**Interaction between glyphosate and dissolved phosphorus on bacterial and eukaryotic
communities from river biofilms**

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Abstract

Since the capacity of river biofilms to degrade glyphosate has been proven to increase when the availability of dissolved phosphorus (P) in water decreases, the present study investigates the diversity responses of bacterial and eukaryotic microbial communities from biofilms in a search for glyphosate-degrader candidates. Glyphosate and P interactions were observed for eukaryotic communities, the highest community richness and diversity being preserved at low concentrations of glyphosate and P. This trend marked by glyphosate was also observed in the structure of eukaryotic communities. Therefore, phosphorus and glyphosate had a synergistic effect in decreasing the richness and diversity of eukaryotes species in biofilms. However, species richness and diversity in bacterial communities were not affected by glyphosate, though shifts in the structure of these communities were concomitant with the degradation of the herbicide. Bacterial communities capable of using glyphosate as P source were characterized by increases in the relative abundance of certain Bacteroidetes, Chloroflexi, Cyanobacteria, Planctomycetes and alpha-Proteobacteria members. Glyphosate-degrader candidates found in natural river biofilms can be further isolated for better understanding of glyphosate degradation pathways, and used as bioremediation strategies in heavily contaminated sites.

Keywords

AMPA; alpha-Proteobacteria; Cyanobacteria; mineralization; soluble reactive phosphorus; microcosm.

1 Introduction

Since the middle of the twentieth century, anthropic activities continuously introduce a huge diversity of xenobiotic compounds into the environment. The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES, 2018) report a continuous strong decline in biodiversity in Europe and Central Asia, partly explained by intensive agriculture and forestry practices used to increase the provision of food and biomass-based fuels. The interaction between anthropic activities and climate change places rivers as among the most threatened ecosystems in the biosphere both in terms of species extinction and losses in ecosystem services. While the vulnerability of animal and plant biodiversity to global change has been demonstrated in freshwater ecosystems (*e. g.* Strayer and Dudgeon (2010)), the response of microbial biodiversity to these impacts is often neglected.

Glyphosate (N-(phosphonomethyl) glycine) is a systemic herbicide inhibiting aromatic amino acid biosynthesis in plants. Since first introduced in the early 70's, and as a result of its high weed prevention efficiency, glyphosate use has increased rapidly (Duke, 2015). This herbicide has become the most frequently used active substance in most countries worldwide (Benbrook, 2016), as well as in France (AGRESTE, 2018). Consequently, glyphosate and its main primary metabolite AMPA (aminomethyl phosphonic acid) have become among the compounds most frequently quantified in river waters (43% and 63% quantification frequency in France from 2012 to 2016, respectively) (NAIADES, 2018). Glyphosate use is authorised in Europe (included in Annex I to Directive 91/414/EEC on 2002/07/01 by Commission Directive 2001/99/EC) and 192 glyphosate-based formulations are currently approved in France (E-Phy, 2018). However, the broad contamination of the environment by glyphosate has recently prompted the authorities to question the extension of its authorisation, by taking into account its potential harmful side effect to human and ecosystems health (Davoren and Schiestl, 2018; Van Bruggen et al., 2018).

Once in rivers, glyphosate can interact with microbial biofilms which have a certain sensitivity to this herbicide. Biofilms are natural consortia of microorganisms composed of bacteria, algae, fungi and protozoa embedded in an extracellular matrix protecting them against environmental stressors such as nutrient starvation and pollutants (Besemer, 2015). Bonnineau et al. (2012) showed a stronger decrease in photosynthetic efficiency in shade-adapted biofilms (half maximal effective concentration $EC_{50} = 11.7 \text{ mg L}^{-1}$) than in high-light adapted biofilms ($EC_{50} = 35.6 \text{ mg L}^{-1}$) exposed to glyphosate. Accordingly, light history can influence the structure and composition of phototrophic populations in biofilms, and thus modulate their photosynthetic response to the herbicide. Other studies also revealed a negative impact of glyphosate in terms of phototrophic community growth when exposed to $10 \text{ } \mu\text{g L}^{-1}$ of glyphosate (Bricheux et al., 2013) and significant decreases in biomass, primary production and heterotroph community respiration when exposed to 2.5 g L^{-1} of glyphosate (Shaw and Mibbayad, 2016). Within phototrophic communities, diatoms appear as the most sensitive group to the herbicide, while cyanobacteria and chlorophyte are the least sensitive (Vera et al., 2010). However, exposure of river biofilms to low concentrations of glyphosate ($<100 \text{ } \mu\text{g L}^{-1}$) did not significantly affect algal and bacterial growth (Carles et al., 2019) and even slightly increased their biomass production (Klátyik et al., 2017). A recent study has provided evidence of the involvement of glyphosate in phosphorus (P) recycling by riverine biofilms (Carles et al., 2019). Phosphorus-starved biofilms are capable of mineralising glyphosate and therefore use the P contained in the herbicide molecule ($\text{C}_3\text{H}_8\text{NO}_5\text{P}$) for metabolism. However, P enriched biofilms tended to transform glyphosate into AMPA ($\text{CH}_6\text{NO}_3\text{P}$) the latter accumulating in the media, as well as in biofilm (Fernandes et al., 2019), thus contributing to P eutrophication of river waters.

