Title: Resistance, resilience, and functional redundancy of freshwater microbial communities facing multiple agricultural stressors in a mesocosm experiment

Running title: Bacterioplankton responses to agricultural stress

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Abstract

Freshwater biodiversity is threatened by fertilizers and pesticides from agricultural sources. Microbial communities can be resistant (*i.e.*, community composition stays largely the same) or resilient (*i.e.*, composition changes but then returns to its initial state) to these contaminants. Even after changes in composition, communities may continue to support ecosystem processes due to functional redundancy, in which different taxa carry out the same process, such that the process is maintained even after some taxa are lost. To test the extent of resistance, resilience, and functional redundancy in aquatic bacterial communities (bacterioplankton) faced with agricultural stressors, we exposed freshwater mesocosms to two commonly used pesticides: the herbicide Roundup (glyphosate) and the neonicotinoid insecticide imidacloprid, alone or in combination, and in high or low nutrient backgrounds. Over the 42-day experiment, we tracked bacterial density with flow cytometry, functional composition with Biolog EcoPlates, and taxonomic diversity with culture-independent 16S rRNA gene amplicon sequencing. We show that only glyphosate, but not imidacloprid or nutrients, measurably changed community structure. Despite this change, metabolic capabilities were maintained, suggesting functional redundancy. We further show that communities are resilient at broad, but not fine phylogenetic levels: the precise amplicon sequence variants (ASVs) driven below the limit of detection by glyphosate stress do not return, but tend to be replaced by relatives within the same genus. Together, our results show that bacterioplankton are broadly resistant, resilient, and redundant faced with severe agricultural stressors on weekly time scales, but that high doses of glyphosate can have longer-lasting effects on fine-scale diversity.

Introduction

Bacterioplankton are drivers of energy and nutrient cycling in freshwater ecosystems [1,2]. These microbial communities can be affected by environmental disturbances [3,4] potentially influencing ecosystem processes in lakes and rivers. Yet exactly how these disturbances affect microbial community structure and function is not well understood [4,5].

When faced with a disturbance, microbial community composition may remain largely unaltered, a phenomenon known as community resistance [5]. Alternatively, a disturbed microbial community may recover over time, a process known as community resilience [5]. Both resistance and resilience confer community stability after a disturbance, allowing ecosystem processes (*e.g.*, community metabolism, nutrient cycling, etc.) to be maintained.

If community composition is permanently altered by a disturbance, ecosystem processes may remain unaffected if the altered community is able to perform the same set of functions as the original community. This phenomenon is known as functional redundancy [4]. Functional redundancy is thought to be common in microbial communities, as most metabolic pathways controlling biogeochemical cycles are found across different clades [2]. It has been suggested that spatial and environmental heterogeneity (or fluctuations) and biotic interactions (*e.g.*, predation) are required to explain the widespread redundancy in biochemical pathways [6]. It is also likely that communities are partially redundant (for certain general functions like respiration or biomass production), but complementary (non-overlapping functions) for very specific functions encoded by unique taxa [7]. The prevalence of, and reasons for, microbial community

resistance, resilience, and functional redundancy are still debated [4,5] particularly in response to novel anthropogenic disturbances which often involve combinations of stressors.

Agricultural expansion and intensification are main drivers of global environmental change in both terrestrial and aquatic ecosystems [8–10]. Chemicals derived from agricultural landscapes, such as fertilizers and pesticides, are among the main sources of freshwater pollution [11], leading to eutrophication [12,13] and biodiversity loss [14–16]. Agricultural runoff usually contains a mixture of nutrients, herbicides, and insecticides that may interact to affect aquatic microbial species [17] and communities [18,19]. The effects of nutrient pollution in freshwater communities are well understood [20,21], but their potential interaction with other agricultural pollutants such as pesticides deserves further study.

The herbicide glyphosate, mainly formulated commercially as Roundup, and the neonicotinoid insecticide imidacloprid are among the most commonly used pesticides worldwide [22,23]. They can be found in surface waters at concentrations up to 430 μg/l [24,25] and 320 μg/l [26] for glyphosate and imidacloprid respectively. There are reports of glyphosate concentrations in surface waters up to several milligrams per litre, particularly in cases of accidental spills, overwater application, or applications followed by extreme storms [27]. Reported concentrations of both pesticides are highly variable, but on average glyphosate is found at higher concentrations than imidacloprid in freshwater [26,28]. Currently, the Canadian Water Quality Guidelines for the protection of aquatic life recommend a maximum aquatic glyphosate concentration of 800 μg/l for long-term exposures and 27 000 μg/l for short-term exposures [29], while for imidacloprid, there is an interim guideline of 0.23 μg/l maximum concentration in

water bodies [30]. Note, however, that these criteria were developed based on toxicity tests with eukaryotic organisms, such that it remains unclear how bacterial communities would respond to these concentrations considered "safe for aquatic life".

Glyphosate is a broad-spectrum synthetic phosphonate herbicide that acts by inhibiting the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS) involved in the shikimate pathway, responsible for the biosynthesis of aromatic amino acids essential to plants, fungi, and microbes [31]. It is used for weed control mainly in agriculture, but also applied in lower volumes to non-crop areas such as roadsides, parks and domestic gardens [32,33]. Glyphosate and its metabolite aminomethylphosphonic acid (AMPA) can be transported from soil to surface waters by runoff or leaching from agricultural landscapes. Soil properties, such as pH and phosphate concentration, influence whether glyphosate and AMPA remain adsorbed to soil particles or are carried away in their dissolved form [34,35]. Frequency, dose, and timing of glyphosate application combined with climate conditions (*e.g*. precipitation patterns), hydrology, and soil type influence how fast and often glyphosate enters waterways, usually as a discontinuous input rather than a continuous exposure [34]. Some microorganisms are resistant to glyphosate either by expressing an insensitive form of the target enzyme [36,37] or by metabolizing the molecule and using it as a phosphorus source [38].

