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1 2	Glyphosate-degrading behaviour of five bacterial strains isolated from stream biofilms
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30	Running head: Glyphosate as phosphorus source
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32	Keywords: herbicide, AMPA, sarcosine, degradation, C-P lyase, periphyton.
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#### 34 SUMMARY

The present study investigates the individual degrading behaviour of bacterial strains isolated 35 from glyphosate-degrading stream biofilms. In this aim, biofilms were subjected to 36 enrichment experiments using glyphosate or its metabolite AMPA (aminomethyl phosphonic 37 acid) as the sole phosphorus source. Five bacterial strains were isolated and taxonomically 38 39 affiliated to Ensifer sp. CNII15, Acidovorax sp. CNI26, Agrobacterium tumefaciens CNI28, Novosphingobium sp. CNI35 and Ochrobactrum pituitosum CNI52. All strains were capable 40 41 of completely dissipating glyphosate after 125 to 400 h and AMPA after 30 to 120 h, except for Ensifer sp. CNII15 that was not able to dissipate glyphosate but entirely dissipated AMPA 42 43 after 200 h. AMPA dissipation was overall faster than glyphosate dissipation. The five strains degraded AMPA completely since formaldehyde and/or glycine accumulation was observed. 44 During glyphosate degradation, the strain CNI26 used the C-P lyase degradation pathway 45 since sarcosine was quantitatively produced, and C-P lyase gene expression was enhanced 46 30× compared to the control treatment. However, strains CNI28, CNI35 and CNI52 47 accumulated both formaldehyde and glycine after glyphosate transformation suggesting that 48 both C-P lyase and/or glyphosate oxidase degradation pathways took place. Our study shows 49 different and complementary glyphosate degradation pathways for bacteria co-existing in 50 51 stream biofilms.

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#### 53 1. INTRODUCTION

Streams and rivers account for the most impacted ecosystems on the planet since they have 54 been heavily exploited by humans and often used as final receptors of pollution (Meybec 55 2003). Urbanization, industry, land-use changes and watercourse alterations are the main 56 proximate causes at the origin of river ecosystems changes which results in physical habitats 57 and water chemistry alterations, and shifts in aquatic biodiversity, among other consequences 58 (Malmqvist and Rundle 2002). Alterations in water chemistry have existed for millennia in 59 streams and rivers (Meybeck and Helmer 1989) and the increasing global chemical pollution 60 61 of surface waters with largely unknown short and long-term effects on aquatic life and on human health is one of the key problems facing humanity (Schwarzenbach et al. 2010). 62 63 Therefore, the necessity of limiting threats of problematic chemicals at their source instead of costly remediation processes have been widely proposed in the scientific literature 64

(Vörösmarty *et al.* 2010, Schwarzenbach *et al.* 2010) and suggested in national and
international policies (Ecophyto II+ 2018, Lamichhane *et al.* 2016, Directive 2009/128/EC).
Because biofilms constitute a highly reactive surface area for the sorption and metabolism of
contaminants in running waters (Araya *et al.* 2003, Lawrence *et al.* 2001, Tien *et al.* 2013),
their study to alleviate specific water pollution problems becomes a potential solution.
However, few studies have investigated the natural potential of aquatic biofilms for the
removal of chemicals (*e.g.* Wang *et al.* 2016b, Carles *et al.* 2019).

72 Glyphosate (N-[phosphonomethyl] glycine) has become the most commonly used 73 herbicide worldwide (Benbrook 2016, Duke and Powles 2008) with about 600 to 750 74 thousand tonnes used annually in the world and an expected 740 to 920 thousand tonnes to be 75 used by 2025 (Maggi et al. 2020). The potential of glyphosate retention in topsoil layers is high due to its strong sorption on clay, iron and aluminium oxide particles (Borggaard and 76 77 Gimsing 2008, Okada et al. 2016, Rampazzo et al. 2013, Sidoli et al. 2016, Vereecken 2005). Consequently, the immediate leaching of aqueous glyphosate residue is relatively low. 78 79 However, 98 % of residues adsorbed onto these soil minerals are prone to remobilize if specific agricultural practices are applied (i.e. tillage and ploughing in combination with 80 81 irrigation and soil pH variations, Maggi et al. 2020). Yang et al. (2015) showed that up to 14 % of applied glyphosate is transported by runoff and suspended load from clay loam soil with 82 a rain intensity of 1 mm/min for 1 h. Therefore, glyphosate and AMPA can reach streams and 83 rivers through chronic and/or peak exposure events, the latter occurring over hours to a few 84 days depending on the intensity and duration of rain events (Daouk et al. 2013). Glyphosate 85 and AMPA concentrations during peaks can reach up to hundreds of micrograms per litre, 86 although basal concentrations rarely exceed a few micrograms per litre (Battaglin et al. 2014, 87 Carles et al. 2019). Also, it must be noted that other sources of AMPA such as detergents, fire 88 89 retardants, anti-corrosives and anti-scaling agents can enter streams and rivers through 90 wastewater treatment plant effluents (Grandcoin et al. 2017, Nowack 2003, Struger et al. 2015, Studnik et al. 2015). Generalized exposure to glyphosate makes aquatic microbial 91 92 communities in sediments prone to acquire tolerance (Bonnineau, unpublished data) and develop transformation and/or mineralization capacities in periphyton (Carles et al. 2019, 93 94 Artigas et al. 2020).

Glyphosate degradation is essentially driven by microorganisms (Mallat and Barceló,
1998). Up to now, two main biodegradation pathways have been described (Figure 1): i) the
glyphosate oxidase pathway (or AMPA pathway), which involves the cleavage of the C-N
bond of the glyphosate molecule by glyphosate oxidoreductase or glycine oxidase enzymes

(+FAD), releasing both AMPA and glyoxylate. Glyoxylate is further metabolized into glycine 99 or via the glyoxylate cycle into microbial biomass and carbon dioxide (Borggaard and 100 Gimsing 2008), while AMPA can be degraded by C-P lyase enzymes, releasing inorganic 101 phosphorus and methylamine, the latter being degraded into formaldehyde and incorporated 102 into microbial biomass. And ii) the C-P lyase pathway (or sarcosine pathway), which involves 103 the cleavage of the C-P bond of the glyphosate molecule by C-P lyase enzymes (encoded by 104 the phn operon, Hove-Jensen et al. 2014), releasing both inorganic phosphate and sarcosine. 105 Sarcosine may further degrade into glycine and formaldehyde by sarcosine oxidase to finally 106 107 incorporate the microbial biomass. The prevalent glyphosate degradation pathway employed by bacteria is the sarcosine pathway (13 strains) followed by the AMPA pathway (8 strains) 108 and some strains are able to use both AMPA and sarcosine pathways (4 strains) (Zhan et al. 109 2018). In addition, molecular dynamics simulation studies confirmed the stability of the 110 111 enzyme-substrate complex for glyphosate oxidoreductase and C-P lyase demonstrating that both enzymes could be preferred in glyphosate biodegradation (Bhatt et al. 2021). 112