Herbicide tolerance can use either low cell permeability or the occurrence of detoxifying enzymes or degradative pathways. Research addressing glyphosate tolerance has been mostly

101 focused on microbial populations rather than on multi-species biofilms. At the population level,
102 contrasted responses to glyphosate were obtained depending on the isolated strain. The
103 herbicide tends to favour the biofilm formation of *Pseudomonas aeruginosa* (Lima et al., 2014)
104 while no significant effect was observed on the biofilm formation of *Clavibacter michiganensis*
105 ssp. *sepedonicus* (Perfileva et al., 2018). Glyphosate tolerance has been explained in
106 *Escherichia coli* by the overexpression of a membrane transporter involved in drug efflux,
107 which reduces intracellular accumulation of the herbicide (Staub et al., 2012). Bacteria could
108 also overcome some of the harmful effects of glyphosate by increasing the production of
109 molecules that scavenge free radicals (Liu et al., 2013). A strong tolerance of several
110 cyanobacterial strains has also been pointed out in cyanobacterial strains possessing an
111 insensitive form of the glyphosate target EPSPS (5-enol-pyruvyl-shikimate-3-phosphate
112 synthase (Forlani et al., 2008)). Finally, the biodegradation of glyphosate by bacterial strains
113 also highlights the capacity of microorganisms to use this herbicide for their growth. The
114 relationships between glyphosate biodegradation is well described for soil and sludge
115 microorganisms (Sviridov et al., 2015). Among various glyphosate-degrading microorganisms,
116 bacteria play the most pivotal role, and the processes of glyphosate biodegradation have
117 therefore been well studied (Zhan et al., 2018). Most of the glyphosate-degrading strains
118 described so far use the herbicide as a source of phosphorus, which implies that they possess
119 enzymes cleaving the C–P bond. Two main pathways for enzymatic biodegradation of
120 glyphosate have therefore been found in bacteria: direct cleavage of the C–P bond, yielding
121 sarcosine and inorganic phosphorus (sarcosine pathway), and cleavage of the C–N bond,
122 yielding glyoxylate and AMPA (AMPA pathway) (Sviridov et al., 2015). Microorganisms that
123 use glyphosate as a phosphorus source via the sarcosine pathway possess the enzymatic CP-
124 lyase complex (Hove-Jensen et al., 2014). The genes encoding the corresponding enzymes are
125 assembled into the *phn* operon, which is widespread among bacterial species such as

Achromobacter sp. MPS 12A (Sviridov et al., 2012) and *Arthrobacter* sp. GLP-1 (Pipke et al., 1987). In the other main glyphosate biodegradation pathway (AMPA pathway), the herbicide molecule is first attacked by the enzyme glyphosate oxidoreductase (GOR), which cleaves the C–N bond in the glyphosate molecule, leading to stoichiometric quantities of AMPA and glyoxylate (Sviridov et al., 2015). In most glyphosate-degrading bacteria, glyoxylate enters the glyoxylate bypass of the Krebs cycle, while AMPA is exported into the extracellular space. This pathway has been extensively studied in *Ochrobactrum anthropi* GPK 3 (Sviridov et al., 2012), and was also described in several other bacteria such as *Pseudomonas* sp. SG-1 (Talbot et al., 1984) and *Agrobacterium radiobacter* SW9 (Mcauliffe et al., 1990).

