Souza et al., 2020; Mohaupt et al., 2020; Ulrich, Pfannerstill, et al., 2021), and herbicides can exert adverse effects on non-target organisms such as crustaceans, fish and algae (De Brito Rodrigues et al., 2017; Spycher et al., 2018; Wijewardene, Wu, Qu, et al., 2021). On a community level, macrophytes, phytoplankton and bacteria form metaorganism-like synergies by mutual nutrient supply (Kazamia et al., 2016), hence, shifts in macrophytes due to herbicide contamination may be followed by

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microbiome changes. Looking at the direct effects of herbicides on aquatic bacterial communities, the current database is more than limited. DeLorenzo et al. (2001) reviewed, besides other studies, the findings of Peterson et al. (1994) where *Cyanobacterium* spp. was significantly inhibited by metazachlor. In soil, inhibitory effects of metazachlor and flufenacet on *Azotobacter* spp., organotrophic bacteria, Actinomycetes and fungi have been reported (Baćmaga et al., 2014; Tomkiel et al., 2019). By contrast, Muturi et al. (2017) found no effects of other herbicides (atrazine; glyphosate) on bacteria.

This study aimed to investigate the ecological dynamics of natural freshwater microbiomes in microcosm experiments that were exposed to different concentrations of herbicides over 28 days. We hypothesised that sub-inhibitory concentrations of herbicides interact with microbiomes, either by direct or indirect (plant-community-driven) selection. For that purpose, data were related to phytoplankton communities that were previously studied in the same microcosm (Wijewardene et al., 2022). To clarify whether microbiome shifts were due to direct or indirect interaction, we investigated the direct effects of flufenacet or metazachlor on aquatic bacterial isolates by in vitro experiments (MIC determination and growth curve assay). In detail, we wanted to investigate (i) whether and at which concentration level pesticides affect natural freshwater microbiomes, (ii) which diversity indices were affected at phylum- and ASV-level, (iii) whether effects differ between the two substances metazachlor and flufenacet, (iv) whether these effects may be based on a direct mode of action, without involving plant communities.

EXPERIMENTAL PROCEDURES

Experimental design

Sampling of water

Water samples from a pond (54°44'20" N, 9°35'42" E) in the Winderatter Lake nature reserve area in northern Schleswig-Holstein were taken on the morning of 17 August 2020. Applied herbicides were not detected in that conservation area by pre-measurements. The experimental setup, the physico-chemical conditions and the nutrients added, which were monitored daily, were described by Wijewardene, Wu, Hörmann, et al. (2021). In addition to the original experiment (containing 87 glass vessels of 2.4 L volume), 21 additional glass vessels of 500 mL were filled on d0, meant to control the equal distribution of original bacterial communities after dividing the water into the vessels). These vessels were labelled according to the pipetting

order (control A d0, d2, d14, d28; control B d0, d2, d14, d28; Met05-A d0, d2, d14, d28; Met05-B d0, d2, d14, d28), so they had herbicide labels although not exposed to herbicides. To the other vessels, herbicides were added on d0 in triplicates at concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$ as described by Wijewardene, Wu, Hörmann, et al. (2021). Triplicates of the original water sample were taken after transport to Kiel University (d-1). In addition, three non-exposed controls per date were exposed to the same environmental conditions at the same time as were controls. Subsequently, 21 samples, each, were collected 2, 7, 14 and 28 days (d2, d7, d14, d28) after herbicide exposure. After collection, biofilms were resuspended by mechanical pressure, using a sterile glass pipette and 500 mL of the original water sample. That suspension was provided in total and stored at -20°C until molecular biological analysis.

Laboratory procedures

DNA extraction

For water extraction, water column samples (450 mL) were first filtered in three steps (2–4 μm : pleated cellulose filters, Macherey&Nagel, Düren, Germany; 0.45 μm , 0.2 μm : Polyethersulfon, Sarstedt, Nümbrecht, Germany) after thawing. DNA was extracted (i) from the last filter, using a modified protocol that combined the DNeasy Power Water Kit (Qiagen, Hilden, Germany) with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described by Djurhuus et al. (2017) and (ii) from the sedimented organic material that remained after pipetting most of the water (450 of 500 mL). For that extraction of sedimented organic material ('vessel sediment'), the organic material was mechanically scratched from the bottom of vessels with the help of a glass pipette and was then transferred to a 50 mL tube, where it was allowed to sediment again. The supernatant was discarded, and the sedimented material was transferred to 1.5 mL tubes and centrifuged at $13,000\times g$. Again, the supernatant was discarded. Following that, extraction of the pellet was performed using the DNeasy Power Soil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, maximum of 250 mg were transferred to a bead tube, and the extraction procedure was based on six steps: homogenisation, mechanical lysis by bead beating (bead size 0.7 mm garnet), inhibitor removal, binding of the DNA to the silica membrane, wash process and an elution step. Elution was completed by adding 100 μL of nuclease-free water to finally elute into a new tube, followed by centrifugation at $10,000\times g$ for 30 sec. As an extraction control, PCR-grade water—labelled H_2O —was included in extraction and sequencing.

Illumina amplicon sequencing

Amplification of the V1-V2-hypervariable-region of the bacterial 16S-rRNA-gene was performed by one-step-PCR using barcoded V1-V2-primers (Kozich et al., 2013) as described elsewhere (Schwenker, Friedrichsen et al., 2022). In total 218 samples (including extraction controls) were submitted for sequencing (IKMB, Kiel, Germany). A no-template control and a mock community DNA standard (Zymo Research Europe GmbH, Freiburg, Germany) with predefined microbial species were included. Equimolar libraries were sequenced using the paired-end MiSeq reagent kit v3 (2×300 bp chemistry) on the MiSeq platform (Illumina Inc., San Diego, CA, USA) according to Caporaso et al. (2012).

Quantitative PCR of 16S-amplicons

16S-amplicon contents were quantified in vessel sediment material by SYBR-quantitative PCR (qPCR) using V1-V2 primers as used by Kozich et al. (2013) but without barcoding. SYBR-qPCR reaction was performed using Luna Universal qPCR Master-Mix (New England Biolabs, Frankfurt am Main, Germany) on a QuantStudio5 cycler (Thermo Fisher Scientific, Dreieich, Germany). Each reaction mixture contained 5 µL of Master-Mix (0.25 µL of each primer) and 4.5 µL of DNA template for a final volume of 10 µL. Temperature-time-conditions were 95°C for 60 sec, followed by 45 cycles at 95°C for 15 sec and 60°C for 30 sec. Melt curves were recorded by applying 95°C for 15 sec, 60°C for 1 min, followed by dissociation at 0.1°C/s and 95°C for 15 sec. *Staphylococcus aureus* ATCC 29213 (DSM 2569) with six copies of 16S-rRNA-genes (<https://genomes.atcc.org/genomes/21eea9803c88405a>) was used as a standard in concentrations from 1.5×10^7 to 1.5×10^3 cfu mL⁻¹. Concentrations of unknowns were calculated as copy numbers by multiplying ATCC-29213-equivalents as detected by qPCR with the number of six.