113



**Figure 1**. Glyphosate and AMPA degradation pathways in the environment.

The occurrence of either of these pathways in aquatic systems is still unknown, though 116 projections indicate that the oxidative pathway prevails in soils worldwide (Maggi et al. 117 2020). Up to now, most of the bacterial strains studied are capable to convert glyphosate to 118 AMPA (Pseudomonas sp. SG-1 (Talbot et al. 1984), Agrobacterium radiobacter SW9 119 (Mcauliffe et al. 1990) and Ochrobactrum anthropi GPK 3 (Sviridov et al. 2012)), even in 120 bacteria that have never been exposed to glyphosate previously, possibly highlighting 121 widespread detoxification mechanisms (Sviridov et al. 2015). Furthermore, the C-P bond has 122 been reported to be cleaved by several other strains (Achromobacter sp. MPS 12A (Sviridov 123 124 et al. 2012) and Arthrobacter sp. GLP-1 (Pipke et al. 1987)). This highlights the ability of microbes to use both glyphosate and AMPA as a carbon (C) and phosphorus (P) source for 125 126 their metabolism, although this has been shown to be closely linked with dissolved nutrient 127 availability in water (Carles et al. 2019, Hove-Jensen et al. 2014, Sviridov et al. 2014, Zhang 128 and van der Donk 2012, Bazot and Lebeau 2008). However, it must be noted that most of the knowledge so far comes from bacteria isolated from soils and activated sludge while few 129 130 studies have investigated glyphosate degradation pathways by bacteria in streams and rivers (Zhan et al. 2018, Jacob et al. 1988). 131

In order to improve our knowledge on glyphosate and AMPA biodegradation potential 132 in streams, we used glyphosate-degrading biofilms (Carles et al. 2019) in enrichment cultures 133 using glyphosate as the sole P source. Five bacterial strains were isolated and their ability to 134 use glyphosate and AMPA as P source investigated. Each strain was tested separately for 135 glyphosate and AMPA degradation and compared to a control supplemented with K<sub>2</sub>HPO<sub>4</sub>. 136 Glyphosate and its metabolites (AMPA, sarcosine, formaldehyde and glycine) were analysed 137 during experiments in order to identify the degradation pathway(s) used by each strain. 138 Because our study is the first literature report of an Acidovorax sp. strain capable to degrade 139 glyphosate, we focused on the molecular mechanisms of the strain Acidovorax CNI26 140 responsible for glyphosate/AMPA degradation. We first hypothesize faster degradation of 141 AMPA than glyphosate since AMPA is easier to degrade and less toxic for aquatic 142 143 microorganisms compared to glyphosate. Accordingly, the biomass growth of strains will also be faster with AMPA than glyphosate. Second, the Acidovorax CNI26 strain is expected to 144 145 rapidly overexpress the C-P lyase gene in the presence of AMPA, while overexpression on both C-P lyase and glyphosate oxidase genes can be displayed in the presence of glyphosate. 146 The expression of these two genes will depend on glyphosate and AMPA concentration 147 variation in the media throughout the experiment. The objectives of this study were i) to 148 149 analyse the glyphosate and AMPA-degrading behaviour of five bacterial strains isolated from

stream biofilms and ii) to investigate the pathway employed by the strain *Acidovorax* CNI26
to degrade glyphosate and AMPA by targeting the C-P lyase and glyphosate oxidase genes.

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#### 153 2. MATERIALS AND METHODS

154 2.1 Chemicals and media

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Glyphosate (N-[phosphonomethyl] glycine (purity  $\geq$  98 %)), AMPA (aminomethyl 156 phosphonic acid (purity  $\geq$  99 %)) and K<sub>2</sub>HPO<sub>4</sub> (purity  $\geq$  98 %) were purchased from Sigma 157 Aldrich (France). Acid phenol, TRIzol<sup>®</sup> and TURBO DNAse were purchased from 158 ThermoFisher Scientific (France). MOPS<sup>CN</sup> medium (P-free), pH 7.4, was made up of: 40 159 mM MOPS (3-(N-Morpholino)propanesulfonic acid), 4 mM tricine, 10 µM FeSO<sub>4</sub>, 276 µM 160 K<sub>2</sub>SO<sub>4</sub>, 0.5 µM CaCl<sub>2</sub>, 525 µM MgCl<sub>2</sub>, 50 mM NaCl, 30 mM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O, 4 µM 161 H<sub>3</sub>BO<sub>3</sub>, 0.3 µM CoCl<sub>2</sub>.6H<sub>2</sub>O, 0.1 µM CuSO<sub>4</sub>.5H<sub>2</sub>O, 0.8 µM MnCl<sub>2</sub>.4H<sub>2</sub>O, 0.1 µM 162 ZnSO<sub>4</sub>.7H<sub>2</sub>O, 0.3 µM Thiamine HCl, 22 mM Glucose, 9.5 mM NH<sub>4</sub>Cl. MOPS<sup>CN</sup>agar plates 163 were prepared by adding 1.5 % (w/v) agar into the liquid media. 164

165

166 2.2 Enrichment cultures and isolation of bacteria

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Glyphosate- and AMPA-enrichment cultures as described by Batisson et al. (2009) were run 168 with stream biofilms from Carles et al. (2019). From this experiment, we selected river 169 biofilms (namely, Ups\_LowP\_LowG and Dws\_LowP\_HighG), having proven their strong 170 capacity to dissipate glyphosate without AMPA accumulation in the media, for subsequent 171 isolation of glyphosate/AMPA-degrading strains. Biofilms were scraped, pooled, and 172 173 suspended in 2 mL of sterile 0.8 % NaCl solution. Biofilm suspensions were then centrifuged (13,000 g, 10 min at 4 °C), and the pellet was washed three times with 2 mL of sterile 0.8 % 174 NaCl solution in order to remove glyphosate or AMPA residues. Biofilm pellets were 175 suspended in 2 mL of sterile 0.8 % NaCl solution and homogenised. This biofilm suspension 176 was used as the inoculum for enrichment cultures. 177