Specific microbial populations involved in glyphosate degradation in natural river biofilms are largely unknown. Therefore, the present study sought to determine the interaction effect of glyphosate and dissolved phosphorus on the structure and composition of bacterial and eukaryotic communities in biofilms and identify taxa potentially involved in glyphosate degradation. Mi-seq Illumina sequencing analyses have been performed on biofilm samples collected from the previous study by Carles et al. (2019). This work studied contrasted biofilms from an upstream site (not exposed to glyphosate) and a downstream site (chronically exposed to glyphosate) exposed to different phosphorus (low_P versus high_P) and glyphosate (low_G versus high_G) concentrations in water. Taxonomic groups in biofilms linked to glyphosate biodegradation have been described and compared to the known glyphosate-degrading microorganisms present in the literature. Our main hypotheses were: i) the structure and composition of microbial communities in biofilms, and especially that of algal populations, will be modified with increased glyphosate concentrations in water; and ii) low dissolved phosphorus availability would favour the development of glyphosate-degrading taxa in biofilms, while high dissolved phosphorus availability would eventually favour the proliferation of taxa capable of transforming the glyphosate into AMPA.

2 Materials and Methods

2.1 Experimental design

A microcosm study was conducted in the laboratory with natural biofilms grown in the field at the end of spring 2017, as described by Carles et al. (2019). Briefly, biofilms grown on glass slides at an upstream site (Ups) and a downstream site (Dws) of the Artière River (Puy-de-Dôme region, France) were placed in 24 microcosms consisting of 20 L glass aquariums. Half of the aquaria received Ups biofilms, while the other half received Dws biofilms. After two weeks of biofilms' acclimation to microcosms in their corresponding river water, each type of biofilm (Ups and Dws) was subjected to the four experimental conditions in triplicate depending on the water phosphorus concentration (LowP = 100 $\mu\text{g P L}^{-1}$ and HighP = 1000 $\mu\text{g P L}^{-1}$ and glyphosate concentration (LowG = 10 $\mu\text{g L}^{-1}$ and HighG = 100 $\mu\text{g L}^{-1}$). Briefly, water from the upstream site of the Artière (low dissolved phosphorus concentration and absence of glyphosate; (Rossi et al., 2019)) was used to fill all microcosms which were later contaminated by phosphorus (K_2HPO_4 , ACS reagent) and glyphosate (PESTANAL[®], analytical standard) depending on the experimental conditions described above. These experimental conditions correspond to 1x and 10x the mean of actual concentration of glyphosate in surface waters from France. The experimental conditions in our microcosm experiment were therefore chosen to assess realistic environmental contamination conditions. Neither photolysis nor adsorption of glyphosate in the microcosms were observed during the experiment (Carles et al., 2019). Water and biofilm analyses were carried out at days 0, 2, 4, 6, 13, and 27 post-contamination. One glass slide (28 × 83 mm) was removed from each aquarium at each sampling time and scraped in sterile conditions. Scraped biofilms were conserved frozen (- 20 °C) until DNA analysis. Dissolved nitrate and phosphorus concentrations in water from aquariums were maintained

throughout the experiment by adding adequate salt solution volumes of 1 M KNO₃ and 1 M K₂HPO₄ respectively. Further details of physico-chemical and microbial parameters measured in this experiment can be found in Carles et al. (2019).

2.2 Nucleic acids extraction and sequencing

Nucleic acid extraction was carried out on selected dates (depending on glyphosate biodegradation results from Carles et al. (2019)): days 0, 6, 13 and 27. Total DNA extraction was performed from 1 mL of scraped biofilm suspension using the FastDNATM Spin Kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's recommendation. Quantification and quality checking of extracted DNA was carried out using a Nanodrop (260/230 and 260/280 ratios; NanodropTM 2000, Wilmington, DE, U.S.A.) and electrophoresis on 1% agarose gel.