Minimum inhibitory concentrations in aquatic bacteria

Three strains of *Pseudomonas* (*P.*) *alcaligenes*, *Paenibacillus amylolyticus* and *Microbacterium hominis* had been previously cultured from water samples of the same lake as used in the microcosm experiment and had been identified by MALDI-TOF MS (Autoflex 3 Smartbeam, Bruker Daltonics GmbH, Bremen, Germany). Strains were tested for inhibitory effects of metazachlor and flufenacet by standardised macrodilution as described elsewhere (Schwenker, Schotte, & Hölzel, 2022). Herbicide concentrations of 50 µg to 50 g L⁻¹ were prepared in 1:10 steps

(original concentrate: 500 g L⁻¹). Minimum inhibitory concentrations (MIC) were determined after incubation at 30°C for 24 h. Suspensions from all tubes (irrespective of whether they had increased in turbidity or not) were transferred to solid agar after incubation for MIC determination, to determine the extent of inhibition and to ensure purity. *P. alcaligenes* was further tested by growth curve assessment at concentrations of 50 g L⁻¹ (flufenacet), 2.5 g L⁻¹ (metazachlor, flufenacet) and 50 µg L⁻¹. Herbicides were diluted in water of standardised hardness (WSH), for macrodilution. Late-exponential-growth phase cultures were diluted 1:1 in double-concentrated Trypticasein Soy Broth (TSB; Carl Roth GmbH & Co. KG, Karlsruhe, Germany), followed by three 1:10 dilution steps in quarter-strength Ringer solution (BR0052G, Thermo Fisher Dreieich, Germany). Of this suspension, 1 mL was used to inoculate a mixture of 24 mL 2× TSB with 25 mL WSH ± herbicide in a wide-neck flask. Flasks were slightly covered with aluminium foil and incubated at 30°C on an incubator shaker. For quantification, 500 µL suspension was pipetted to 4.5 mL of Ringer solution. The 1:10 dilution was then thoroughly homogenised and 100 µL was spirally plated out onto Columbia agar containing 5% sheep blood (Thermo Fisher Scientific, Dreieich, Germany).

Bioinformatic analysis

Microbiome data were processed using the DADA2 workflow version 1.10 for large datasets resulting in abundance tables of amplicon sequence variants (ASV) (Callahan et al., 2016). Briefly, sequences were processed separately and finally collected into a single abundance table per dataset and checked for chimaeras. ASVs were taxonomically annotated by the naïve Bayesian classifier provided in DADA2 and the Ribosomal Database Project (Wang et al., 2007).

Statistics

Statistical analyses were performed using R-software version 4.0.2 (R Development Core Team, 2020), in which the figures were created using R-package *ggplot2* (Wickham, 2016). Alpha (taxonomic) diversity indices, such as phyla/ASV richness, the Shannon-Wiener index and evenness (Shannon, 1963), and the Simpson index (Simpson, 1949) were calculated by function *div* in the R-package *ecolooop* (Guo, 2019). Significant differences in alpha diversity indices among samples at each sampling time were identified by the ANOVA test/Kruskal–Wallis test. Variations among different treatments and sampling times (beta-diversity) were analysed by calculating the relative abundance of phyla/ASV (R Development Core Team, 2020) and by performing the principal coordinate analysis (PCoA, based on the Bray–Curtis method) with Hellinger-

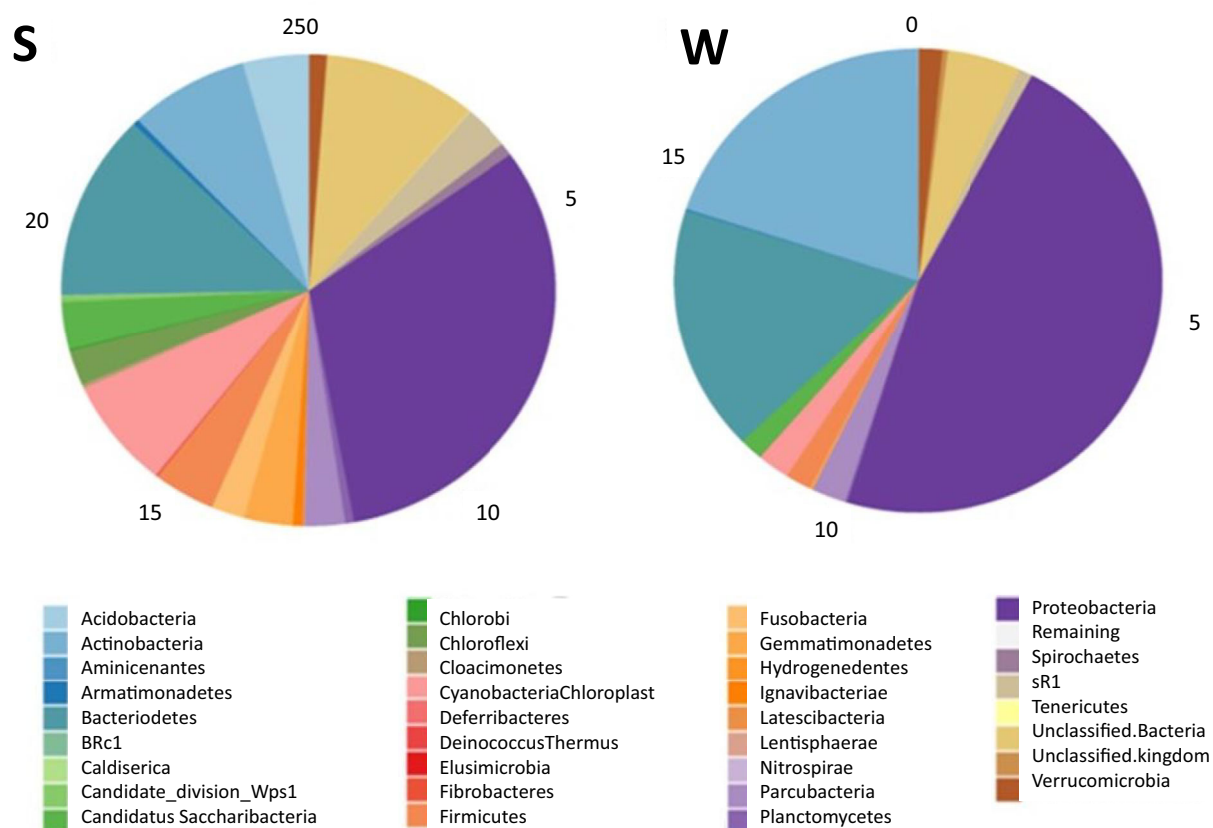


FIGURE 1 Phyla detected in sedimented organic material (S) and in filter-retentates of water (W). Reads were pooled among all replicates.

transformed data using R-package *vegan* (Oksanen et al., 2016).

RESULTS

Absolute abundance and bacterial quantity

Water column

Mean bacterial reads of the water column samples were below 100 on d-1 and below 50 on d0, d14 and d28, below 4000 on d7, but above 25,000 on d2. All phyla found in water were present in the glass vessel sediment material, but not vice versa (Figure 1). Only a minor part of the water column samples (11/108 samples) resulted in successful sequencing. Thus, further analysis was only performed for vessel sediment material, apart from Bray–Curtis analysis (Figure S1A,B).

Vessel sediments

Looking at absolute abundances in vessel sediments (Figure 2), the duration of the experiment was negatively associated with sequencing success: while only 4 out of

84 sample replicates from d1 to d7 had less than 5000 reads, 15 out of 42 sample replicates from d14 to d28 had less than 5000 reads, most of them ($n = 13$) below 500. Of those, the majority ($n = 11$) was taken 29 days after initially sampling the pond and belonged to control or low concentrations of herbicides. Results were compared to 16S-copy number as quantified by qPCR, pointing to the fact that unsuccessful sequencing was rarely related to low contents of 16S-amplicons: Only two d7-samples of Met50-assays were significantly lowered in 16S-amplicon concentrations (below the limit of quantification at 10^3 copies mL^{-1}). All other samples with less than 500 reads had concentrations above 10^7 copies mL^{-1} . Mean 16S-amplicon concentrations in sedimented organic material were $1.10 \pm 0.58 \times 10^8$ copies mL^{-1} in the original sample on d-1, and $7.89 \pm 5.67 \times 10^6$ copies mL^{-1} (Flu5) to $6.00 \pm 7.10 \times 10^7$ copies mL^{-1} (Met05) in the samples of d0. Final concentrations were $8.99 \pm 7.57 \times 10^6$ copies mL^{-1} (Met5) to $0.95 \pm 1.10 \times 10^8$ copies mL^{-1} (control).