A volume of 125  $\mu$ L of each biofilm suspension was added to 12.5 mL of MOPS<sup>CN</sup> media supplemented with glyphosate (0.5 mM) in 50 mL flasks. Phosphorus contained in the glyphosate molecule was the only source of P in the culture media. Two additional enrichment steps were carried out every 3 weeks by sub-culturing 125  $\mu$ L of the culture into

12.5 mL of fresh medium containing 2 and 4 mM of glyphosate, respectively. During the 182 enrichment culture steps, the flasks were incubated at room temperature in an orbital shaker at 183 100 rpm in the dark. The cultures were sampled at the beginning (day 0) and the end (day 21) 184 of each enrichment step to determine glyphosate concentration (see details in section 2.4). At 185 the end of the third enrichment step, cultures were spread for bacterial isolation on MOPS<sup>CN</sup> 186 agar plates containing the same glyphosate concentrations as in liquid MOPS<sup>CN</sup> (4 mM). Pure 187 colonies were tested separately for their capacity to transform glyphosate and AMPA in liquid 188 MOPS<sup>CN</sup> media containing 0.5 mM of glyphosate. Liquid cultures were incubated and 189 sampled for glyphosate and AMPA concentration determination at the same incubation 190 conditions described above. Among the isolates, 5 pure strains degrading glyphosate and/or 191 AMPA in MOPS<sup>CN</sup> medium were selected for further chemical and molecular 192 characterization. 193

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195 2.3 Identification and phylogenetic analysis of bacteria

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The identification of the 5 strains degrading glyphosate and/or AMPA was carried out by 16S
rDNA gene sequencing as described by Batisson *et al.* (2009). The sequences were deposited
in GenBank under the accession numbers MN017826, MN017827, MN017828, MN017829
and MN017830, corresponding to isolates CNII15, CNI26, CNI28, CNI35 and CNI52,
respectively.

202 Phylogenetic analysis based on 16S rDNA gene sequences was carried out by multiple 203 alignments (Clustal  $\Omega$ ) followed by the construction of a phylogenetic tree (MEGA 6.0 204 software). In addition to the 16S rDNA sequences of the 5 newly isolated strains (present 205 study), sequences from previously isolated glyphosate-degrading strains were also included in 206 the analysis. Only one sequence per genus was included in the analysis, either the 16S rDNA 207 gene sequence of the glyphosate-degrading strain described, or the sequence of a 208 corresponding genus/species if the 16S rDNA degrading strain sequence was not available.

- 209
- 210 2.4 Biodegradation tests with bacteria
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The use of glyphosate, AMPA and  $K_2HPO_4$  as P sources by the five strains was determined separately. These strains were inoculated at 10<sup>5</sup> cell mL<sup>-1</sup> in 120 mL of MOPS<sup>CN</sup> liquid media supplemented with 0.132 mM of P-equivalents of glyphosate, AMPA or  $K_2HPO_4$  in 225mL

polyethylene flasks. Non-inoculated media served as abiotic controls. Each experiment was 215 carried out in triplicate. The cultures were incubated in the dark at room temperature and 100 216 rpm agitation for 2 weeks. Six samplings were performed ( $6 \times 1$  mL) to monitor strain growth 217 (Optical Density  $OD_{\lambda} = 600 \text{ nm}$ ) and variation in concentrations of glyphosate, AMPA, K<sub>2</sub>HPO<sub>4</sub>, 218 sarcosine, formaldehyde and glycine over time. The same experiment was repeated later on, 219 in the exact same conditions and 5 mL of culture was recovered to assess gene expression 220 kinetics of C-P lyase and glyphosate oxidase in the CNI26 strain. All samples were kept 221 frozen at -20 °C before glyphosate and glyphosate-metabolite concentration analysis. 222

223

224 Bacterial Abundance: Bacterial abundance during biodegradation tests was estimated in triplicate using a conversion factor obtained from the correspondence curves between optical 225 density at 600 nm and cell counting on flow cytometry. For each of the five bacterial strains, 226 correspondence curves were obtained by following the growth of one colony in 10 mL of 227 MOPS<sup>CN</sup> liquid media supplemented with 0.132 mM of K<sub>2</sub>HPO<sub>4</sub> every 24 hours during one 228 week. Samples were first measured for  $OD_{600}$  and then fixed with formaldehyde (2 %, final 229 230 concentration) before flow cytometry analysis. Twenty-five microliters of fixed bacterial suspension was diluted 10-fold in TE buffer (10 mM Tris, 1 mM EDTA) and stained with 2.5 231 232 µL of SYBR Green I before counting bacterial cells with a BD FACSCalibur flow cytometer (15 mW at 488 nm, Becton Dickinson, USA). Correspondences obtained were 1 AU =  $5 \times 10^8$ 233 cell mL<sup>-1</sup> for CNI15,  $2.8 \times 10^8$  cell mL<sup>-1</sup> for CNI26,  $4.2 \times 10^8$  cell mL<sup>-1</sup> for CNI28,  $5 \times 10^8$  cell 234 mL<sup>-1</sup> for CNI35 and  $8.2 \times 10^8$  cell mL<sup>-1</sup> for CNI52. 235

Glyphosate and metabolites concentration: Glyphosate and AMPA concentrations were determined in triplicate from 1 mL of culture media following the method of Wang *et al.* (2016a) with some modifications. Briefly, standards of glyphosate and AMPA (0-100  $\mu$ M) prepared on MOPS<sup>CN</sup> liquid media and culture samples were derivatized with

9-fluorenylmethylchloroformate (FMOC-CI) and analysed by HPLC (Waters Inc., MA, 241 U.S.A) coupled with a Diode Array Detector (DAD) detector set at  $\lambda = 265$  nm, and a reverse 242 243 phase column (Phenomenex Kinetex EVO C18, 5 µm, 150 x 4.6 mm) at 22 °C. The mobile phase was made up of 5 mM ammonium acetate pH 9 (Solvent A) and methanol (Solvent B) 244 at a flow rate of 1 mL min<sup>-1</sup>, linear gradient 0–5 min: 80 % A; 5–11 min: 80-30 % A; 11– 245 16 min: 30 % A; 16–20 min: 30-80 % A; 20–30 min: 80 % A. Glyphosate and AMPA were 246 detected at 2.8 and 12.4 minutes retention time. Sample taken in triplicate were injected twice. 247 Glyphosate and AMPA quantification in the strain's biomass was measured at the end of 248 249 experiments following the method described in Carles et al. (2019).