Imidacloprid is a neonicotinoid insecticide available in various commercial formulations and commonly administered in the control of piercing-sucking insects on crops worldwide [39,40]. As it is systematically active in plants, it can be applied to seeds, foliage, or directly onto soil [41]. Leaching or runoff of imidacloprid from soils to water bodies may occur, depending on the

soil type, moisture, and organic matter content [42]. Imidacloprid is water soluble, facilitating its movement into surface and groundwater, while its rapid degradation combined with its discrete applications in fields result in pulsed exposures to aquatic systems [43]. At least 13 bacterial isolates from soil have been reported to degrade imidacloprid, resulting in a variety of metabolites, some even more toxic and persistent than imidacloprid [44].

In this study, we describe a mesocosm experiment with lake-derived bacterioplankton to test the effects of pulse applications of glyphosate and imidacloprid on bacterial community density, composition and carbon metabolic profiles. To identify interactive effects of agricultural stressors, we applied these pesticides alone or in combination, and in the presence or absence of nutrient enrichment simulating fertilizer pollution. None of these treatments affected bacterial cell densities, and only high glyphosate treatments caused dramatic shifts in community composition, with no apparent interactive effects with other stressors. None of the stressors caused detectable changes in carbon metabolic profiles, consistent with functional redundancy despite the glyphosate-induced shifts in community composition. Defined at broad phylogenetic categories, community composition returned close to its initial state 24 days after the first glyphosate pulse – indicating resilience to glyphosate stress. Despite this broad resilience, the precise strains (defined here as amplicon sequence variants; ASVs) initially present in the community did not return and were replaced with close but non-identical relatives. Thus, glyphosate induces long-term changes in fine-scale community composition. Together, these results show that microbial community responses to complex combinations of anthropogenic stressors may involve resistance to some stressors (imidacloprid and nutrients), but resilience to others (glyphosate).

Methods

Experimental design and sampling

We conducted a mesocosm experiment for 56 days (of which the first 42 days are reported here) at the Large Experimental Array of Ponds (LEAP) platform at McGill University's Gault Nature Reserve in Québec, Canada (45°32'N, 73°08'W). The methodological details of this experiment have been described in Fugère et al. [45], which focused on community rescue in eukaryotic phytoplankton. The experiment involved two phases of pesticide treatments. Here we only consider phase I of the experiment (days 0-42), not phase II (days 43-56) in which "lethal" doses of stressors were applied (as the definition of lethal concentrations were specific to eukaryotic phytoplankton). The experimental ponds were each filled with 1 000 l of water from Lake Hertel (45º32' N, 73º09' W), a pristine lake unaffected by agricultural pollution. We used 48 ponds in total, divided into groups that received either a low amount of nutrients (**Fig. 1**) to maintain a mesotrophic lake intake with a target total phosphorus (TP) concentration of 15 μg/l (similar to what is typically found in Lake Hertel [46]), or a high amount of nutrients simulating an eutrophic pond with a target TP concentration of 60 μg/l. As detailed in Fugère et al. [45], nutrient solutions containing nitrate (KNO_3) and phosphate (KH_2PO_4 and K_2PO_4) were added to manipulate nutrients while preserving the same N:P molar ratio (33:1) found in the lake. Within each nutrient treatment, some of the ponds received varying amounts of the herbicide Roundup or the insecticide imidacloprid, separately or in combination, in a regression design with seven levels of pesticide concentration plus controls with no pesticide addition (**Fig. 1**). The seven levels of target concentration were: 0.04, 0.1, 0.3, 0.7, 2, 5.5 and 15 mg/l for glyphosate acid, the active ingredient in Roundup Super Concentrate (Monsanto, St-Louis, MO, USA) and 0.15, 0.4, 1, 3, 8, 22 and 60 μg/l for imidacloprid (Sigma-Aldrich, Oakville, Canada).

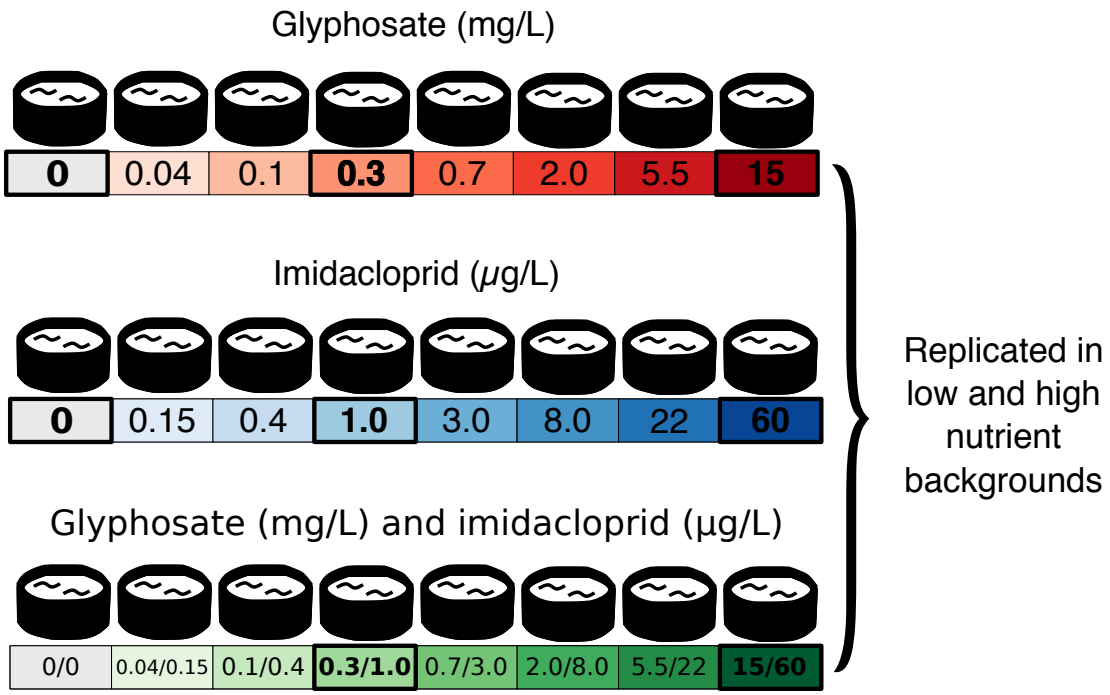


Figure 1. Agricultural stressors applied alone or in combination to the Large Experimental Array of Ponds (LEAP). In total, 48 ponds were filled with 1 000 l of lake water and received two pulses of glyphosate and imidacloprid, alone or in combination, and under two different nutrient enrichment scenarios. The experiment lasted 42 days and pesticides were applied on days 5 and 33. Bold outlined boxes indicate ponds sampled for DNA extraction and 16S rRNA gene amplicon sequencing.