Further sample processing and sequencing was performed by the University of Illinois Keck Center (Urbana, USA). Briefly, sequencing (MiSeq bulk 2 x 250 bp, 10M-20M paired reads on Illumina MiSeq platform) yielded a total of 4,117,394 reads (Eukaryotes) and 13,244,386 reads (Prokaryotes). Specific primers used for PCR were Euk_1391f (5'-GTACACACCGCCCGTC) / EukBr (5'-TGATCCTTCTGCAGGTTACCTAC) for Eukaryotes (Gilbert et al., 2014) and V4_515F_New (5'-GTGYCAGCMGCCGCGGTAA) / V4_806R_New (5'-GGACTACNVGGGTWTCTAAT) for Prokaryotes (Caporaso et al., 2011). Sample inference from amplicon data was carried out using DADA2 pipeline (Version 1.8, <https://bioconductor.org/biocLite.R>). The DADA2 pipeline (Callahan et al., 2016) consists of amplicon sequence variants (ASVs) inference, replacing the coarser and less accurate OTU clustering approach (Callahan et al., 2017). ASV methods have demonstrated sensitivity and specificity as good as or better than OTU methods and better discriminate ecological patterns (Amir et al., 2017; Callahan et al., 2016; Edgar, 2016; Eren et al., 2015, 2013; Tikhonov et al.,

201 2015). Sequence variants and their sample-wise abundances were generated from the
202 demultiplexed fastq files after removing substitution and chimera errors. Taxonomic
203 classification was carried out via a native implementation of the RDP naive Bayesian classifier,
204 and species-level assignment to 16S rRNA gene fragments by exact matching. The
205 corresponding sequence files were deposited in NCBI's Sequence Read Archive (SRA
206 BioProject ID, PRJNA596462).

208 2.3 Data analyses

209 Data analyses were performed using RStudio (Version 1.1.456) implemented with the
210 Phyloseq package (version 1.24.0). After rarefaction, bacterial and eukaryotic datasets were
211 composed of samples containing the same number of reads (41085 and 11315 reads,
212 respectively). Rarefaction curves were plotted using the ggplot2 package (version 3.0.0) from
213 community analyses made with the vegan package (version 2.5-2). Shannon index and Chao1
214 species richness were determined with Phyloseq package. PERMANOVA tests were carried
215 out on the weighted unifracs distances matrix of bacterial and eukaryotic communities using the
216 R package “vegan”. After testing homogeneity in bacterial and eukaryotic datasets dispersion,
217 the adonis function was used to test the null hypothesis to see if experimental treatments shared
218 similar centroids.

219 The correlation between each ASV abundance and the glyphosate or AMPA concentration
220 at each sampling time (days 0, 6, 13, and 27) has been established for bacteria and Eukaryotes
221 using the Spearman's rank correlation test. Then, the 10 highest negatively correlated ASVs
222 with glyphosate concentration ($r < -0.69$) were included in 16S and 18S phylogenetic analyses,
223 in comparison to the sequences of known isolated glyphosate-degrading strains (or closest
224 sequences retrieved from the GenBank database). The phylogenetic analysis was carried out by

multiple alignments (Clustal Ω) followed by the construction of phylogenetic trees using MEGA X software (Kumar et al., 2018).

Analyses of variance were carried out using RStudio (Version 1.1.456). The fixed factors (modalities) were as follow: Site (Ups, Low P-content and Dws, High P-content); Glyphosate in water (LowG and HighG concentration); dissolved P availability in water (LowP and HighP water); Time (6, 13 and 27 days). The influence of these factors was tested on various dependent variables linked to diversity indexes (Chao1 and H') and shifts in microbial community structure (mean rate of change) were assessed using three/four-way ANOVA followed by separate post hoc comparisons (Tukey's test, $P < 0.05$). The four-way ANOVA includes the factors described above and Time (6, 13 and 27 days). The normality and homogeneity of variance were checked prior to ANOVA tests (Shapiro's and Levene's tests, respectively, $P < 0.05$) and data not normally distributed were transformed using logarithmic, square, square root or Box-Cox functions.

3 Results and Discussion

3.1 Glyphosate and/or Phosphorus effect on communities structure

3.1.1 Initial community structure and composition of biofilms

The total number of ASVs identified by 16S and 18S rRNA genes sequencing was 41085 and 11315, for Bacteria and Eukaryotes respectively. Each microbial community was well covered by the sequencing, as attested by the inflections in the rarefaction plots of the different biofilm samples (Figure S1).