250 Sarcosine, glycine and formaldehyde (MAK073-1KT, MAK261-1KT and MAK131-251 1KT, respectively) concentrations were determined fluorometrically in triplicate from 1 mL of culture media using assay kits purchased from Sigma Aldrich and following the 252 253 manufacturer's recommendations. Measurements were performed on a Spark 10M (Tecan, Switzerland) multimode microplate reader. Excitation/emission wavelengths were set up to 254 255 535/587 nm for sarcosine and glycine concentration determination and 370/470 nm for 256 formaldehyde concentration determination. Glycine concentration was also determined in K<sub>2</sub>HPO<sub>4</sub> and AMPA treatments as background control since glycine is a common metabolite 257 produced by bacteria. The orthophosphate ( $PO_4^{3-}$ ) concentration was determined in triplicate 258 from 1 mL of culture media using a spectrophotometer (890 nm, Genesys<sup>TM</sup> 20, 259 ThermoSpectronic, NY, USA) according to the protocol of Murphy and Riley (1962). 260

261

C-P lyase and glyphosate oxidase genes in CNI26: The genome of the strain CNI26 was 262 sequenced and the diversity of C-P lyase and glyphosate oxidase genes was analysed to 263 design specific primers for further gene expression experiments. The shotgun genomic library 264 265 of CNI26 was prepared with the Hyper Library construction kit from Kapa Biosystems 266 (Roche). The library was quantified by qPCR and sequenced on one MiSeq Micro flow cell for 251 cycles from each end of the fragments using a MiSeq 500-cycle sequencing kit 267 version 2. The program used to perform de-novo genome assembly was Spades, version 268 3.11.1. The size of the assembly was 5.75Mb which means that the approx. genome coverage 269 was 270×. The assembly was fragmented in 89 scaffolds. We took the longest scaffold (700 270

kb) and compared it to the closest sequences retrieved in GenBank database using BLAST. 271 The top hit was CP003872.1 Acidovorax sp. KKS102. The genome assembly was annotated 272 with Prokka v 1.3. Annotation results reveal the presence of one glycine oxidase gene copy 273 and two C-P lyase pathway-encoding operons including genes for: transport proteins 274 (phnCDE), known enzymatic activity in phosphonate catabolism (phnIJMLN), auxiliary 275 polypeptides involved in phosphonates catabolism (phnGHKL) (Hove-Jensen et al. 2014). 276 The qPCR primers were specifically designed in this study for the strain Acidovorax sp. 277 CNI26 using Primer3Plus (Untergasser et al. 2007). The primers used were CP-lyasesF (5'-278 CCA CCC YTT TGA RGT GCA GC-3') and CP-lyasesR (5'-CCT GGC AAT AGT CSG 279 AG-3') for the C-P lyase gene expression and GlyoxF (5'-CTA CAT CGC GCC CAA ACA 280 AG-3') and GlyoxR (5' ATT GTG TGG CCA GCT CCA G 3') for glycine oxidase 281 expression. 282

283 RT-qPCR analysis on glyphosate-degrading genes was performed using total RNA extracted from strain CNI26 exposed to glyphosate, AMPA and K<sub>2</sub>HPO<sub>4</sub> as P source in 284 285 triplicate. RNA samples were taken at the middle and the end of the exponential decay phases of glyphosate (100 and 124 hours) and AMPA (36 and 48 hours) dissipation curves observed 286 287 in the previous biodegradation experiment. Five millilitres of the CNI26 bacterial culture were quickly sampled from each experimental condition, mixed with RNAlater® solution (1:1 v:v) 288 and stored at 4 °C until RNA extraction. Before RNA extraction, bacteria were washed twice 289 with 1X PBS. Total RNA was extracted according to the method described by Toledo-Arana 290 et al. (2009). Briefly, bacteria were mechanically lysed with the PreCellys 24 system (Bertin 291 Technologies, Montigny le Bretonneux, France) at a speed of 6.5 m s<sup>-1</sup> for two consecutive 292 cycles of 30 s. After acid phenol and TRIzol® extraction, total RNA was precipitated with 293 isopropanol and treated with 10 units of TURBO DNase. After a second phenol-chloroform 294 extraction and ethanol precipitation, RNA pellets were suspended in DEPC-treated water. The 295 296 efficiency of the DNase treatment was checked by PCR using the 16S BAC338f/515r primers (Borrel et al. 2012). RNA concentration in the extracts was quantified with the Qubit system 297 (Thermo Fisher Scientific). Extracted RNA was then converted into cDNA with the 298 SuperScript III Reverse Transcriptase kit (Invitrogen, USA) using Random Primers 299 (Invitrogen) and according to the manufacturer's instructions. qPCR was performed in a 300 CFX96 touch Real-Time PCR detection system (BioRad, USA) using the MESA GREEN 301 qPCR Master Mix Plus kit (Eurogentec) coupled with white qPCR96-well plates (Eurogentec) 302 in a final volume of 15 µL and using the C-P lyase and glycine oxidase primers described 303 above. The qPCR conditions were as follows: initial denaturation at 95 °C for 5 min, 40 304

cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 15 s and elongation at
72 °C for 20 s. C-P lyase and glycine oxidase gene expressions were normalized using the 16S
rRNA (BAC338f/515r) as the housekeeping gene.

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309 2.5 Data treatment and statistical analyses

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Bacterial growth during biodegradation experiments for each of the 5 isolated strains wasfitted to the Gompertz growth model (SGompertz) according to the following equation:

313

$$Bt = Maxe^{-e^{(-mg(x-Max50))}}$$

314

Where *Bt* is the bacterial density (in cells mL<sup>-1</sup>) at time *t*, *Max* is the maximum amplitude of the curve (cells mL<sup>-1</sup>),  $Max_{50}$  is the time required (hours) for bacterial density to increase 50 % of the initial value and  $m_g$  is the growth rate coefficient (hours<sup>-1</sup>). Direct comparisons between treatments (glyphosate, AMPA) and control (K<sub>2</sub>HPO<sub>4</sub>) for the *Max*<sub>50</sub> parameter was achieved by the ratio  $Max_{50}$  treatment/ $Max_{50}$  control calculation. A ratio value < 1 indicates lower  $Max_{50}$  values in the treatment (faster growth) than in the control, whereas a ratio > 1 indicates higher  $Max_{50}$  values in the treatment (slower growth) than in the control.