Treatment ponds received two pulses of pesticides (at days 5 and 33 of the experiment) while nutrients were applied weekly to maintain a "press" treatment. We sampled all ponds at six different timepoints: one before any pesticide application (day 0); three between pulse 1 and pulse 2 applications (days 6, 14, and 29); and two timepoints after the second pulse (days 34 and 42). Day 0 corresponds to August 17th, 2016. All mesocosms were filled with lake water 99 days prior to the experiment, on May 11th, 2016, to allow the communities to acclimatize to the mesocosm setting. Biweekly water changes (10% of total volume) between May and August

153 ensured a continuous input of organisms tracking seasonal changes in the source lake
154 community. We collected water from each mesocosm with integrated PVC tubing samplers (2.5
155 cm diameter), stored in dark Nalgene bottles (1 l) previously washed three times with pond
156 water. To avoid cross contamination, we sampled each pond with a separate sampler and bottle.
157 We kept bottles in coolers while sampling and then moved them to an on-site laboratory, where
158 they were stored at 4 °C until processing as described below.
159
160 *Nutrient and pesticide quantification*
161 We sampled 40 ml of unfiltered pond water in acid-washed glass tubes, in duplicates, for TP and
162 total nitrogen (TN) quantification. Samples were processed at the GRIL analytical laboratory at
163 the Université du Québec à Montréal following standard protocols [47]. TN concentration was
164 determined using the alkaline persulfate digestion method coupled with a cadmium reactor [48]
165 in a continuous flow analyzer (OI Analytical, College Station, TX, USA). TP was estimated
166 based on optical density in a spectrophotometer (Biocrom Ultrospec 2100pro, Holliston, MA,
167 USA) after persulfate digestion through the molybdenum blue method. Glyphosate and
168 imidacloprid concentrations were determined in the Department of Chemical Engineering at
169 McGill University. The concentration of glyphosate in the ponds was measured using a method
170 described in our earlier work [45]. Briefly, 1 l water samples were acidified to a pH < 3 with
171 sulfuric acid and frozen at -20 ºC until analysis. The samples were analyzed by liquid
172 chromatography coupled to mass spectrometry using an Accela 600-Orbitrap LTQ XL (LC–
173 HRMS, Thermo Scientific). The method for glyphosate quantification was based on heated
174 electrospray ionization (HESI) in negative mode, acquisition in full scan mode (50-300 m/z) at
175 high resolution (FTMS = 30,000 m/Dz) and the use of an ion trap to perform targeted data

176 acquisition for the product ion spectra (MS2) and generate identification fragments. The limits of
177 detection and quantification of glyphosate were 1.23 and 4.06 μg/l, respectively. Imidacloprid
178 concentrations were measured using the same LC-HRMS system but using positive HESI mode
179 and a mass range of a mass range of 50 to 700m/z. The detection and quantification limits for
180 this method were 1.44 and 4.81 μg/l, respectively.
181
182 *Estimating total bacterial density through flow cytometry*
183 To estimate total density of bacterial cells, we fixed 1 ml of the 1 L sampled pond water with
184 glutaraldehyde (1% final concentration) and flash froze this subsample in liquid nitrogen. We
185 stored samples in -80 ºC until they were processed via a BD Accuri C6 flow cytometer (BD
186 Biosciences, San Jose, CA, USA). After samples naturally thawed, we prepared dilutions (1:25
187 or 1:50) with Tris-EDTA buffer (Tris-HCl 10 mM; EDTA 1 mM; pH 8) and aliquoted in two
188 duplicate tubes. Samples were then stained with Syto13 Green-Fluorescent Nucleic Acid Stain
189 (0.1 v/v in DMSO; ThermoFisher S7575) and incubated in the dark at room temperature for 10
190 min. Every day, to validate the equipment calibration, we ran BD TruCount Absolute Count
191 Tubes (BD Biosciences) prior to sample processing. Samples were run until reaching 20 000
192 events, at a rate of 100-1 000 events/s. Bacterial density was estimated based on flow duration,
193 flow rate, and sample dilution. We calculated the average bacterial density for each pair of
194 analytical duplicates with a coefficient of variation (CV, *i.e.*, ratio between the standard
195 deviation and average of the duplicate values) smaller than 0.08; samples were processed again
196 when the CV was higher than 0.08.
197
198 *Carbon substrate utilization patterns*

199 We used Biolog EcoPlate® assays (Hayward, CA, USA) to infer community-level utilization of
200 dissolved organic carbon by bacteria. For all treatments and at each sampled timepoint, we added
201 125 µl of unfiltered pond water to each well of the Biolog plates, containing 31 different
202 dissolved organic carbon substrates in triplicate wells. We measured the optical density in each
203 well as a proxy for bacterial respiration of the substrates, with substrate utilization causing a
204 concomitant reduction of the redox-sensitive tetrazolium dye (with colour intensity measurable
205 via absorbance at 590 nm). Plates were incubated in the dark at room temperature and well
206 absorbance was measured daily until an asymptote was reached (up to two weeks). For each
207 daily measurement, an average well colour development (AWCD) was calculated. We selected
208 substrate absorbance values of the plate measurements with AWCD closest to the reference
209 value of 0.5, as suggested in [49] to correct for inoculum density. We then calculated the blank-
210 corrected median absorbance of each substrate at each sampled timepoint.
211
212 *DNA isolation, 16S rRNA gene amplification and sequencing*
213 We selected a subset of ponds for DNA isolation and subsequent analyses (outlined in bold in
214 **Fig. 1**) to assess community responses at the extremities and the middle of the exponential
215 gradient. From each timepoint and nutrient treatment subarray, we chose two control ponds
216 (beginning of the gradient, no pesticide addition), ponds with the third lowest concentration
217 (middle of the gradient) of each or both pesticides (1 µg/l imidacloprid or/and 0.3 mg/l
218 glyphosate), and ponds with the highest concentration (end of the gradient) used in the
219 experiment for each or both pesticides (60 µg/l imidacloprid or/and 15 mg/l glyphosate). We
220 selected ponds with high concentrations of pesticides with the intention of maximizing the
221 chance of detecting a response from the bacterial community. That said, even the highest