At the beginning of the experiment, a total of 29 and 22 bacterial phyla were detected in upstream and downstream biofilms respectively (Figure 1A and S2A). Unclassified sequences represented less than 0.3% of the total number of ASVs in both biofilm types. Globally, the

bacterial communities were dominated by 3 phyla, representing 83.8% of the total number of ASVs, including Proteobacteria (59.4%), Cyanobacteria (16.3%) and Bacteroidetes (8.1%) (Figure 1A). These three phyla are generally dominant in stream biofilms (Battin et al., 2016), Proteobacteria often being the most common (Liao et al., 2019; Peng et al., 2018).

However, some differences were observed depending on the site on which the biofilm was grown. Indeed, the environmental template between upstream and downstream sites was consistently different; the downstream site presented higher concentrations than the upstream site in terms of dissolved inorganic phosphorus (0.32 and 0.03 mg P L⁻¹, respectively), organic carbon (12.97 and 6.50 mg DOC L⁻¹ respectively), glyphosate (0.25 and 0 µg L⁻¹ respectively) and AMPA (1.08 and 0.09 µg L⁻¹ respectively) (see Rossi et al. 2019). A total of 21 phyla were common to both sites, with 8 phyla specific to upstream and 1 phyla specific to downstream. Moreover, Cyanobacteria were more frequent in downstream (25.9%) than upstream biofilms, likely due to the higher phosphorus concentration in the downstream site compared to the upstream one. Cyanobacteria are also able to tolerate glyphosate, which has higher concentrations downstream than upstream. This tolerance has been explained by the presence of a resistant form of EPSPS in cyanobacteria and the ability of some strains to metabolize glyphosate (Arunakumara et al., 2013; Drzyzga and Lipok, 2018; Forlani et al., 2008). Inversely, Bacteroidetes was less common in downstream (4.2%) than upstream (12%) biofilms. It has already been demonstrated that members of the Phyla Bacteroidetes are sensitive to glyphosate, as in the case of human (Dechartres et al., 2019) and honey bees (Motta et al., 2018) gut microbiota.

For the eukaryotic communities at day 0, a total of 21 and 18 phyla were detected in upstream and downstream biofilms respectively (Figure 1B and S2B). Unclassified sequences accounted for 40.7% (upstream) and 24.4% (downstream) of the total number of ASVs. Globally, eukaryotic communities were dominated by 4 phyla, accounting for 58.1% of the total

number of ASVs (Figure 1B). These phyla were Ochrophyta (23.5%), Chlorophyta (13.7%), Ciliophora (10.5%) and Annelida (10.4%). A total of 14 phyla common to both biofilm types were detected, while 6 phyla were only detected in the upstream biofilm and 4 in the downstream one. For example, Ciliophora was one of the dominant phyla in the upstream biofilm (20.3%), whereas it was almost absent from the downstream one (0.6%). Different sensitivity levels of Ciliates with respect to glyphosate have been reported in the literature (Bonnet et al., 2007; Everett and Dickerson, 2003; Tsui and Chu, 2003), indicating that, in the present study, the presence of glyphosate by itself cannot explain the variation of Ciliophora abundance between upstream and downstream biofilms.

Conversely, Annelida and Chlorophyta were well represented in the downstream site (20.8% and 26.5%, respectively), while they absent or only accounted for 0.8% in the upstream site respectively. Both phyla are known to tolerate eutrophic conditions quite well, which is consistent with the higher nutrient and organic carbon concentrations found in the downstream site compared to the upstream site (Rossi et al. 2019). Moreover, these phyla seem to be insensitive to glyphosate. The few studies available in the literature exposed organisms to unrealistic (excessively high) glyphosate concentrations. For example, a study showed some adverse effects on two species of Annelids at concentrations that greatly exceed the recommended field application rate of glyphosate (García-Torres et al., 2014). Besides, high concentrations of glyphosate (order of mg L^{-1}) decreased the abundance of Chlorophytes in the periphyton (Gonzalez et al., 2019).

Differences between downstream and upstream eukaryotic communities can be explained by xenobiotics pressure, as well as other chemical stressors, such as increased availability of dissolved carbon and nutrients (Van Horn et al., 2011) which support the “paradox of enrichment” phenomenon in aquatic microbial communities (*e. g.* Tubay et al. (2013)).