Dissipation kinetics of glyphosate, AMPA and K<sub>2</sub>HPO<sub>4</sub> molecules in the media during biodegradation experiments were fitted to a dose-response sigmoidal model (DosResp), according to the following equation:

325

$$Ct = Cf + \frac{Ci - Cf}{1 + 10^{(DT50 - x)md}}$$

326

where Ct is the molecule concentration ( $\mu$ M) at time t, Ci the initial concentration of the 327 molecule ( $\mu$ M), Cf the final concentration of the molecule ( $\mu$ M), DT<sub>50</sub> the time required to 328 decrease the concentration of the molecule by 50 % of its initial concentration value (hours) 329 and  $m_d$  the dissipation rate coefficient (hours<sup>-1</sup>). Direct comparison between treatments 330 (glyphosate and AMPA) and control ( $K_2$ HPO<sub>4</sub>) for the  $DT_{50}$  parameter was achieved by the 331 ratio  $DT_{50}$ \_treatment/ $DT_{50}$  control calculation. A ratio < 1 indicates lower  $DT_{50}$  values in the 332 treatment (faster dissipation) than in the control, whereas a ratio > 1 indicates higher  $DT_{50}$ 333 values in the treatment (slower dissipation) than in the control. 334

All fittings were performed using the OriginPro 2016 software (Origin Lab Corporation,USA).

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Statistical differences related to the bacterial growth kinetics parameters (*Max, Max<sub>50</sub>* and  $m_g$ ) or glyphosate, AMPA and K<sub>2</sub>HPO<sub>4</sub> dissipation kinetics parameters (*DT<sub>50</sub>* and  $m_d$ ) among the five isolated bacterial strains were assessed using multiple Kruskal-Wallis tests followed by separate pairwise comparison tests using the Tukey's contrast. The factors tested were strain (CNII15, CNI26, CNI28, CNI35 and CNI52), P-source (glyphosate, AMPA and K<sub>2</sub>HPO<sub>4</sub>) and their interaction (strain × P source).

C-P lyase and glyphosate oxidase gene expression was expressed as the relative quantity of
copies of genes in each of the P-source treatments (glyphosate, AMPA and K<sub>2</sub>HPO<sub>4</sub>)
following the model of Pfaffl (2001):

347

Relative quantity 
$$= \frac{E^{-CqS1Gi}}{E^{-CqS1GR}}$$

348

where *E* is the primer efficiency, CqSiGi is the threshold cycle for the gene of interest (i.e. C-P lyase or glycine oxidase) and CqSiGR is the threshold cycle for the housekeeping gene (16S). Statistical differences between sampling times within glyphosate and AMPA treatments were performed for each gene separately using the Kruskal-Wallis test.

All the statistical analyses were computed using the R software Version 4.0.3 at the alpha threshold of 0.05.

355

356

357 **3. RESULTS** 

### 359 *3.1 Bacterial growth and taxonomic affiliation*

360

Most of the strains were able to grow in the presence of K<sub>2</sub>HPO<sub>4</sub>, glyphosate or AMPA as 361 unique P source, except CNII15 in the presence of glyphosate (Table 1, Figure S1). P 362 treatments had a significant effect on the growth dynamics of bacterial strains; the lowest time 363 required to increase bacterial density by 50 % (Max<sub>50</sub>) was observed with K<sub>2</sub>HPO<sub>4</sub> (48.3  $\pm$  5.3 364 days on average for the 5 strains), followed by AMPA ( $80.3 \pm 21.9$  days) and glyphosate 365  $(168.1 \pm 37.9 \text{ days})$  (Kruskal-Wallis test, P < 0.001; Table 2).  $DT_{50}/DT_{50 \ K2HPO4}$  ratio 366 calculations confirmed the same trend; AMPA treatment displayed lower ratio values ranging 367 from 0.95 to 1.49 (except CNII15, ratio = 4.32) compared to the glyphosate treatment ranging 368 from 2.22 to 4.61 (Table 1). 369

Strain growth also varied within P treatments (Strain  $\times$  P treatment, Table 2). 370 According to the Max<sub>50</sub> results, strain CNI28 grew faster in the presence of K<sub>2</sub>HPO<sub>4</sub>, strains 371 372 CNI26 and CNI28 grew faster in the presence of AMPA, and finally, strain CNI26 grew faster in the presence of glyphosate (Tukey's test, P < 0.05; Table 1). Maximal bacterial density 373 374 (Max) was not different among P treatments (P = 0.44), although an interaction effect existed 375 between strains and P treatments (Table 2). Comparatively, the highest Max values in the presence of K<sub>2</sub>HPO<sub>4</sub>, AMPA and glyphosate were observed for strains CNI35, CNII15 and 376 CNI52, respectively (Tukey's test, P < 0.05; Table 1). No statistical differences were 377 observed for growth rates  $(m_g)$  among P treatments (P = 0.10) but strain x P treatment 378 interaction existed for K<sub>2</sub>HPO<sub>4</sub> and AMPA (P < 0.05 both, Table 2). The highest  $m_g$  values in 379 the presence of K<sub>2</sub>HPO<sub>4</sub> and AMPA were observed for CNI52 and CNI28, respectively (Table 380 1). The strong variability of  $m_g$  values among strains in the presence of glyphosate resulted in 381 non-statistical differences. 382

Four out of the five isolated strains showed an affiliation with  $\alpha$ -proteobacteria: *Ensifer* sp. (CNII15), *Agrobacterium tumefaciens* (CNI28), *Novosphingobium* sp. (CNI35) and *Ochrobactrum pituitosum* (CNI52), whereas only one showed affiliation with  $\beta$ proteobacteria: *Acidovorax* sp. (CNI26) (Figure 2). The majority of known glyphosatedegrading strains in  $\alpha$ - and  $\beta$ -proteobacteria use glyphosate as the source of P, whereas few exceptions use the herbicide as the source of C (*Agrobacterium tumefaciens* P5 and *Achromobacter* sp. CH1, Figure 2).