Beta-diversity: Relative abundance

Figure 3 depicts the changes in relative abundance over time. The most striking changes are seen in

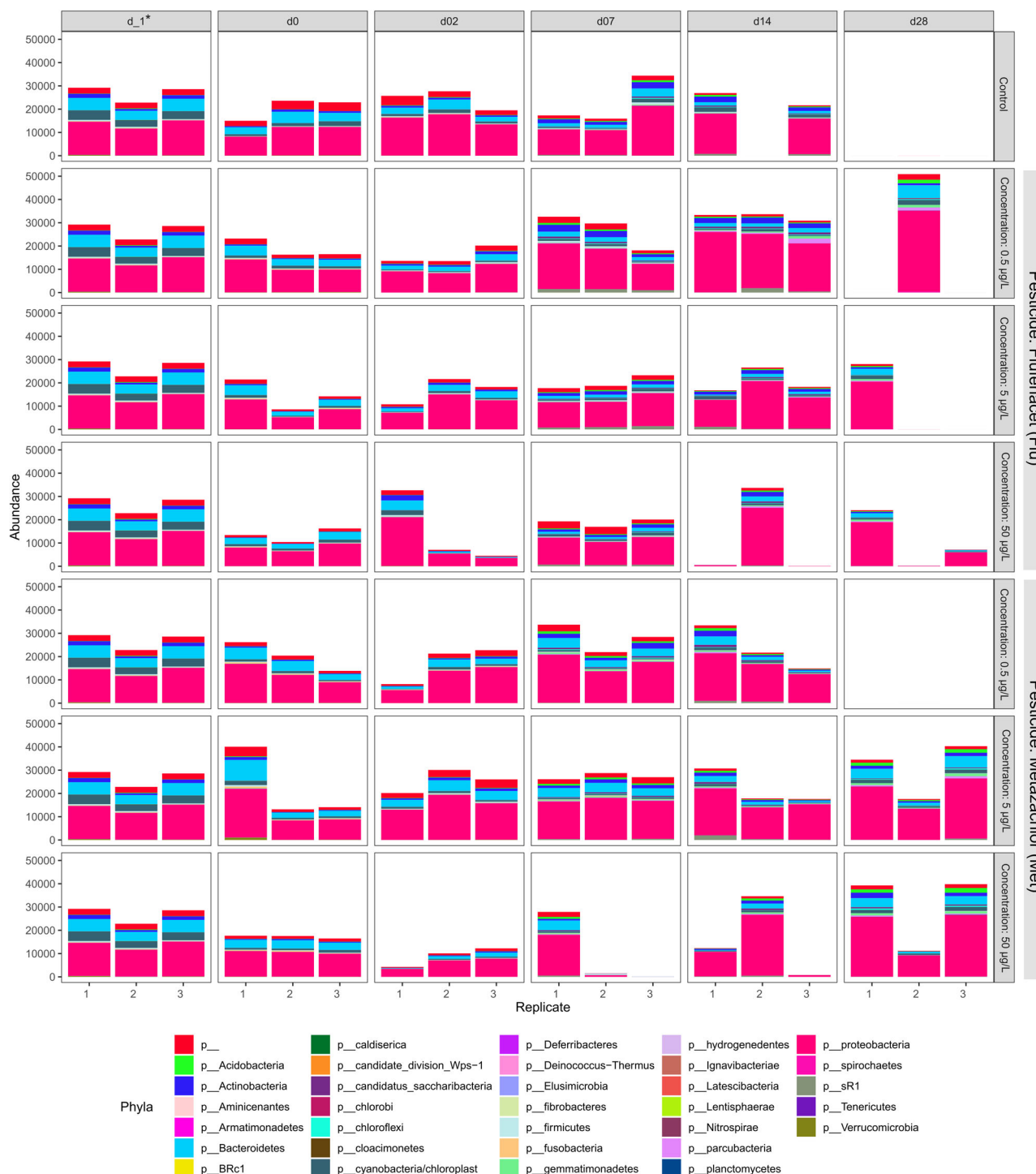


FIGURE 2 Absolute abundance of phyla in vessel sediments across two different herbicide treatments over exposure time. Two herbicides, metazachlor (Met) and flufenacet (Flu), were applied in different concentrations, resulting in samples with herbicide concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$ and one control sample (three replicates in each sample). Sampling times: d_1* = at sampling, before dividing the water samples into vials (*resulting in the same control data for all substances and concentrations; data repeated in each line for intuitive reading), d0 = within the vessel, before exposure, d2, d7, d14, d28 = 2, 7, 14, 28 days after exposure. All phyla/phyla candidates are shown.

low-read samples that are prone to huge and arbitrary shifts. Disclosing low-read samples from detailed description, some trends remain obvious on phylum level: *Actinobacteria* increased with time until d7, partly also on d14, in the control and in all

concentrations of Flu0.5 and Met0.5. *Bacteroidetes* decreased within the first 7 days in the control and Flu, while they decreased not earlier than d7 to 14 in Met. *Cyanobacteria/chloroplast* DNA decreased after transfer to the vessels in all assays. However,

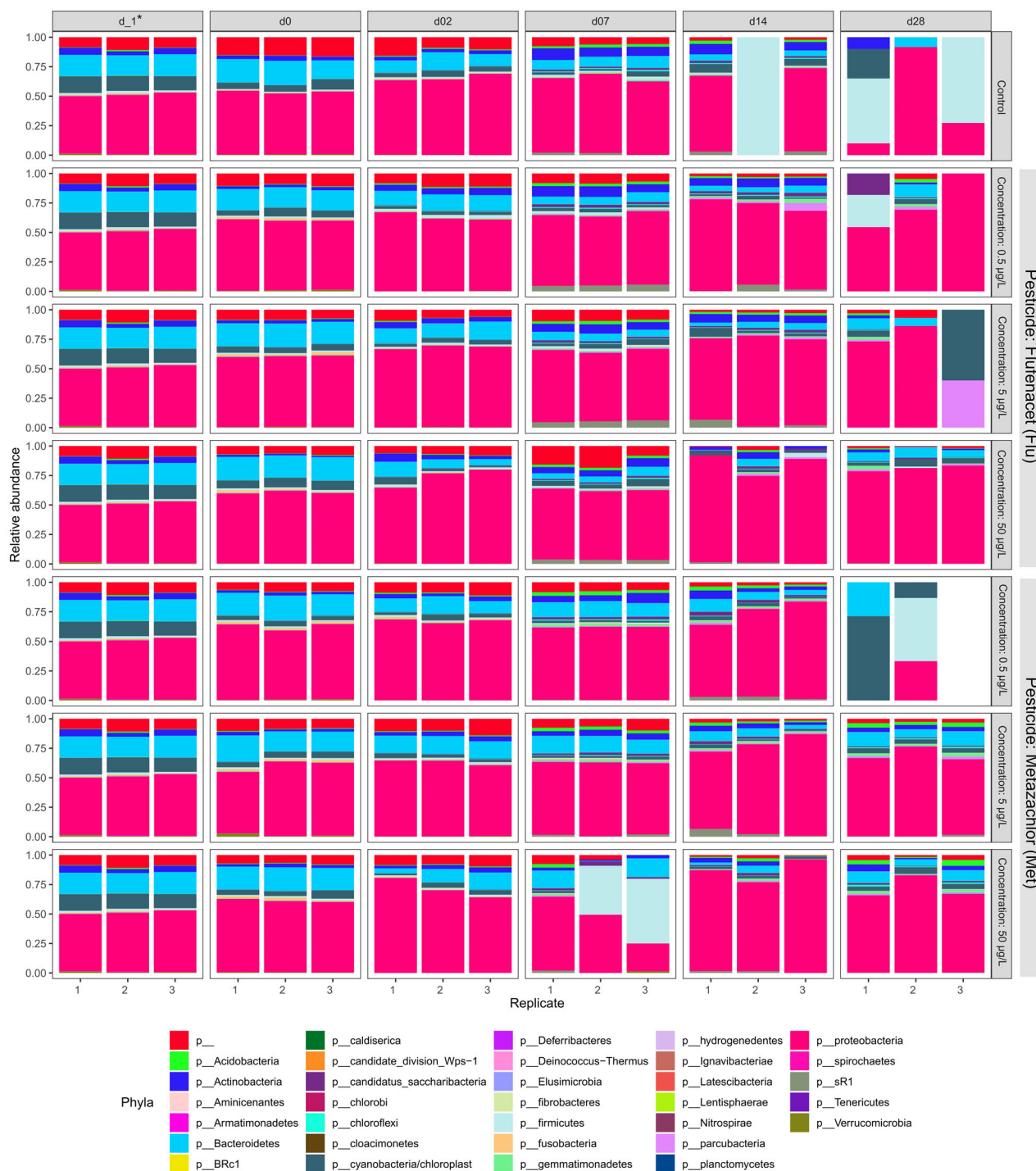


FIGURE 3 Changes in the relative abundance of phyla I vessel sediments across two different herbicide treatments over exposure time. Two herbicides, metazachlor (Met) and flufenacet (Flu), were applied in different concentrations, resulting in samples with herbicide concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$ and one control sample (three replicates in each sample). Sampling times reported as: d_1* = at sampling, before dividing the water samples into vials (*resulting in the same control data for all substances and concentrations; data repeated in each line for intuitive reading), d0 = within the vessel, before exposure, d2, d7, d14, d28 = 2, 7, 14, 28 days after exposure. All phyla/phyla candidates are shown.