222 concentrations are below the criterion from the Canadian Water Quality Guidelines for short
223 term exposures, available for glyphosate exposure [29], allowing us to ask whether changes in
224 bacterial communities can be detected at concentrations considered safe for aquatic eukaryotes.
225 In total, we sampled 16 of the 48 experimental ponds at each timepoint for DNA sequencing.
226 After sampling 1 l of pond water as previously described, we immediately filtered 250 ml on
227 0.22 μm Milipore hydrophilic polyethersulfone membranes (Sigma-Aldrich, St. Louis, USA) and
228 stored filters in -80 ºC until DNA extraction. We extracted and purified total bacterial genomic
229 DNA from frozen filters using the PowerWater DNA Isolation Kit (MoBio Technologies Inc.,
230 Vancouver, Canada). We quantified genomic DNA with a Qubit 2.0 fluorometer (ThermoFisher)
231 and used 10 ng to prepare amplicon libraries for paired-end sequencing (2 x 250 bp) on a MiSeq
232 instrument (Illumina, San Diego, CA, USA). We performed a two-step polymerase chain
233 reaction (PCR) targeting the V4 region of the 16S rRNA gene, with primers U515_F and
234 E786_R, as described in [50]. After amplification (step 1 PCR) and barcode attachment (step 2
235 PCR), amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter Life
236 Sciences, Indianopolis, IN, USA). Fragment size was confirmed through agarose gel and
237 quantified on a NanoDrop microvolume spectrophotometer (ThermoFisher, Waltham, MA,
238 USA). Libraries were then pooled at equimolar ratio, denatured and sequenced, using the MiSeq
239 reagent Kit V2 with 500 cycles. The 96 samples were split into two different sequencing runs,
240 each of them including PCR negative controls (water used in the PCR reactions), extraction
241 blanks (result of the DNA extraction obtained from clean filters), and a mock community DNA
242 sample containing identified 16S rRNA clone libraries. In one of the runs we used a custom
243 mock composed of 16S rRNA clone libraries from freshwater lake samples [50] and in the other

244 we used the American Type Culture Collection MSA-1002 mock community (ATCC, Manassas,
245 VA, USA).
246
247 *Sequence data processing*
248 We used idemp (https://github.com/yhwu/idemp) to demultiplex barcoded sample fastq files
249 from 16S rRNA deep sequencing data and cutadapt to remove remaining Illumina adapters [51].
250 We then used the DADA2 package [52] in R to filter and trim reads, infer amplicon sequence
251 variants (ASVs), remove chimeras and finally obtain a matrix of ASV counts in each sample for
252 each MiSeq run independently. Excluding mock communities, extraction blanks and PCR
253 controls, we obtained 1 787 412 raw reads in the first run and 4 702 355 in the second run, of
254 which we retained, respectively, 1 565 021 and 4 188 644 non-chimeric reads. PCR negative
255 controls and extraction blanks produced 214 non-chimeric reads in total; these were excluded
256 from downstream analyses as we only included samples with a minimum of 6 000 reads. Of the
257 30 expected sequences from the custom mock [50], DADA2 found 25 exact sequence matches,
258 producing 5 false negatives and 7 false positives (for a total of 32 sequences). In the ATCC
259 mock, 23 of the 24 expected sequences were found, with only 1 false negative but 10 false
260 positives (for a total of 33 sequences). We concatenated DADA2 abundance matrices from each
261 MiSeq run and then used TaxAss [53] to assign ASV taxonomy with a database specifically
262 curated for freshwater bacterioplankton, FreshTrain [54], and GreenGenes [55], with a minimum
263 bootstrap support of 80% and 50%, respectively. After performing a multiple sequence
264 alignment with the R package DECIPHER [56], we constructed a maximum likelihood
265 phylogenetic tree using the phangorn package following recommendations made by [57]. For
266 subsequent analyses, we imported the ASV abundance matrix together with the environmental

267 and the taxonomy data as an object in the phyloseq package [58] in R. We removed sequence
268 data identified as mitochondria or chloroplast DNA and normalized read counts [59] using the
269 DESeq2 package [60]. We calculated the estimated absolute abundance (EAA) of ASVs per
270 sample by multiplying their relative abundance by the total bacterial cell counts found in the
271 sample through flow cytometry [61].
272
273 *Statistical analyses*
274 To assess resistance and resilience to experimental treatments, we assessed changes (or lack of
275 change) in bacterial community composition using Principal Coordinate Analysis (PCoA) and
276 Principal Response Curves (PRCs), as explained in detail below. We conducted all statistical
277 analyses in R version 3.5.1 [62]. We used the function glmer.nb() from the lme4 package [63] to
278 build generalized linear mixed models (GLMM) and test whether nutrient or pesticide treatments
279 affected the overall bacterial density throughout the experiment. We considered time and the
280 measured concentrations of glyphosate, imidacloprid, TP and TN as fixed effects, and pond as a
281 random effect.
282
283 To quantify the extent of functional redundancy along one axis of niche space, we compared
284 community carbon substrate utilization patterns using Bray-Curtis dissimilarity of the Biolog
285 metabolic profiles, and represented this variability in a non-metric multidimensional scaling
286 (NMDS). To assess differences in community taxonomic composition, we calculated weighted
287 Unifrac distances [64] and Jensen-Shannon divergence among the subset of samples selected for
288 DNA analyses and represented them in principal coordinate analysis (PCoA) bidimensional
289 plots. We further performed a series of permutational analyses of variance (PERMANOVA)

based on weighted Unifrac distances, followed by a conservative Bonferroni correction for multiple hypothesis testing to test the effect of treatments (as factors) on community taxonomic composition at four sampled timepoints: at day 0 (before any treatment was applied), day 6 (immediately after the first pulse), day 29 (immediately before the second pulse) and day 42 (last day of the experiment, after the second pulse).