Figure 2. Phylogenetic analysis of the five bacterial strains isolated from stream biofilms, 391 together with the known bacterial glyphosate-degrading strains based on 16S gene sequence 392 (Table S1). Only one sequence per genus has been included in the present analysis, either the 393 16S rDNA gene sequence of the glyphosate-degrading strain described, or the sequence of a 394 corresponding genus/species if the degrading strain sequence was not available (NA). The 395 alignment was performed with ClustalW. The tree was constructed with the Neighbor-Joining 396 397 (N = 1000 bootstrap replicates) using MEGA6. Scale bar = 0.05 substitution per site. Bootstrap percentages  $\geq 50$  % are indicated near tree nodes. For the glyphosate-degrading 398 strains, the metabolism of glyphosate (source of carbon (C), nitrogen (N) and phosphorus (P)) 399 and the main metabolite (AMPA (A), Sarcosine (S), formaldehyde (F), glycine (G) or 400 unknown (U)) are also indicated on the right. 401

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			C	N P	Metabolite	Source	Reference
0.05		87 Burkholderia sp. AQ5-13 (KX792234.1)	σ	0	U	GP-soil	(Manogaran et al. 2017)
		98 Burkholderia pseudomallei ATCC 23343 (NR_043553.1)	le l	0	U	GP-soil	(Glass et al. 2006)
		Burkholderia vietnamiensis AQ5-12 (KX792233.1)	na	0	U	GP-soil	(Manogaran et al. 2017)
		100 Acidovorax sp. CNI26 (MN017827)	nan	0	S/F/G	River-biofilm	(this study)
		65 Achromobacter sp. CH1 (HQ619222.1)			A/S/U	GP-soil	(unpublished)
		99 Alcaligenes sp. PGBS001 (EU622578.1)	<u> </u>	0	S	Cyanobacteria	(unpublished)
		Stenotrophomonas maltophilia IAM 12423 (NR_041577.1)	•	•	A	GP-soil	(Nourouzi et al. 2011)
		100 Enterobacter cloacae ST23 (KU049660.1)	2	0	S	Rhizosphere	(Kryuchkova et al. 2014)
					U	GP-soil	(Sabullah et al. 2016)
		62 Pseudomonas fluorescens SG-1 (KU291443.1)	ono	0	A	Soil	(Carles et al. 2017)
	77	100 Pseudomonas sp. GA09 (KP671492.1)			A	GP-soil	(Zhao et al. 2015)
Γ		99 Pseudomonas sp. GA07 (KP671491.1)	d-7		A	GP-soil	(Zhao et al. 2015)
		<sup>85</sup> 7 <i>Pseudomonas</i> sp. GC04 (KP671493.1)		•	А	GP-soil	(Zhao et al. 2015)
		Novosphingobium sp. CNI35 (MN017829)		0	F/G	<b>River-biofilm</b>	(this study)
		88 Ochrobactrum pituitosum CNI52 (MN017830)	P	0	F/G	<b>River-biofilm</b>	(this study)
		<sup>100</sup> Ochrobactrum sp. GDOS (JF831448.1)	Le l	.0	Α	GP-soil	(Hadi et al. 2013)
		97 Sinorhizobium meliloti LU12 (EU182657.2)	Prin	0	S	Unknown	(unpublished)
		38 <b>Ensifer sp. CNII15</b> (MN017826)	olle	0	F/G	River-biofilm	(this study)
		74 Agrobacterium tumefaciens CNI28 (MN017828)	Id-1	0	F/G	River-biofilm	(this study)
		99 Agrobacterium tumefaciens P5 (KX255008.1)	٠ (		A/S	Activated-sludge	(unpublished)
		68 Bacillus cereus CB4 (JN887351.1)			A	GP-soil	(Fan et al. 2012)
		100 Bacillus subtilis BS 15 (KX783560.1) Firmicute	s		U	Rhizosphere	(Yu et al. 2015)
		Geobacillus caldoxylosilyticus S1812 (NR_028708.1)			A	Industry	(Ahmad et al. 2000)
L .	66	82 Arthrobacter atrocyaneus (X80746.1)		0	A	Cell-collection	(Pipke and Amrhein et al. 1988)
		Arthrobacter sp. R-II-1 (AB017541.1)	2	0	S	Unknown	(unpublished)
		100 Streptomyces lusitanus OUCT 16-27 (MH532549.1)		0	U	Cell-collection	(unpublished)
		95 Streptomyces sp. YP127 (MF102228.1)		0	S	Activated-sludge	(unpublished)
1		Spirulina laxissima SAG 256.80 (DQ393278.1) Cyanobacteria	а	0	U	Cell-collection	(unpublished)
99		Flavobacterium sp. CC-JY-6 (DQ239767.1) Bacteroidetes	s	0	A	Activated-sludge	(unpublished)

404 **Table 1**. Microbial growth estimated parameters (*Max*, *Max*<sub>50</sub>, *m<sub>g</sub>*) and model fitting (R-square) are represented for each microbial strain 405 subjected to glyphosate, AMPA and Phosphorus (control) treatments. Pairwise comparisons tests (Tukey's contrast, P < 0.05) on growth 406 parameters were run between (uppercase letters) and within (lowercase letters) treatments. N.S means not statistically significant and N.D means 407 that data did not fit the dose-response sigmoidal model.