they still contributed to the absolute abundance of the controls and Flu5 in a considerable share until d14–d28. The main share belonged to *Proteobacteria* in all assays at all times (except low-read

samples), starting from above 50%. During the experiment, the share of *Proteobacteria* increased further, to more than 75% on d14—the highest in Met50 and Flu50.

Looking at class level, the original sample was dominated by *Betaproteobacteria*, followed by *Gammaproteobacteria*, together with chloroplasts and unclassified phyla. Flavobacteria, *Actinobacteria*, *Sphingobacteria*, *Alphaproteobacteria*, *Bacteroidia*, *Cytophagia*, *Clostridia* and *Fusobacteria* also contributed to the original total abundance in shares worth mentioning. Samples that appear to differ quite clearly from controls were again biased by low-read samples (d7, Met50; d14, control; d28, control, Flu05, Flu5, and Met05). *Alphaproteobacteria* quickly started to increase to similar shares compared to *Betaproteobacteria* one day after sampling from the pond and dominated from d2 on. *Bacteroidia* and *Clostridia* widely vanished between d7 and d14. Deviating from all other samples, Met5 and Met50 were the only assays where *Acidobacteria* and *Cyanobacteria* increased remarkably on d28. Distributions on class level are visualised in Figures S2 and S3.

Beta-diversity: PCoA

In PCoA, samples were labelled either by substance (Figure 4A) or sample date (Figure 4B). The different labels depict that microbiome changes in the microcosm were mainly dependent on the course of time. All sediment samples that clearly deviated from the day-based clusters belonged to the experiments with the highest concentrations of pesticides. That was still true when PCoA was run on ASV-level (see Figure S4A,B).

Alpha-diversity indices of microbiome data in samples with different herbicide concentrations (day-wise)

Because sequencing success steeply declined for assays run until d14 and d28, these two dates were excluded from alpha-diversity analysis.

Day 0

D0 samples aimed to monitor the equal distribution of bacterial communities to the different vessels. That was successfully confirmed on the phylum level by Shannon and Simpson diversity indices (Figure 5). However, phyla richness of controls differed significantly from 'Met05'—which was a control here. Evenness was significantly—but arbitrarily—higher in controls, compared to those vessels filled after vessels that were later exposed to Flu05, Met05 and Met5. On the ASV level, only Simpson diversity differed significantly between controls and assays that were later exposed to herbicides (Figure S5).

Day 2

After 2 days, the phyla richness in the control had fully recovered, while microcosms with high concentrations of metazachlor and flufenacet (Flu5, Flu50, Met50) had a significantly lower number of detectable phyla, compared to the control and compared to d0 samples (Figure 6). Lower alpha diversity was also reflected in a significantly lower Shannon diversity index of Met50. In contrast to d0-controls, evenness was significantly higher in Flu05. Diversity indices on the ASV level mostly mimicked trends on the phylum level, with the lowest alpha-diversity values for Flu50 and Met50. Only evenness showed a very different picture, with the highest values for Met50 and Flu50 (Figure S6).

Day 7

On d7, alpha diversity showed somehow conflicting trends: phyla richness in the control decreased again, phyla richness of Flu50 remained low, while phyla richness in Met50 differed considerably between replicates (but had partly recovered, compared to d2). Shannon diversity indices of the control and Met50 were significantly lower, compared to Flu05 and Flu5. The same was true with the Simpson diversity index in the control (Figure 7). On ASV level, Flu50 differed significantly in richness and evenness when compared to Met5 (Figure S7).

Inhibitory effects of herbicides on cultured freshwater bacteria

In the MIC assay, flufenacet inhibited *Microbacterium hominis* at a MIC of 50 mg L⁻¹, *P. alcaligenes* at 500 mg L⁻¹, and *P. amylolyticus* at 5 g L⁻¹. MIC for metazachlor was 500 mg L⁻¹ in *M. hominis* and 5 g L⁻¹ in the other strains. Turbidity-based assessment of MIC was hampered by the high turbidity of the non-inoculated herbicide-medium-mixture, so MIC values were confirmed by plating. All tubes assessed as 'turbid' resulted in bacterial growth. At metazachlor concentrations equalling the apparent MIC, *M. hominis* and *P. amylolyticus* were still inhibited when plated to solid medium, while *P. alcaligenes* was able to regrow on solid medium after cultivation from a non-turbid tube with 5 g L⁻¹ metazachlor, being inhibited from regrowth only after incubation at 50 g L⁻¹. That ability to regrow was even more pronounced after flufenacet incubation, where it occurred for all bacteria at concentrations of 10-fold (*P. amylolyticus*) to 100fold MIC (*M. hominis*, *P. alcaligenes*).

In a growth-curve-assay with 50 g L⁻¹ flufenacet (Figure 8), *P. alcaligenes* evolved resistance in one assay, while it was completely inhibited in the second