We built principal response curves (PRCs) to assess the effects of the treatments on functional and taxonomic composition as a function of time [65]. PRCs are a special case of partial redundancy analysis (pRDA) in which time and treatments, expressed as factors, are used as explanatory variables of the model, while community composition is the multivariate response matrix. Time is considered as a covariable (or conditioning variable) whose effect is partialled out, and changes in community composition in the treatments over time are always expressed as deviations from the control at each timepoint. We tested for the significance of the first displayed PRC axis by permuting the whole time-series of each pond and comparing the permuted data to the experimental data using the function permutest() from the vegan package in R [66]. For each PRC model, we evaluated the proportion of variance (inertia) explained by the conditional variable (time) and the constrained variable (treatments), as well as the proportion of explained variance per axis (the eigenvalue of each RDA axis divided by the sum of all eigenvalues). We used these values to decide which PRC model best explained the variation in the data. Before performing PRCs for the taxonomic dataset, we transformed the abundance matrix using the Hellinger transformation [67].

Results

Bacterial cell density and carbon metabolic profiles are robust to stressors

We first tested the effects of pesticides and nutrient pollution on bacterial cell density and carbon utilization profiles. None of the experimental treatments (alone or in combination) significantly affected overall bacterial density (**Fig. 2**).

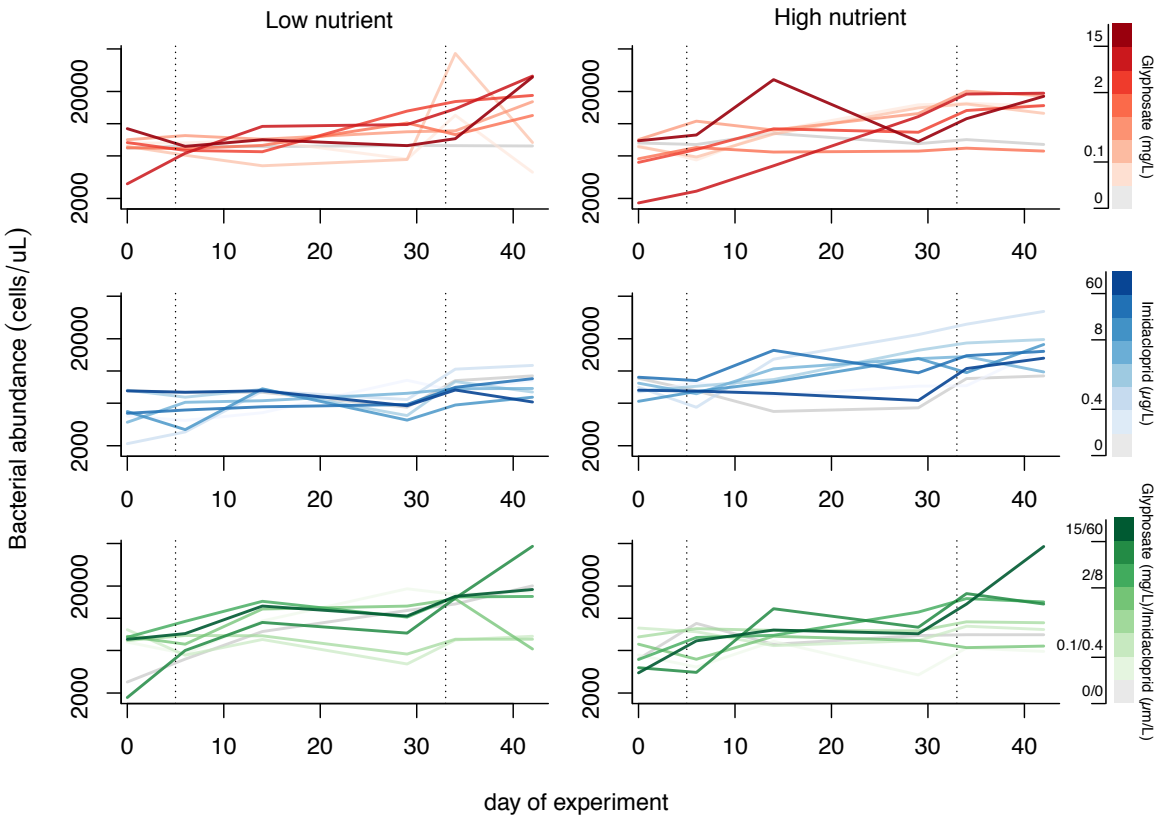
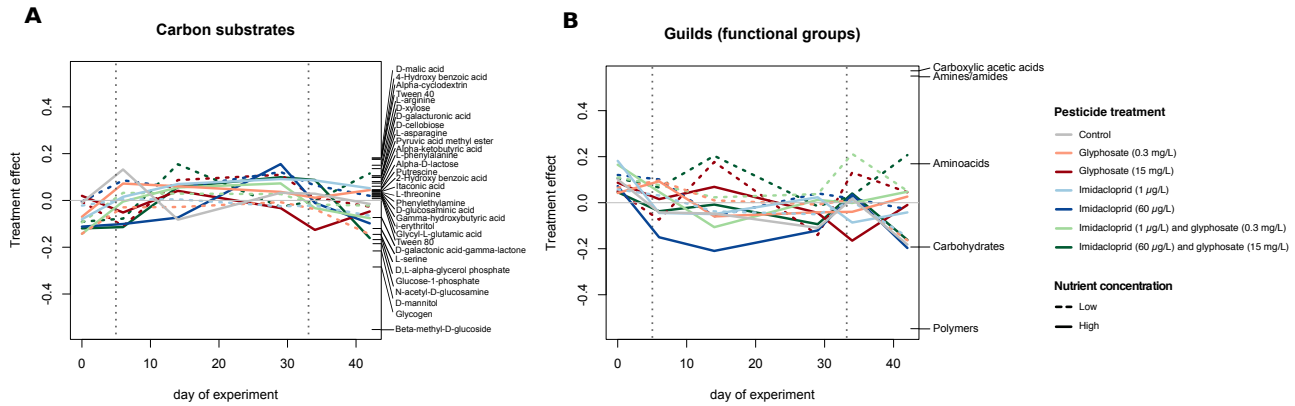


Figure 2. Bacterial cell density is unaffected by environmental stressors. Total bacterial cell counts (per uL) are plotted over time in ponds with low and high nutrient addition. Dashed vertical lines indicate pulse application dates. Ponds with both glyphosate and imidacloprid follow the same gradient pattern as treatments with either of these pesticides applied alone.