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	Strain	Growth model para	meters						
		Max (Cell mL <sup>-1</sup> )		$Max_{50}$ (Hours <sup>-1</sup> )		$m_g$ (Hours <sup>-1</sup> )		R <sup>2</sup>	Max <sub>50</sub> /Max <sub>50</sub> _K <sub>2</sub> HPO <sub>4</sub>
	CNII15	$6.22E8 \pm 7.57E7$	(b)	$38.50\pm0.13$	(d)	$0.075 \pm 0.003$	(c)	$0.992 \pm 0.002$	1
	CNI26	$2.00E8 \pm 6.21E6$	(e)	$45.17\pm0.78$	(c)	$0.048 \pm 0.003$	(d)	$0.996\pm0.001$	1
$K_2HPO_4$	CNI28	$4.26E8 \pm 3.80E6$	(d)	$36.79 \pm 1.21$	(e) (C)	$0.107\pm0.011$	(a)	$0.993 \pm 0.001$	1
112111 0 4	CNI35	$6.76E8 \pm 1.13E7$	(a)	$63.97 \pm 0.72$	(a)	$0.088 \pm 0.002$	(b)	$0.954\pm0.011$	1
	CNI52	$4.95\text{E8} \pm 2.30\text{E7}$	(b)	$57.16\pm0.39$	(b)	$0.114\pm0.013$	(ab)	$0.867\pm0.017$	1
	CNII15	6.59E8 ± 1.50E7	(a)	159.11 ± 1.45	(a)	$0.037 \pm 0.002$	(c)	$0.992 \pm 0.003$	$4.32 \pm 0.02$
	CNI26	$2.34E8 \pm 1.56E6$	(d)	$42.91 \pm 0.30$ (	(d)	$0.050\pm0.002$	(b)	$0.993 \pm 0.001$	$0.95 \pm 0.02$
AMPA	CNI28	$4.20E8 \pm 5.59E6$	(c)	$(N.S) 43.60 \pm 0.66$	(d) (B)	$0.107\pm0.011$	(a) (N.S)	$0.979\pm0.006$	$1.19 \pm 0.06$
	CNI35	$5.14E8 \pm 1.14E7$	(b)	$95.73 \pm 1.74$	(b)	$0.097\pm0.005$	(a)	$0.992 \pm 0.004$	$1.49 \pm 0.04$
	CNI52	$5.64\text{E8} \pm 3.69\text{E7}$	(ab)	$60.06\pm0.47$	(c)	$0.099\pm0.019$	(a)	$0.957\pm0.014$	$1.05\pm0.01$
	CNII15	N.D		N.D		N.D		N.D	N.D
	CNI26	9.21E7 ± 2.15E6	(c)	$116.53 \pm 2.62$	(c)	$0.111\pm0.017$		$0.988 \pm 0.001$	$2.58 \pm 0.03$
Glyphosate	CNI28	$3.99E8 \pm 4.27E7$	(b)	$134.58 \pm 10.42$	(b) (A)	$0.015\pm0.002$		$0.973 \pm 0.004$	$3.66 \pm 0.24$
• •	CNI35	$5.48E8 \pm 2.88E7$	(ab)	$294.58\pm25.68$	(a)	$0.066\pm0.046$	(IN.S)	$0.996 \pm 0.002$	$4.61 \pm 0.42$
	CNI52	$5.59E8 \pm 7.70E7$	(a)	$126.65\pm2.56$	(b)	$0.041 \pm 0.004$		$0.981 \pm 0.001$	$2.22\pm0.06$

410 **Table 2**. Results of the Kruskal-Wallis tests performed on bacterial growth model parameters (*Max*, *Max*<sub>50</sub> and *m<sub>g</sub>*) and dissipation model 411 parameters ( $DT_{50}$  and  $k_d$ ). The tested factors are P treatments (K<sub>2</sub>HPO<sub>4</sub>, AMPA or glyphosate) and the interaction between strains (CNII15, 412 CNI26, CNI28, CNI35, CNI52) and P treatments.  $X^2$  statistic scores, degrees of freedom (*Df*) and *P*-values were provided for each source of 413 variation. Bold values indicate significant differences at P < 0.05.

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Kruskal-Wallis		Dissipation model parameters						Growth model paraemters								
Testedfactors		$m_d$			$DT_{50}$			$m_g$			Max <sub>50</sub>			Max		
		$X^2$	Df	p value	$X^2$	Df	p value	$X^2$	Df	p value	$X^2$	Df	p value	$X^2$	Df	p value
P source		22.88	2	< 0.001	22.19	2	< 0.001	4.54	2	0.10	21.77	2	< 0.001	1.61	2	0.44
	K <sub>2</sub> HPO <sub>4</sub>	12	4	< 0.05	12.9	4	< 0.05	12.4	4	< 0.05	13.5	4	< 0.01	13.5	4	< 0.01
Strain x	AMPA	12.63	4	< 0.05	12.9	4	< 0.05	10.9	4	< 0.05	13.03	4	< 0.05	12.7	4	< 0.05
	Gly	8.64	3	< 0.05	6.58	3	0.08	6.89	3	0.07	9.35	3	< 0.05	8.69	3	< 0.05

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Initial concentrations in P-equivalents of  $K_2$ HPO<sub>4</sub>, AMPA and glyphosate averaged 117.1  $\pm$ 419 4.3  $\mu$ M (C<sub>i</sub>) and final concentrations dropped to 0  $\mu$ M (C<sub>i</sub>) for all P treatments and strains 420 (Table 3). Accumulation of glyphosate and/or AMPA in strain biomass at the end of 421 experiments was < 0.5 % of the initial amount of glyphosate and/or AMPA added. All the 422 strains were able to use either of the above-mentioned compounds as sole P source, except 423 CNII15, which was not able to use glyphosate (Figure 3). However, differences in dissipation 424 kinetics between P treatments as well as among strains x P treatments were observed (Table 425 2). Average  $DT_{50}$  values for K<sub>2</sub>HPO<sub>4</sub> and AMPA (34.8 ± 5.7 and 67.4 ± 22.1 hours, 426 respectively) were higher compared to that of glyphosate (165.1  $\pm$  46.2 hours; Tukey's test, P 427 < 0.05; Table 3). This was confirmed by  $DT_{50}/DT_{50 \ K2HPO4}$  ratio calculations which displayed 428 average ratio values ranging from 1.02 to 1.85 for AMPA and 2.22 to 6.60 for glyphosate. 429 Dissipation rates  $(m_d)$  were also higher for K<sub>2</sub>HPO<sub>4</sub> (0.53 ± 0.21 hours<sup>-1</sup>) and AMPA (0.29 ± 430 0.12 hours<sup>-1</sup>) compared to glyphosate (0.04  $\pm$  0.01 hours<sup>-1</sup>) (Tukey's test, P < 0.05; Table 3). 431 The CNII15 strain was the only exception for slow AMPA dissipation reaching a 432 433  $DT_{50}/DT_{50 \ K2HPO4}$  ratio of 5.12 and  $m_d$  of 0.05 ± 0.018 hours<sup>-1</sup>.

The strain x P treatment interaction on the  $DT_{50}$  parameter revealed faster AMPA dissipation by CNI26 and CNI28 strains (Tukey's test, P < 0.05) and faster glyphosate dissipation by CNI26 and CNI52 strains, although not statistically significant (P > 0.05, Table 3). Dissipation rates ( $m_d$ ) differed between strains in the same manner that  $DT_{50}$  did. For instance, the highest AMPA dissipation rates were found in CNI26 and CNI28 (0.662 and 0.464 hours<sup>-1</sup>, respectively) while the highest glyphosate dissipation rates were found in CNI26 and CNI52 (0.055 and 0.056 hours<sup>-1</sup>, respectively) (Tukey's test, P < 0.05).