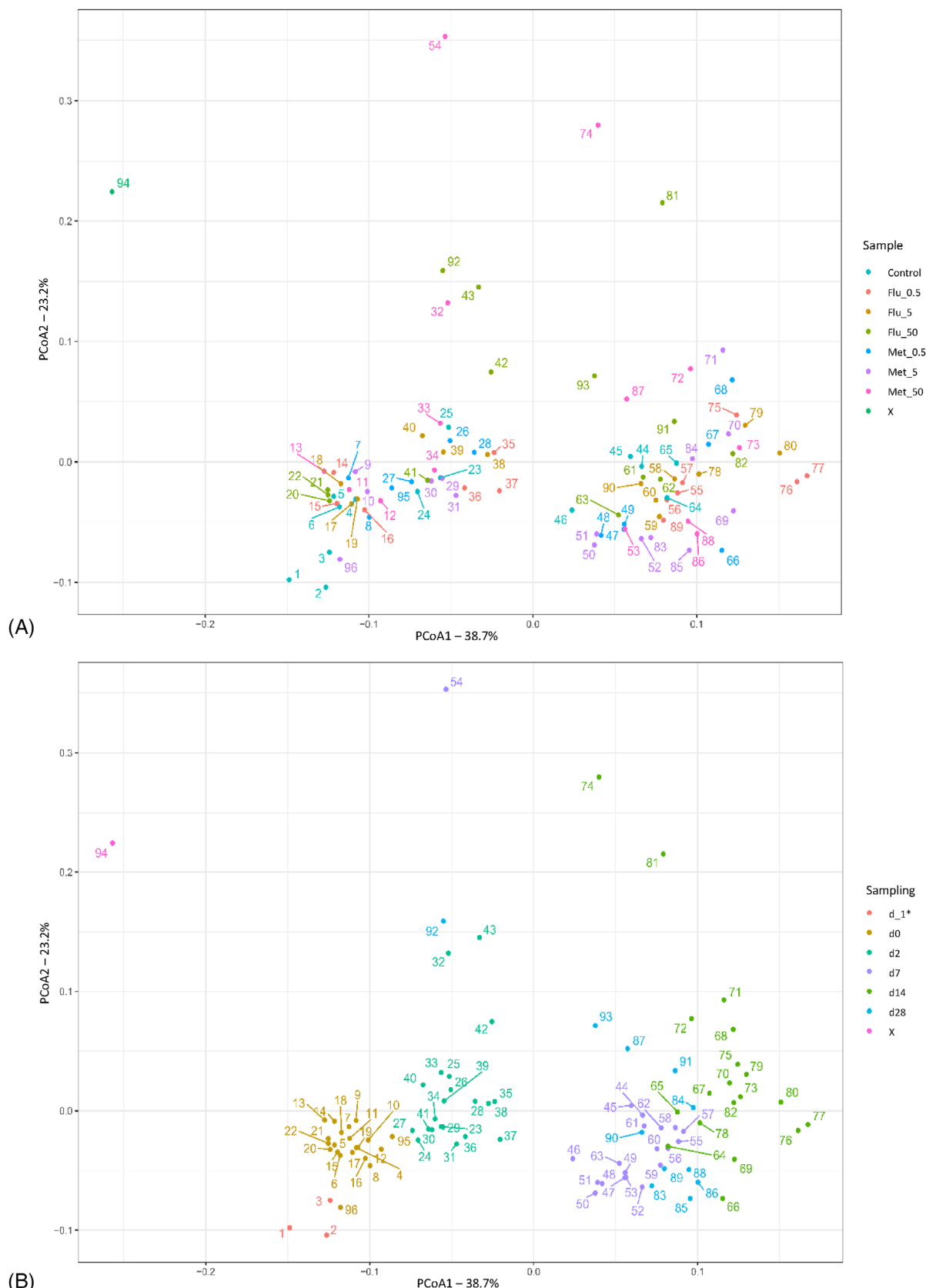


FIGURE 4 (A, B) PCoA of aquatic microcosm microbiomes on phylum level (Bray–Curtis-distance). Unassigned phyla and samples <500 reads were excluded. Six time points, two herbicides (flufenacet, metazachlor) and three concentrations (0.5, 5 and 50 $\mu\text{g L}^{-1}$). (A) Colour-labelling according to sample type. Note that samples 1–23 were not exposed to herbicides, irrespective of colour. Control = Not exposed to herbicides. X = extraction control. (B) Colour-labelling according to sample date. d_1* = at sampling, before dividing the water samples into vials (*resulting in the same control data for all substances and concentrations; data repeated in each line for intuitive reading), d0 = within the vessel, before exposure, d2, d7, d14, d28 = 2, 7, 14, 28 days after exposure. X = extraction control.

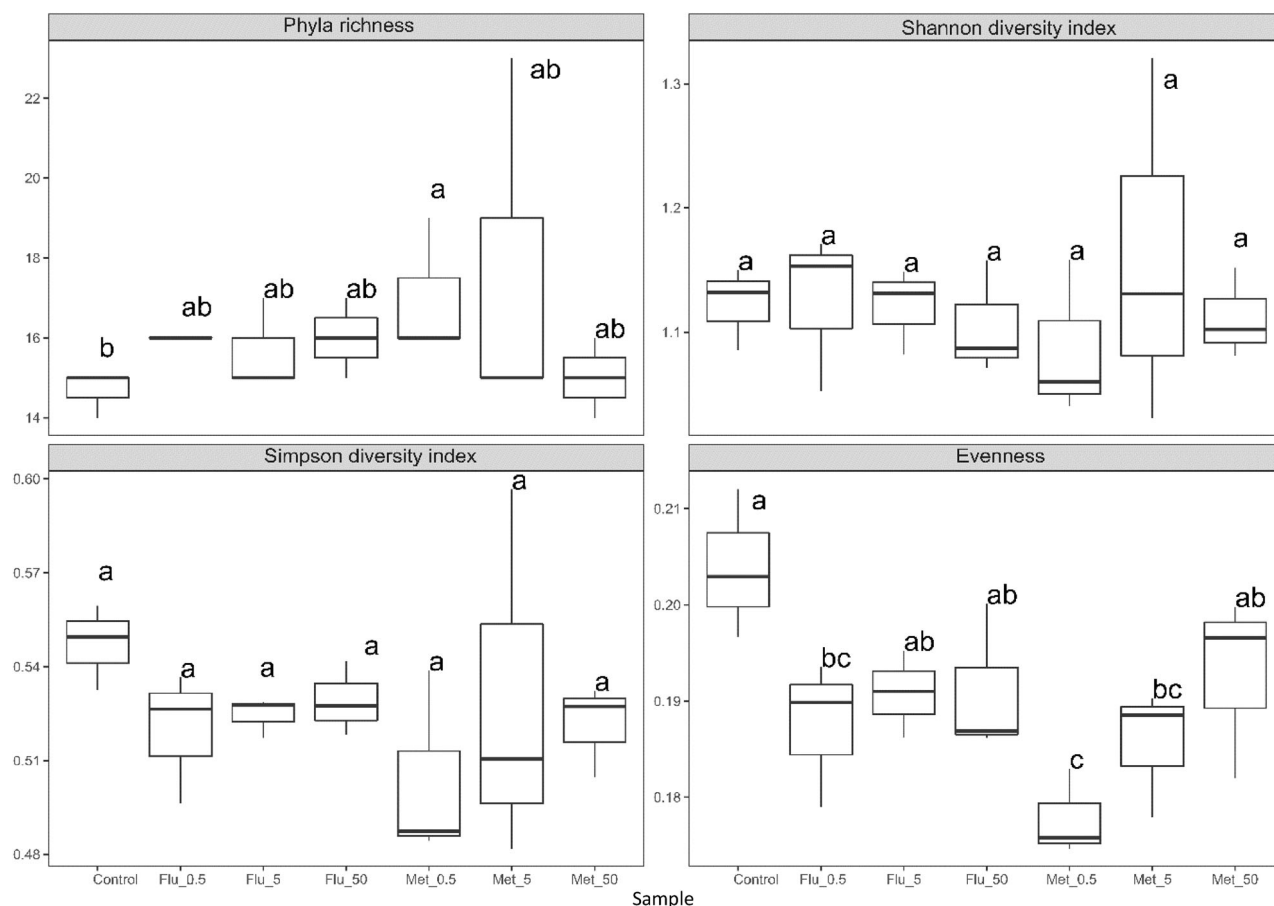


FIGURE 5 Alpha-diversity measures on phylum level for d0 controls which were afterwards exposed to herbicides: Phyla richness, Shannon diversity index, Simpson diversity index, Evenness. Three replicates per assay. Control = Not exposed to herbicides. Flu = flufenacet, Met = metazachlor. 0.5, 5, 50: concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$. The boxes denote interquartile ranges with the median as a black line. Outliers not shown.

assay, as it would have been expected inferring the results of MIC-value determination. With 2.5 g L^{-1} flufenacet and an inoculum of 2×10^4 cfu/mL, the two-fold assays had a flattened slope during the exponential growth phase, compared to the control, and one of the two assays showed a prolonged lag phase. A flattened slope was still seen with 50 $\mu\text{g L}^{-1}$ flufenacet. The metazachlor assay (2.5 g L^{-1}) resembled the flattened slope of the corresponding flufenacet growth curve, but no prolonged lag phase was discerned, and no effects were found with 50 $\mu\text{g L}^{-1}$ metazachlor.

DISCUSSION

Freshwater aquatic microbiomes

In our study, we investigated the reaction of a typical freshwater community towards graded concentrations of two herbicides, flufenacet and metazachlor. Microcosm experiments were strengthened by in vitro experiments meant to elucidate causal, direct interaction of herbicides

and selected bacteria (mainly *P. alcaligenes*). With regard to the question of whether our pond water samples were suitable to represent aquatic communities, we compared all detected (candidate) phyla with the literature. We found that all 32 bacterial (candidate) phyla had been detected in freshwater or freshwater sediments before (Baricz et al., 2020; Chaya et al., 2019; Cremer et al., 2022; Davis et al., 2009; Farag et al., 2014; Huang et al., 2018; Kadnikov et al., 2018; Kaushik et al., 2014; Lu et al., 2019; Morrison et al., 2017; Newton et al., 2006; Tang et al., 2014) or had been associated with freshwater-bound hosts (Jaffe et al., 2022). Information on mean phyla numbers in freshwater environments is surprisingly hard to find and phyla numbers range between two (tropical reservoir; Kaushik et al., 2014) and 40 (freshwater sediment, Wang et al., 2012). Proceeding from phylum number to relative abundance, the relative abundance of *Proteobacteria* was higher in the original sample used in this study than in other freshwater data (Lee & Eom, 2016), which might be explained by the summer season. Huang et al. (2018) found a similar dominance of *Proteobacteria* in July.