The GLMM with the best fit revealed that neither date (p=0.566), glyphosate concentration (p=0.887), imidacloprid concentration (p=0.982), TP (0.851), or TN (0.366) could explain the

326 variation in bacterial density. Stressors did not affect community carbon utilization patterns
327 either, as evidenced by the metabolic profiles characterized over time across experimental
328 treatments (**Fig. S1**). None of the stressors caused significant changes in the utilization of any of
329 the 31 unique carbon substrates tested (**Fig. 3A**). We grouped the 31 carbon compounds into
330 chemical guilds, such as carbohydrates, polymers, amino acids, and amines/amides [68,69], and
331 still did not find any differences in substrate use among treatments (**Fig. 3B, Fig. S1B and D**).
332 Overall, these results indicate that the tested environmental stressors did not significantly affect
333 bacterial density or community-level metabolic capabilities.
334



335
336 **Figure 3. Community metabolic profiles are unaffected by environmental stressors.**
337 Principal response curves (PRCs) of selected treatments show no significant difference between
338 controls and pesticide treatments when microbial communities are described according to (A)
339 their ability to metabolize 31 different carbon substrates, or (B) after grouping these compounds
340 based on carbon functional groups. Weights of each tested compound are shown along the right
341 Y axis. For comparison with the community structure PRCs (Fig. 5), the metabolic profiles PRCs
342 were calculated based on the subset of samples for which DNA was extracted.
343

344 *Glyphosate is the major driver of altered community composition*

345 We next asked whether environmental stressors affected bacterial community composition. We
346 used two complementary measures of community structure: (1) weighted UniFrac distances to

track changes in community composition, weighted for phylogenetic branch lengths and changes in relative abundance, and (2) Jensen-Shannon distances to assess changes in community composition at the finest possible resolution, tracking amplicon sequence variants (ASVs) regardless of their phylogenetic relatedness. We found that neither nutrients nor imidacloprid had a significant effect on either measure of community composition (PERMANOVA $P > 0.05$; **Table S1 and S2**), while glyphosate explained a significant amount of the variation in both measures (R^2 ranging from 0.29 to 0.54, depending on the time since glyphosate application; PERMANOVA $P < 0.05$; **Tables S1 and S2**). Interactions among treatments were not evident (**Tables S1 and S2**); it appears that glyphosate was the dominant driver of community changes, independent of the other stressors.

Community resilience to glyphosate at broad phylogenetic scales

To visualize changes in community composition according to treatments and over time, we used PCoA biplots based on both weighted UniFrac and Jensen-Shannon distances (**Fig. 4**). Glyphosate at the highest tested concentration (15 mg/l) was the main driver of community change regardless of nutrient background. We detected a recovery from this change after the first pulse of pesticides only when using weighted Unifrac (**Fig. 4A**), a metric that takes phylogenetic information into account, but not using Jensen-Shannon distance applied to ASVs (**Fig. 4B**). This suggests that, after the first pulse, the community recovers at a broad phylogenetic scale, with particular ASVs replaced with close but distinct phylogenetic relatives. As such, the community appears to be resilient at a broad phylogenetic scale, but not at a fine scale.