3.1.2 Responses of richness and diversity in microbial communities

At the beginning of the experiment, Ups biofilms showed higher bacterial and eukaryotic species richness (Tukey, $P < 0.05$) and diversity (Tukey, $P < 0.05$) compared to the Dws ones, and this site effect was maintained for Eukaryotes after 6 days of incubation (Table 1 and Figure S3, $P < 0.05$). Moreover, a decrease in eukaryotic species richness ($P < 0.05$) and diversity ($P < 0.05$) was observed over time, as well as for bacteria, in Dws communities (time $\times$ site interaction, $P < 0.01$). Low species richness and diversity in downstream communities can be explained by xenobiotics and nutrients pressure (Van Horn et al., 2011). However, downstream communities that are expected to be more acclimated to glyphosate are those that experience the greatest decrease in eukaryotes richness and diversity after exposure to glyphosate (Table 1).

Dissolved phosphorus treatments were not essentially affecting microbial diversity indices in biofilms (Table 1 and Figure S3C, $P > 0.05$), with the exception of eukaryotic communities, that slightly decreased their species richness under HighP water on certain sampling dates (days 6 and 13) compared to day 0 (Time $\times$ Pwater interaction, $P < 0.01$). However, the structure of both bacteria and Eukaryotic communities responded to dissolved phosphorus treatments at the beginning of the experiment (day 6) compared to later sampling dates (Figure S4; PERMANOVA, Time $\times$ Pwater interaction, $P < 0.05$ and 0.01, respectively).

A higher eukaryotic species richness ($P < 0.001$) and diversity ($P < 0.001$) has been observed after 6, 13 and 27 days for LowG compared to HighG conditions (Table 1 and Figure S3, $P < 0.05$). This trend marked by the glyphosate was also observed in the structure of eukaryotic communities (Figure S4, PERMANOVA Gwater effect, $P < 0.01$, Table S1). Interactions between Pwater and Gwater were observed for Eukaryotic species richness ($P < 0.05$), diversity ($P < 0.01$) but not for the structure of this community (PERMANOVA Pwater $\times$ Gwater interaction, $P = 0.059$, Table S1). The highest diversity was observed in lowP_lowG

treatments for both upstream and downstream communities. According to our results, the interaction (lowP_lowG) was significant for eukaryotic communities. We could conclude that P and glyphosate had a synergistic effect in decreasing eukaryotes species richness and diversity in biofilms. No conclusions can be drawn for prokaryotes.

Neither the species richness nor the diversity of bacterial communities were affected by the two glyphosate concentrations tested ($P = 0.812$ and $P = 0.712$, respectively). PERMANOVA tests revealed also that glyphosate did not significantly modify bacterial communities structure (Gwater effect, $P = 0.054$; Figure S4 and Table S1). The decrease of richness and diversity in the presence of high concentration of glyphosate was only observed for Eukaryotic communities, which are potentially targeted by the herbicide. Indeed, The physiological and biochemical similarities between algae and terrestrial plants suggest that algae would be particularly vulnerable to glyphosate (Annett et al., 2014). Moreover, this impact in HighG water could explain the higher impact in HighP water, in which glyphosate effects are more consistent on days 13 and 27 in Eukaryotic communities.

On the basis of these results, it could be supposed that the most promising area to find glyphosate-degrading strains could be within the bacterial community. Bacterial richness and diversity were weakly modified, so more tolerant to glyphosate, than the Eukaryotic ones. The most promising communities are certainly those exposed to LowP and LowG water. There are two main reasons according to Carles et al. (2019): (i) glyphosate degradation is accelerated when dissolved phosphorus availability in water is low, and (ii) glyphosate is completely removed, without accumulation of AMPA, in this condition.

3.1.3 Shifts in microbial communities structure

Unifrac coordinates were used to calculate the mean rate of change in the structure of the microbial community in experimental conditions over the duration of the experiment (Figure S5). Overall, the rate of change in bacterial communities was much slower than that observed

for the eukaryotic ones. Eukaryotic community structure of biofilms in LowP water changed faster than the HighP one ($P < 0.01$). Moreover, the microbial community structure was modified more quickly for biofilms exposed to LowG compared to HighG (Figure S4, $P < 0.05$ (Bacteria), $P < 0.01$ (Eukaryotes)). The interaction between dissolved phosphorus and glyphosate ($P < 0.05$ (Bacteria), $P < 0.001$ (Eukaryotes)) revealed that both type of biofilms (Ups and Dws) undergo the highest rate of change when exposed to LowP and LowG (Figure S5).