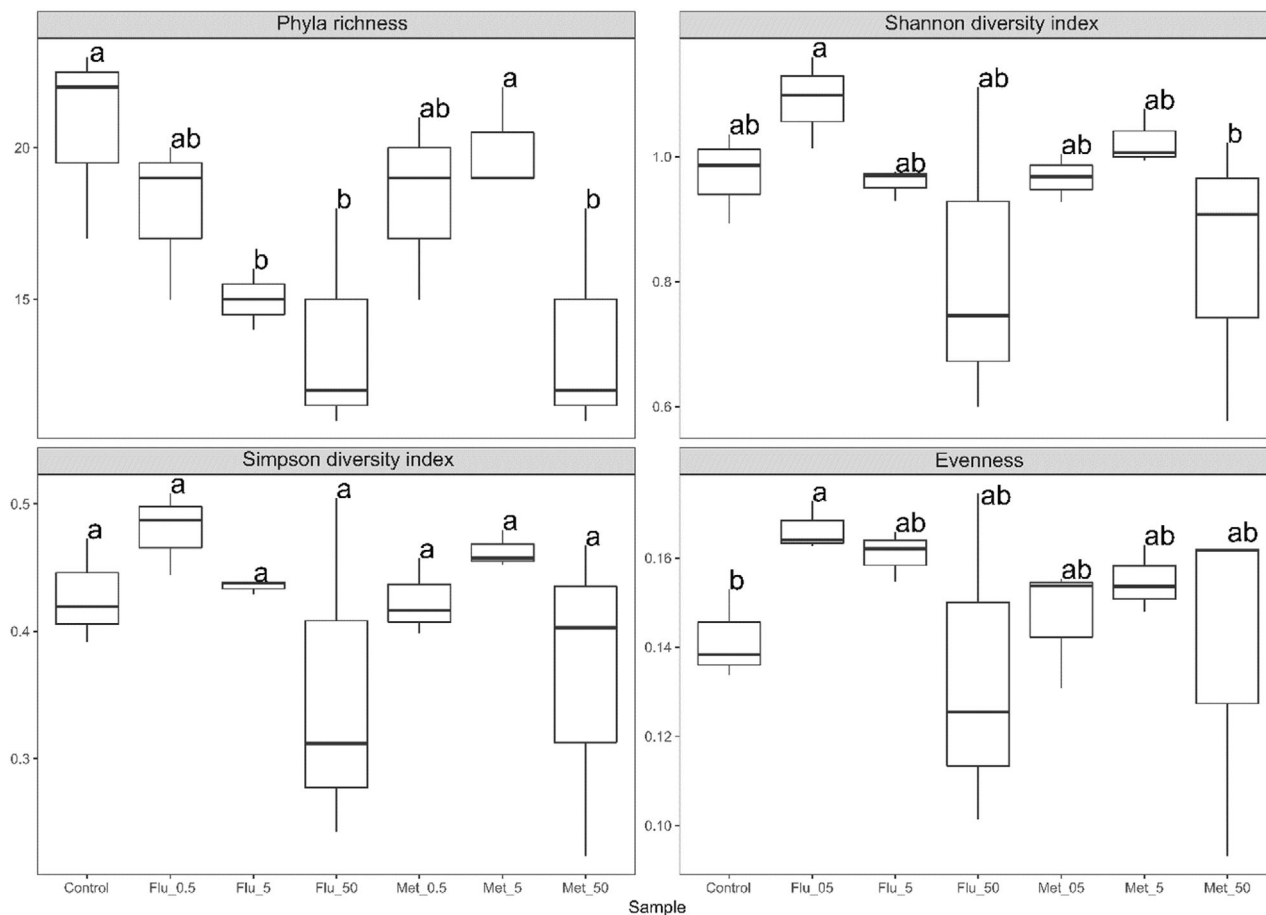


FIGURE 6 Alpha-diversity measures on phylum level for assays that had or had not been exposed to herbicides for 48 h: Phyla richness, Shannon diversity index, Simpson diversity index, Evenness. Three replicates per assay. Control = Not exposed to herbicides. Flu = flufenacet, Met = metazachlor. 0.5, 5, 50 = concentrations of 0.5, 5, and 50 $\mu\text{g L}^{-1}$. The boxes denote interquartile ranges with the median as a black line. Outliers not shown.

Experimental limitations

We used triplicates of 2.4 L volume since it is known that initially similar aquatic communities can follow ‘alternative, yet stable trajectories’ even in the absence of selectors in a size-dependent manner (Pagaling et al., 2017). Bottleneck events are expected to have strong effects in evolutionary experiments (Mahrt et al., 2021), so it is important to point out that we had one unavoidable bottleneck event at the very beginning, when we had to divide the water into vessels. Connected to that, we observed effects on some, but not all alpha-diversity indices in d0-samples, none of which had been exposed to herbicides yet.

At least one replicate was lost from d7 following. At first glance, 16S-amplicon concentrations as revealed by qPCR hardly explained low sequencing success, since values were comparable to d2 and d7. However, we could not use primers adapted to samples rich in plant DNA, thus, high concentrations of 16S-amplicons might be explained by high concentrations of plant DNA instead of bacterial DNA (Dorn-In et al., 2015) or

an unfavourable ratio of both. For some assays (e.g., in control, Flu5, Met05), this hypothesis matches a high proportion of chloroplasts instead of bacteria in the clean reads. Moreover, it also matches the fact that assays with high concentrations of herbicides appeared conserved to a certain extent. However, sequencing success from water and sediment samples might have also been hindered by unsuccessful barcoding (Filges et al., 2019).

As the bacterial reads of the water column samples were very low, we did not take these sequencing results for in-depth analysis. Others also reported difficulties in the extraction of water samples (Horáková et al., 2008).

One striking exception for low concentrations in the water column is seen on d2, with a mean count of 25,000 reads. We assume that this is a result of initial planktonic bacterial growth (as also seen in cultural results, data not shown), initiated by the mixing procedure on day 0, while bacteria were not yet fixed in biofilms. At the following time points, a fixation in biofilms might have resulted in declining read numbers for planktonic bacteria in the water column (from <4000