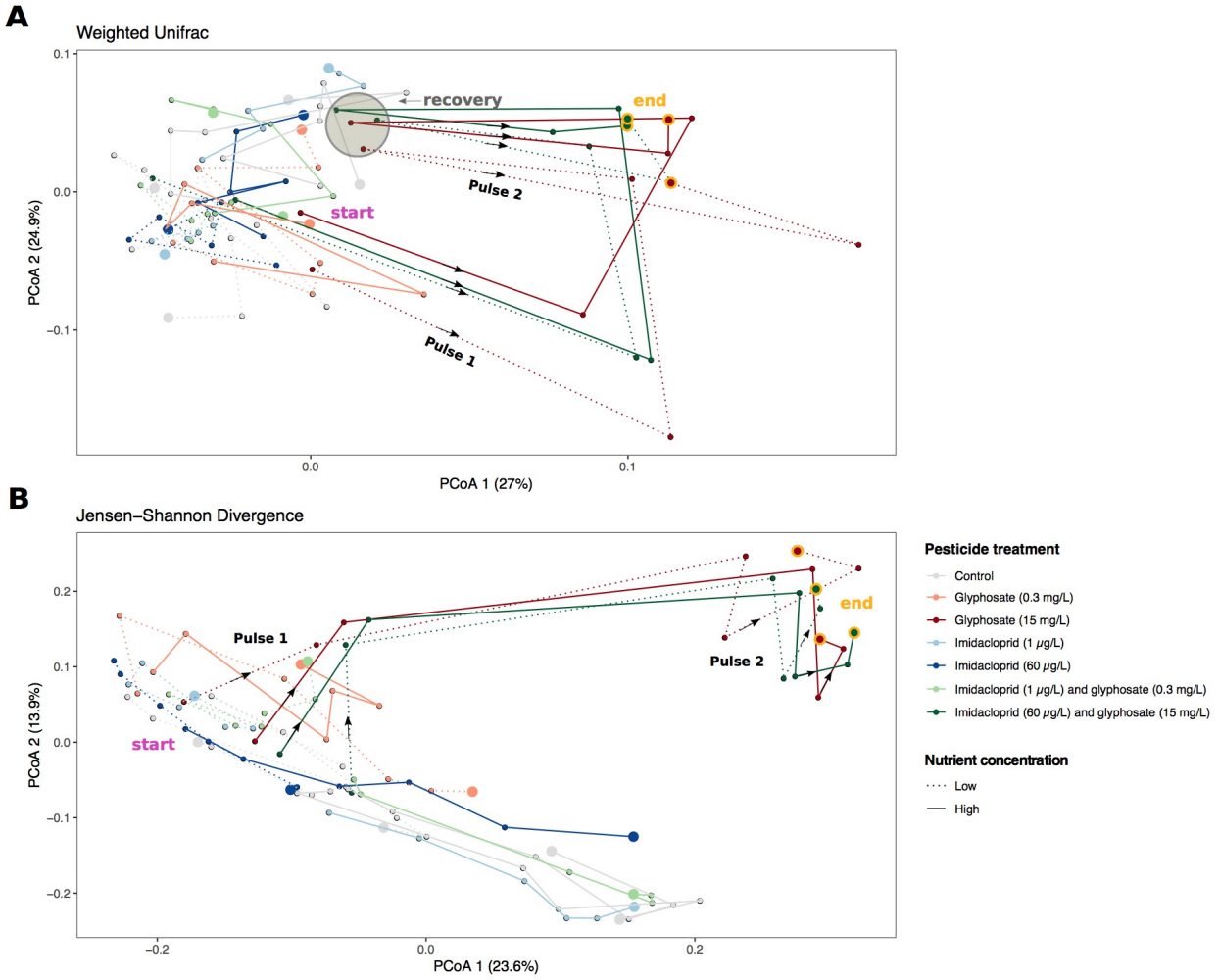


Figure 4. Community resilience to glyphosate stress at broad but not fine phylogenetic resolution. PCoA ordinations were built based on (A) weighted Unifrac distance or (B) Jensen-Shannon Divergence calculated on the estimated absolute abundance (EAA) matrix after DESeq2 normalization. The lines group each treatment time series respecting the chronological order (days of experiment) and to distinguish the first from the last day of experiment, the last time point is represented as a larger circle. The first and second pulse applications of glyphosate are indicated by arrows, which also show the direction of time.

To further assess how resilience varied with phylogenetic scale, we used PRCs to track community changes at the phylum and ASV level, as proxies for broad- and fine-scale phylogenetic structure, respectively. Given that nutrient inputs did not significantly affect

community composition (**Tables S1 and S2**), we built PRC models by combining ponds with the same pesticide treatment, irrespective of nutrient load (**Fig. 5**). This approach captured the same effects as PRCs considering all experimental treatments separately (**Fig. S2**), but facilitated the visualization of pesticide effects. PRCs captured a significant amount of the variation in community responses to nutrient treatments over time, with more variation explained at broader taxonomic levels compared to finer levels (**Table S3**). At the broadest taxonomic scale (phylum), communities showed a clear response to high (15 mg/l) but not low (0.3 mg/l) levels of glyphosate, followed by a recovery (similar to the community in control ponds) before the second pulse (**Fig. 5A**). No effect of imidacloprid was evident, and glyphosate was clearly the most influential treatment. A similar pattern of resilience (response and recovery) was also observed down to the genus level, with weaker recovery at progressively finer taxonomic scales (**Fig. S3**). The recovery was detected 24 days after the first pulse, but not after the second pulse; this does not exclude the possibility of an eventual recovery, but the experiment ended nine days after the second pulse, which was likely insufficient time to observe recovery. Notably, no recovery was observed at the ASV level (**Fig. 5B**), consistent with the community composition analysis (**Fig. 4B**). These results support the idea that high concentrations of glyphosate led to long-lasting community shifts at the ASV level, but community resilience at broader taxonomic or phylogenetic scales. In other words, some ASVs are effectively extirpated over the time frame of our experiment (and at the resolution of our sequencing depth) but are replaced with close relatives such that phylogenetic community composition is maintained.

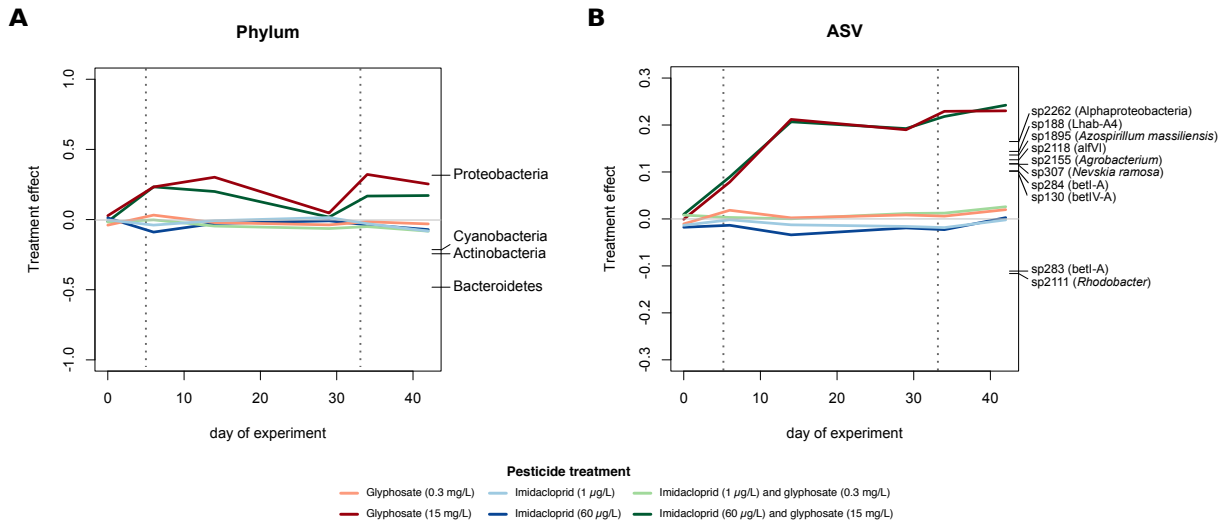


Figure 5. Community resilience to glyphosate at the phylum level but not at the ASV level. Principal response curves (PRCs) show the effect of pesticide treatments over time relative to control ponds at (A) the phylum level or (B) the ASV level. Dashed vertical lines indicate the dates of pesticide application. Only phyla with weights >0.2 and ASVs with weight >0.1 are plotted on the right Y axis to facilitate visualization. The finest available annotated level of taxonomic assignment of each ASV is indicated in panel B. Low and high nutrient treatments were grouped together for clarity, but follow the same pattern when considered separately (Fig. S2).