The lower concentration of glyphosate in water induces the fastest shifts in the microbial community structure. The plasticity of the microbial community structure seems to be higher in the case of low doses of pollutant. Despite the relatively high EC_{50} of glyphosate (11.7 – 35.6 $mg\ L^{-1}$ found by Bonnineau et al. (2012) with regard to the photosynthetic efficiency of biofilm, a negative impact of glyphosate on phototrophic community growth has been observed for biofilms exposed to 10 $\mu g\ L^{-1}$ of glyphosate (Bricheux et al., 2013). The results of our study probably reflect an exposure to a sub-lethal dose enabling for better tolerance to glyphosate. The response was more marked on eukaryotes, which most likely possess the class I of EPSPS, the sensitive form of EPSPS towards glyphosate (Annett et al., 2014). There is also a strong effect of phosphorus, indicating that a higher trophic state can lower the adaptation of microbial communities or override the effect of glyphosate.

In that sense, we confirm our focus on the LowP_LowG condition showing the fastest adaptation capacity in the search for glyphosate degraders. Moreover, it was also shown that the fastest glyphosate dissipation (lowest dissipation times 50%, DT_{50s}) was obtained with biofilms in LowP_LowG microcosms (Carles et al., 2019).

3.2 *Glyphosate-degrading microorganisms in biofilms*

As mentioned above, we further analysed microbial diversity in the most efficient condition for glyphosate degradation without AMPA accumulation (LowP_LowG) taking into account ASVs abundance data from days 6, 13, and 27.

3.2.1 Relationships between ASVs and glyphosate and AMPA

The correlation between each ASV abundance and glyphosate concentration over time has been plotted against the correlation between this ASV abundance and AMPA concentration (Spearman's rank correlation). Then, an additional correlation test revealed a positive linear relationship between ASVs abundance correlations with glyphosate and AMPA (Figure 2), as attested by the values of the Spearman's rank correlation test (Eukaryotes, $\rho = 0.809$, $P < 0.001$; bacteria, $\rho = 0.832$, $P < 0.001$). These results highlight a linear distribution of microorganisms from those characterized by high ASVs abundance in the presence of high concentrations of glyphosate and AMPA (positive Spearman's correlation) to the ones that correspond to high ASVs abundance in the presence of low concentrations of glyphosate and AMPA (negative Spearman's correlation). The last were selected to search for potential glyphosate degradation candidates since they were present at high abundances until the complete depletion of glyphosate and AMPA molecules in the microcosms.

3.2.2 Taxonomic repartition of the correlation between ASVs abundance and glyphosate

3.2.2.1 *Eukaryotes*

The repartition of the correlations between ASVs abundance depends on the Phylum (Figure S6). Some of the most abundant phyla, such as Chlorophyta and Ochrophyta, present a large variation in terms of the correlation between the abundance of their ASVs and the concentration of glyphosate in the water. Only three Phyla (Chlorophyta, Cryptomycota and Ochrophyta) contain ASVs abundance that are highly negatively correlated with glyphosate ($-0.84 <$

Spearman's rank coefficient < -0.69). Seven of the 10 highest negatively correlated ASVs belong to the Chlorophyta phylum. The abundance of Chlorophyta has been seen to increase and those of Bacillariophyceae (diatoms) to decrease in periphytic biofilms treated with the glyphosate formulation Roundup® (Vera et al., 2010).

Eukaryotic microorganisms described so far for their glyphosate-degrading activity mostly belong to the Ascomycota Phylum (Zhan et al., 2018). However, none of the ASVs belonging to the Ascomycota phylum were found in the top ten ASVs negatively correlated with glyphosate (Figure S7). The present study does not suggest a potential role for Ascomycota members in glyphosate degradation since their ASVs abundances were not strongly negatively correlated with glyphosate. One possible explanation is that Ascomycota are weakly represented in the periphyton since they prefer carbon-rich substrates, such as leaf litter or wood, for growth (Voříšková and Baldrian, 2013). Despite the fact that most Ascomycota members able to degrade glyphosate use this herbicide as a phosphorus source, as in the case of the biofilms in this study, AMPA did not accumulate in the media in the LowP LowG condition. The degradation of glyphosate by Ascomycota described in the literature, which seems to be mainly dominated by the production of AMPA (Zhan et al., 2018), should therefore not be predominant within biofilms.