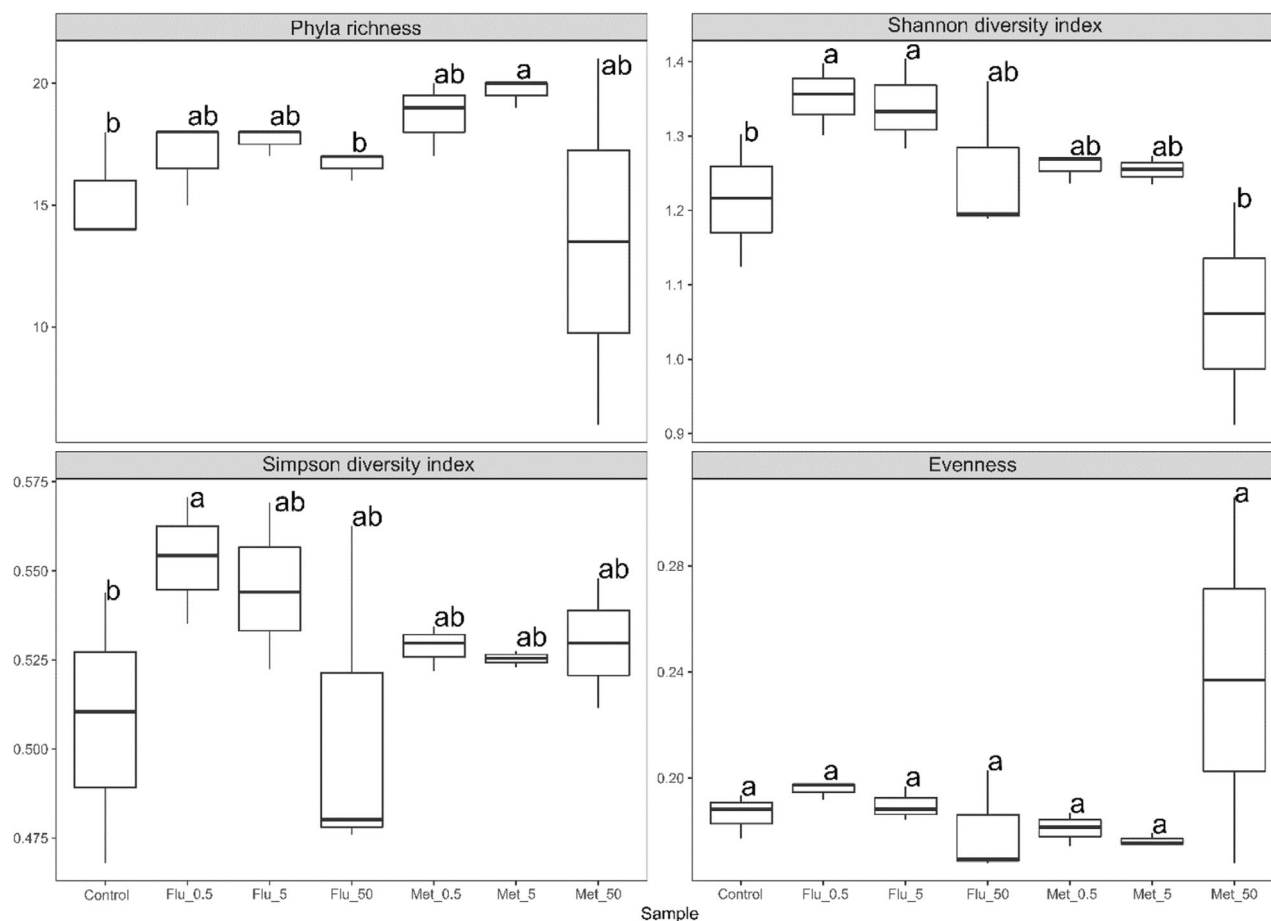


FIGURE 7 Alpha-diversity measures on phylum level for assays that had or had not been exposed to herbicides for 7 days. Phyla richness, Shannon diversity index, Simpson diversity index, Evenness. Three replicates per assay. Control = Not exposed to herbicides. Flu = flufenacet, Met = metazachlor. 0.5, 5, 50 = concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$. The boxes denote interquartile ranges with the median as a black line. Outliers not shown.

reads on day 7 to <50 on days 14 and 28). Such biofilms were visually apparent at the bottom of each vessel at later time points (d7–d28).

The reason for not performing an in-depth analysis with low-count water samples is well illustrated when looking at sediment samples: an apparent increase in the proportion of firmicutes (Figure 3) is only seen with the few low-count sediment samples. There are two possible explanations for that: Of course, spore-forming firmicutes are the last to get lysed in an adverse environment, so we expect their proportion to increase in hostile surroundings. That might explain the high proportion of *Clostridia* in Met50 on d7, which are abundant then, while they are much less apparent in the extraction control. However, the high abundance of bacilli in the extraction control (Figure S2) shows that contamination is also a possible source for finding spore-formers, especially in low microbial biomass samples, which are generally challenging (Dahlberg et al., 2023). Thus, we prefer to treat the high abundance of Firmicutes on d28 (partly also on d14) as an artefact.

Natural occurrence and inhibitory concentrations of herbicides

Herbicide concentrations of 0.5, 5 and 50 $\mu\text{g L}^{-1}$ were chosen for the microcosm experiments. Long-term data from freshwater bodies in the federal state Schleswig Holstein (2011–2021) show permanent pollution of the target compounds of the study with maximum concentrations of up to 11 $\mu\text{g L}^{-1}$ flufenacet and 76 $\mu\text{g L}^{-1}$ metazachlor (LLUR, 2023). MICs of three aquatic bacterial strains, isolated from the same pond as sampled for the microcosm experiment, were clearly above these concentrations. However, it is important to remember that a ‘selective window’ opens at concentrations far lower than MIC (Gullberg et al., 2011). Early signs of inhibition are best depicted by growth curves (Bopp & Lettieri, 2007). Furthermore, flufenacet revealed impaired growth in the early exponential phase even at the lowest tested concentration of 50 $\mu\text{g L}^{-1}$ (Figure 8).

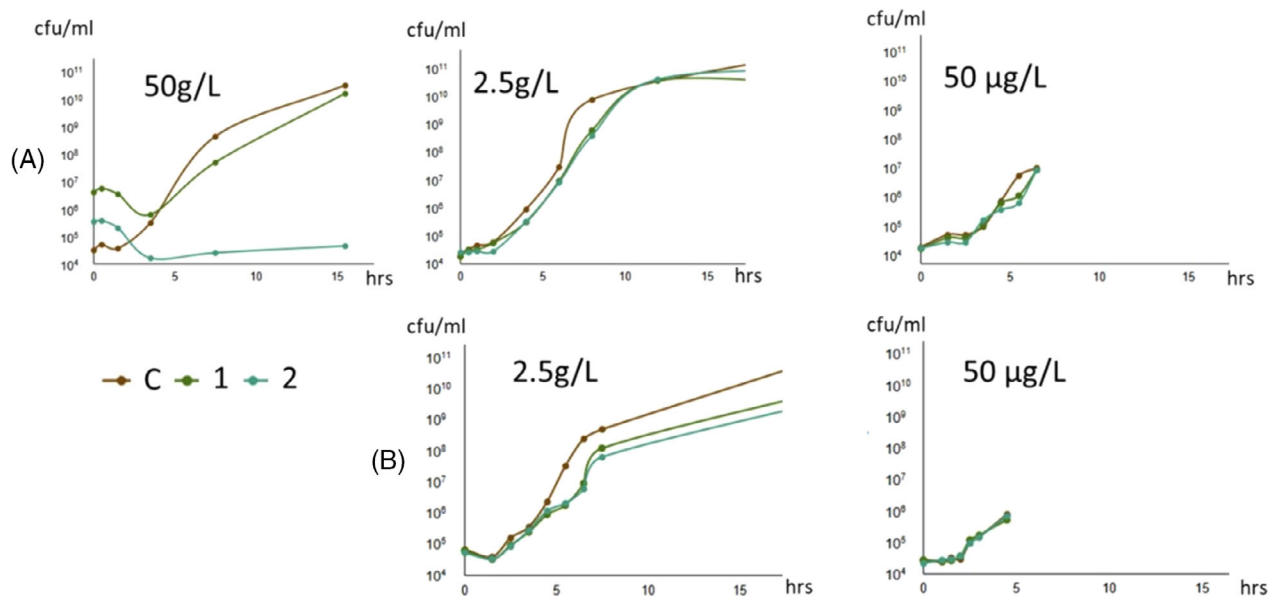


FIGURE 8 Growth curves of *Pseudomonas alcaligenes* in the presence of (A) flufenacet (50 g, 2.5 g, 50 µg L⁻¹) or (B) metazachlor (2.5 g, 50 µg L⁻¹) over time in hrs (hours). Growth is given in Colony forming units per ml (cfu/ml). C = control, 1 and 2 = two-fold assay. Please note that the experiment with 50 g flufenacet L⁻¹ was performed at above-MIC concentration (no growth expected), and that no such experiment was performed with metazachlor.