Agrobacterium *and other proteobacteria are most affected by glyphosate*

To understand which bacterial taxa were most affected by the experimental treatments, we explored their respective weights (also known as species scores; [65]) in each PRC. Proteobacteria was the phylum most positively affected by glyphosate, with a weight of 0.37 along the first PRC axis (**Fig 5A; Table S4**), while the average weight was -0.04. Bacteroidetes, Actinobacteria and Cyanobacteria were the most negatively affected phyla (**Fig. 5A**), with weights of -0.43, -0.26 and -0.24, respectively (**Table S4**). All ten ASVs with absolute weights >0.1 belonged to the phylum Proteobacteria, and except for sp283 and sp2111, they were all positively affected by glyphosate treatment (**Fig. 5B; Table S5**). These ASVs include

Agrobacterium, a genus of Rhizobiaceae of which some members have been shown to use glyphosate as a sole source of phosphorus [70] and to express the *aroA* class II gene, which encodes glyphosate resistance [71]. The positive effects of glyphosate on the order Rhizobiales, the family Rhizobiaceae, and the genus *Agrobacterium* (**Fig. S3**) all further suggest a competitive advantage of members of this lineage under glyphosate stress.

Discussion

The herbicide glyphosate and the insecticide imidacloprid can affect community structure at different trophic levels in a variety of aquatic environments and experimental setups [72–76], but until now their interactive effects on bacterioplankton have been poorly studied. To assess possible interactive effects, we tested how imidacloprid and glyphosate affected bacterial communities of aquatic mesocosms receiving different nutrient inputs in the Large Experimental Array of Ponds (LEAP). We found that glyphosate significantly affected bacterial community composition only at the highest tested concentration (15 mg/l), with no evident interaction with imidacloprid or nutrient load. These results suggest that most bacteria originating from the pristine source environment (Lake Hertel, a mountaintop lake in a nature preserve) were already resistant to these stressors across a wide range of concentrations, and that ecotoxicological criteria (environmental concentrations considered "safe for aquatic life") intended for eukaryotes were adequate to preserve bacterioplankton diversity, density, and metabolic functions.

Despite the marked changes in taxonomic composition driven by glyphosate, the bacterial communities did not change in terms of metabolic profiles throughout the experiment, providing evidence for functional redundancy in carbon utilization potential. This result is consistent with broad-scale ecosystem functions such as respiration and dissolved organic carbon consumption being weakly coupled with microbial species composition [77–79], allowing these functions to remain unaffected by fluctuations in community composition [6].

Our results suggest two mechanisms of stability at play in microbial communities: resistance to imidacloprid, glyphosate, nutrient loading, and their interactions when assessing changes in

453 functional capacities (overall bacterial density and carbon utilization patterns), and resilience in
454 community composition following glyphosate disturbance. The recovery in community
455 composition was only evident by grouping ASVs at more inclusive taxonomic or phylogenetic
456 levels. Glyphosate thus drove a turnover of ASVs which, even after the recovery, are different
457 from the ASVs initially found in the undisturbed community.
458
459 Glyphosate treatments had a strong positive effect on the phylum Proteobacteria, previously
460 found to be positively associated with high concentrations of glyphosate in rhizosphere-
461 associated communities [80] and phytoplankton-associated communities [81]. Multiple species
462 of Proteobacteria can use glyphosate as a source of phosphorus by breaking its C-P bond [38].
463 We identified the Rhizobiaceae *Agrobacterium* – a genus containing species known to be able to
464 degrade glyphosate [38] – as being favoured in the glyphosate treatment in our experiment. The
465 ability to degrade glyphosate may be widespread in the family Rhizobiaceae [70], and
466 *Agrobacterium* have also been found to encode glyphosate-resistant EPSPS genes [37,82]. Thus,
467 while glyphosate may be a stressor for the community at large (*e.g.*, phytoplankton [45]), it may
468 be a resource for some members of *Agrobacterium*. Further genomic and metagenomic analyses
469 of our experimental samples could reveal whether these ecological dynamics are underlain by
470 evolutionary adaptation, and whether community resistance and resilience can be explained by
471 the initial presence of resistant bacteria in the community, or to *de novo* mutations or gene
472 transfer events.
473
474 Together, our data provide evidence of natural resistance to two of the most commonly used
475 pesticides in the world, the herbicide glyphosate and the neonicotinoid insecticide imidacloprid.

We also showed resilience to high doses of glyphosate in bacterial communities that have no known history of contact with the herbicide, as the source water from Lake Hertel is unaffected by agricultural runoff. Finally, our results suggest that whether a stressed community is considered resilient may depend on the phylogenetic depth of the traits required to deal with stress [83], such as pesticide degradation or resistance of the target enzyme.

Mesocosm experiments are incomplete representations of natural ecosystems [84], but they allow us to manipulate and replicate the exposure of complex lake bacterial communities to agricultural chemical pollutants commonly found in freshwaters. Pesticides could have driven changes in bacterial community via direct or indirect mechanisms, such as direct effects on other trophic levels that cascaded down to bacteria via predation or other interactions. In a previous study of the same experiment described here, we found that glyphosate treatment reduced the diversity of eukaryotic phytoplankton, but did not significantly change phytoplankton community composition [45]. It therefore seems unlikely that the dramatic changes in bacterial community composition were driven by phytoplankton. On the contrary, bacterial degradation of glyphosate likely released bioavailable phosphorus, stimulating phytoplankton growth [45]. Further studies will be needed to disentangle how the effects of these stressors cascade through food webs, and how trophic structure influences their effects [85]. As we show, bacterial communities are broadly resistant and resilient to common agricultural pollutants, but it is still likely that pollutants select for resistant bacteria at the sub-genus level [86,87], which can potentially pass on resistance genes to organisms from other trophic levels [88,89].

Data availability

Sequence data corresponding to raw 16S rRNA reads and metadata are available on https://figshare.com/projects/LEAP_MiSeq_fastq/78297.

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Author contribution

N.B.C., V.F., M.-P.H., R.D.H.B., B.E.B., G.B., G.F.F., B.J.S. and A.G. designed the study. N.B.C., V.F. and M.-P.H. collected the data. N.B.C., C.C.Y.X. and V. Y. contributed to the development of laboratory methods. N.B.C. and V.F. analysed data. N.B.C. made the figures and

drafted the manuscript. N.B.C. and B.J.S. wrote the first manuscript draft and all authors contributed significantly to data interpretation and commented on manuscript drafts.

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