3.2.2.2 *Bacteria*

Similarly to the Eukaryotic community, the repartition of the correlation between bacterial ASVs abundance depends on the Phylum (Figure S8). Five of the most abundant phyla (Bacteroidetes, Chloroflexi, Cyanobacteria, Planctomycetes and Proteobacteria) contain ASVs abundance that are highly negatively correlated with glyphosate (Spearman's rank coefficient < -0.71).

All glyphosate-degrading microorganisms isolated so far come from various environments such as rhizosphere, activated-sludge and glyphosate contaminated soils (Sviridov et al., 2015), while none has been isolated from river biofilms. Interestingly, the present study reveals some ASVs potentially linked to glyphosate biodegradation by river biofilms. Some of them are close to strains able to degrade glyphosate, in terms of 16S phylogeny (Figure 3).

For example, one Bacteroidetes member isolated from activated sludge (a *Flavobacterium* strain) is able to use glyphosate as a sole phosphorus source (Balthazor and Hallas, 1986). Alpha-proteobacteria also contains several glyphosate-degrading strains, including the well-studied strain *Ochrobactrum anthropi* GDOS (Hadi et al., 2013). This strain has been isolated from a glyphosate-contaminated soil and is able to use glyphosate as a phosphorus source. Among alpha-proteobacteria, all glyphosate-degrading strains are able to use the herbicide as a sole phosphorus source. Microorganisms that use glyphosate as a phosphorus source possess the enzymatic CP-lyase complex (Hove-Jensen et al., 2014). This enzymatic complex enables an orthophosphate molecule to be released from the glyphosate (sarcosine degradation pathway) or from AMPA (AMPA degradation pathway) (Sviridov et al., 2015). The genes encoding the corresponding enzymes are assembled into the *phn* operon, which is described in *Ochrobactrum anthropi* ATCC 49188 (Hove-Jensen et al., 2014). It is then probable that alpha-proteobacteria candidates for glyphosate degradation in our study (4 ASVs over the ten) use similar enzymatic machinery since AMPA was not an end product in their metabolism.

A general tolerance and a remarkable ability to degrade the glyphosate have also been described for cyanobacteria (Forlani et al., 2008; Lipok et al., 2007). Incubations of *Spirulina platensis* strain C1 with 10 mM glyphosate concentrations resulted in the disappearance of the herbicide in 14 days concomitantly with the appearance of transient peaks of orthophosphate monoester (Forlani et al., 2008). Chloroflexi are also phototrophic bacteria like cyanobacteria but no studies have shown the presence of strains capable of degrading the glyphosate. On the

contrary, a recent publication highlighted that the relative abundance of this phylum tends to decrease in soils treated with glyphosate (Lu et al., 2017).

Overall, the ten bacterial ASVs found in LowP and lowG treatment are potential candidates for further glyphosate-bioremediation experiments. However, it has to be noted that our approach can rule out certain ASVs participating in the degradation of glyphosate but present in low ASV abundance when glyphosate is depleted.

4 Conclusion

Rivers are continuously affected by various stressors, such as habitat degradation, nutrients and xenobiotics pollution, flow regulation and water abstraction. Studying the stressors, not only separately, but also by taking into account their interactions, gives a more realistic picture of the integrity of ecosystems and their endangered components. In the present study, phosphorus and glyphosate induced differential responses in microbial communities of river biofilms. While eukaryotic communities showed strong diversity plasticity when exposed to low glyphosate concentrations, shifts in the structure of bacterial communities were specifically observed during glyphosate dissipation. Understanding the fate of glyphosate in river ecosystems requires further identification of glyphosate-degrading species from natural microbial communities. Enrichment cultures and isolation of glyphosate-degrading strains will contribute to improving bioremediation actions and the restoration of contaminated areas.

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6 Competing interests

The authors declare no competing interests.

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