Changes in microbiome composition and their potential causes

Beta-diversity

In general, the temporal course had more impact on bacterial composition in the microcosm than herbicide exposure, except for a few assays with high concentrations of herbicides (50 µg L⁻¹). Such time-dependent changes might be caused by changes in medium conditions, which were monitored and partly controlled in our experiment (Wijewardene, Wu, Hörmann, et al., 2021), and biofilm formation (Peter et al., 2011), which was mechanically disrupted here. Thus, we hypothesise that time-dependent changes were mainly driven by changes in (i) metacommunities (here: phytoplankton, diatoms, since zooplankton had been eliminated), and (ii) herbicide selection over time of either the microbiome (direct selection) or their host plants (indirect selection). The point ii) is only plausible if changes differ between control and experiments.

i. Changes in metacommunities not driven by herbicides

In an attempt to relate time-course-dependent microbiome changes to metacommunity data, we focused first on plant community data from the control experiments (Wijewardene et al., 2022). Compared to d0, Bacillariophyta increased less pronounced than in herbicide assays, while a much higher increase was seen for Chlorophyta. Chlorophyta microbiomes are

dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Deltaproteobacteria* and *Actinobacteria*. Accordingly, *Gammaproteobacteria* increased over time in the control samples, together with the Chlorophyta, as previously described (Wijewardene et al., 2022).

ii. Direct or indirect selection by herbicides over time

Indirect selection

With regard to a potential selection by herbicides over time, we saw a clear increase of *Alphaproteobacteria* over time in the herbicide assays, compared to the control, together with a significant increase in Bacillariophyta (diatoms) in the same samples (Wijewardene et al., 2022). Diatoms are known to closely interact with bacteria: in a marine environment, they were particularly associated with Alpha- and *Gammaproteobacteria*, but also *Bacteroidetes* (Amin et al., 2012). Interestingly, the plant species that was most pronouncedly selected by both herbicides over time in our assays was *Peridinium willei* (Wijewardene et al., 2022), and the genus *Peridinium* spp. has been reported to intracellularly host *Alphaproteobacteria* (Schweikert & Meyer, 2001).

Direct selection

Looking at our in vitro experiment, *P. alcaligenes*—as a *Gammaproteobacterium*—was more tolerant to both

herbicides than were *Microbacterium hominis* (Actinomycetes) or *P. amylolyticus* (Bacilli). In one of the twofold assays, *P. alcaligenes* even managed to grow in 50 g L^{-1} flufenacet, albeit obviously weaker than the control, while the other assay was inhibited. The molecular basis of that adaption or resistance mechanism has yet to be determined. The experiment was erroneously performed at the above MIC concentration (where no growth at all would have been expected), and the assay in which we observed growth was inoculated with an untypically high inoculum of above $5 \times 10^6 \text{ cfu L}^{-1}$, which favours the presence of resistant mutants (Steels et al., 2000). That ability to adapt or to evolve resistance would, by theory, provide the base for direct selection of *Gammaproteobacteria*; indeed, we saw a clear increase in the ratio of *Gammaproteobacteria* to Bacilli when comparing high concentrations of both herbicides to controls and low concentrations of metazachlor.

Another hint towards direct selection was seen at high concentrations of metazachlor (Met5, Met50), where phylum *Acidobacteria* increased over time. *Acidobacteria* are considered 'k-strategists' (Kielak et al., 2016), following an evolutionary approach where they invest in fewer offspring, but provide them with a relatively high probability of individual survival (Andrews & Harris, 1986). *Acidobacteria* are also known to be highly tolerant to toxic components, which may be linked to their ability to produce exopolysaccharides and a variety of multidrug transporters (Kielak et al., 2016). An interesting case of selection or possible adaptation of bacteria to metazachlor is the increase in *Cyanobacteria* on d28. Although earlier published studies show a decrease in *Cyanobacteria* (Peterson et al., 1994) under metazachlor treatment, some studies show that *Cyanobacteria* can adapt to metazachlor in some way. There is supporting research evidence that *Cyanobacteria* can cope with the challenges of increased herbicide concentrations in freshwater ecosystems through rapid evolutionary processes (Melero-Jiménez et al., 2021). Furthermore, Karier et al. (2017) report on the selective effect of metazachlor on the Chlorophytes that chronically precede the *Cyanobacteria*, from which a proliferation of *Cyanobacteria* occurs in waters. That nicely illustrates how direct and indirect selection effects go hand in hand.

Alpha-diversity

While only minor differences related to beta-diversity were visible between the experiments and between the experiment and control, significant differences were found for alpha-diversity indices. Differences due to unequal distribution in the bottleneck event were controlled and did (initially) not affect Shannon or Simpson

diversity indices, but phyla richness and evenness. In general, alpha-diversity is lowered by selection, since selection gives an advantage to particular strains, increasing their concentration at the expense of others. Isolates of different phylogenies often differ in their intrinsic tolerance to chemicals (Brown et al., 1990). This is also depicted by the different MIC-values of *P. alcaligenes* (*Proteobacteria*), *P. amylolyticus* (Bacillota), and *M. hominis* (Actinomycetota), therefore we consider selection on phylum level as a plausible cause of lowered alpha-diversity.

Concentration dependency of herbicide effects on microbiomes

In general, selection is concentration-dependent, since concentrations that do not affect any of the present phyla will not result in selection (Stanton et al., 2020). Our microcosm study indicates that $50 \mu\text{g L}^{-1}$ flufenacet or metazachlor should be considered as a selective concentration (Figures 6 and 7), while—in this particular community—exposure to $0.5 \mu\text{g L}^{-1}$ did not result in selection. For the intermediate concentration of $5 \mu\text{g L}^{-1}$, the flufenacet assay differed significantly from the control in terms of phylum richness, while the metazachlor assay did not (Figure 6). This matches the fact that exemplarily chosen aquatic bacteria were more susceptible to low concentrations of flufenacet than metazachlor in the in vitro part of our study.

CONCLUSION

Taken together, we saw selective effects of flufenacet and metazachlor on aquatic bacteria, both by cultural in vitro assays and by 16S-amplicon analysis of microbiomes. Effects occurred at concentrations of $50 \mu\text{g L}^{-1}$ with both herbicides, while $5 \mu\text{g L}^{-1}$ was selective only in the case of flufenacet, but not metazachlor. Effects cannot be clearly divided into direct and indirect selection, since major changes in plant communities were observed as well (Wijewardene et al., 2022), which necessarily change microbial communities (and maybe vice versa). Future research should aim to substantiate causal plant-microbe interactions in a more mechanistic way, which was beyond the scope of this study. However, our in vitro results indicate that herbicides have also direct effects on bacteria. The concentrations that were identified as selective were indeed reached as maximum concentrations in regional water bodies. Thus, the environmental effects of these herbicides on aquatic microbiomes seem feasible and might negatively affect ecosystems. In the future, competitive in vitro evolution experiments, co-incubating bacterial isolates from different phyla, might provide deeper insights into herbicide-driven

microbiome evolution. Only functional analysis, for example, performed with metagenome data, will reveal whether microbial ‘change’ is consistent with ‘loss of function’ and might thus be considered as an additional environmental impact of flufenacet and metazachlor.

AUTHOR CONTRIBUTIONS

Lishani Wijewardene: Data curation (equal); formal analysis (equal); methodology (equal); visualization (equal); writing – original draft (lead). **Julia Anna Schwenker:** Visualization (equal); writing – original draft (equal). **Meike Friedrichsen:** Investigation (equal). **Ailina Jensen:** Investigation (equal). **Franziska Löbel:** Formal analysis (equal); investigation (equal). **Tabea Austen:** Formal analysis (equal); investigation (equal). **Uta Ulrich:** Conceptualization (equal); project administration (equal); resources (equal); writing – review and editing (equal). **Nicola Fohrer:** Project administration (equal); resources (equal); writing – review and editing (equal). **Corinna Bang:** Data curation (equal); formal analysis (equal). **Silvio Waschina:** Formal analysis (equal); visualization (equal). **Christina Susanne Hölzel:** Conceptualization (equal); project administration (equal); resources (equal); supervision (equal); writing – original draft (equal); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are provided in full in this paper, except for raw data (amplicon single variants), which are available in the Zenodo repository at <https://zenodo.org/doi/10.5281/zenodo.7828123>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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