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Figure 3. Dissipation kinetics of K<sub>2</sub>HPO<sub>4</sub> (A), AMPA (B) and glyphosate (C) for the five 450 bacterial strains (CNII15, CNI26, CNI28, CNI35, CNI52). Fittings to the dose-response 451 sigmoidal model (DosResp) are represented. Lines correspond to the model with mean 452 parameters values for each condition. The values are means  $\pm$  standard errors (n = 3) of the 453 experimental data. 454







**Table 3.** Kinetics of K<sub>2</sub>HPO<sub>4</sub>, AMPA and glyphosate dissipation for each bacterial strain analyzed (CNII15, CNI26, CNI28, CNI35, CNI52). Values of the model parameters are final concentration (*Cf*), initial concentration (*Ci*), dissipation time 50% (*DT*<sub>50</sub>), dissipation rate ( $m_d$ ) and coefficient of determination (R<sup>2</sup>) are reported as the mean ± standard error. Statistical differences between P treatments (K<sub>2</sub>HPO<sub>4</sub>, AMPA or glyphosate) are indicated by the uppercase letters (A > B > C, Tukey's test, *P*< 0.05) whereas statistical differences between strains are indicated by the lowercase letters (a > b > c > d, Tukey's test, P < 0.05). N.S means not statistically significant and N.D: no fittingto dose-response sigmoidal model.

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	Strain	Dissipatio	on model parameter	'S							
		$Cf(\mu M)$	<i>Ci</i> (µM)	$DT_{50}$ (Hours <sup>-1</sup> )			$m_d$ (Hours <sup>-1</sup> )			R <sup>2</sup>	DT <sub>50</sub> /DT <sub>50</sub> K <sub>2</sub> HPO <sub>4</sub>
	CNII15	$0\pm 0$	$109.44 \pm 1.34$	$28.64 \pm 2.50$	(c)		$0.242 \pm 0.114$	(ab)		$0.999 \pm 0.001$	1
	CNI26	$0\pm 0$	$105.01\pm0.61$	$24.65\pm0.02$	(c)		$0.486 \pm 0.003$	(c)		$1 \pm 0$	1
$K_2HPO_4$	CNI28	$0\pm 0$	$100.67\pm0.70$	$23.65\pm0.02$	(d)	(B)	$0.455\pm0.002$	(b)	(B)	$1\pm 0$	1
	CNI35	$0\pm 0$	$116.24\pm0.26$	$45.93\pm0.03$	(b)		$1.342\pm0.033$	(d)		$1 \pm 0$	1
	CNI52	$0\pm 0$	$111.20\pm2.11$	$51.03 \pm 1.22$	(a)		$0.139 \pm 0.005$	(a)		$0.994 \pm 0.004$	1
	CNII15	$0\pm 0$	$125.36 \pm 7.71$	$144.41 \pm 1.22$	(a)		$0.049 \pm 0.019$	(a)		$0.987 \pm 0.008$	$5.12 \pm 0.47$
	CNI26	$0\pm 0$	$132.55 \pm 14.65$	$29.29 \pm 5.17$	(d)		$0.662 \pm 0.196$	(c)		$0.999 \pm 0.001$	$1.18 \pm 0.21$
AMPA	CNI28	$0\pm 0$	$140.37 \pm 6.93$	$24.15\pm0.15$	(d)	(B)	$0.464\pm0.005$	(c)	(B)	$1 \pm 0$	$1.02 \pm 0.01$
	CNI35	$0\pm 0$	$122.32\pm0.76$	$85.16\pm5.61$	(b)		$0.099 \pm 0.031$	(a)		$0.972\pm0.026$	$1.85 \pm 0.12$
	CNI52	$0\pm 0$	$112.19\pm1.43$	$53.99 \pm 0.39$	(c)		$0.165\pm0.018$	(b)		$0.991 \pm 0.001$	$1.06\pm0.02$
	CNII15	N.D	N.D	N.D			N.D			N.D	N.D
	CNI26	$0\pm 0$	$132.40 \pm 16.76$	$114.14 \pm 2.89$			$0.055 \pm 0.009$	(b)		$0.945 \pm 0.024$	$4.63 \pm 0.12$
Glyphosate	CNI28	$0\pm 0$	$142.96 \pm 34.15$	$129.76 \pm 22.53$		(A)	$0.011\pm0.001$	(a)	(A)	$0.952 \pm 0.014$	$5.49\pm0.96$
Glyphosate	CNI35	$0\pm 0$	$94.69 \pm 6.19$	$303.32\pm18.56$	$(\mathbf{N}.\mathbf{S})$	S) ( )	$0.020\pm0.004$	(a)		$0.989 \pm 0.009$	$6.60 \pm 0.40$
	CNI52	$0\pm 0$	$93.34 \pm 5.85$	$113.33\pm1.13$			$0.056\pm0.005$	(b)		$0.985\pm0.008$	$2.22\pm0.04$

#### 466 *3.3 Metabolite production*

467 In the glyphosate treatment, no AMPA accumulation was observed but varying concentrations of formaldehyde, glycine and sarcosine were detected depending on the strain tested (Figure 468 4A). Sarcosine was only detected in strain CNI26 at 19 % and 23 % of mass balance 469 calculations at the intermediate and final sampling times. Accumulation of formaldehyde at 470 intermediate and final sampling times (5.3 % and 14 %, respectively), and glycine in much 471 lower concentrations (2.0 % and 2.4 %, respectively), were also observed in the CNI26 strain. 472 The metabolite production pattern was rather similar among CNI28, CNI35 and CNI52, 473 474 accumulating more formaldehyde on average (13.8 % on average for the three strains) than glycine (3.2 %) at the final sampling time. As described above, metabolite production was 475 476 negligible for CNII15 subjected to glyphosate since no dissipation was observed (Figure 4A).

In the AMPA treatment, all AMPA was transformed and few metabolites accumulated at the end of the experiment. Metabolites, mostly formaldehyde, were detected at the final sampling time in CNI15, CNI26 and CNI52 (1.16 % on average for the three strains, Figure 480 4B), whereas strains CNI28 and CNI35 accumulated both glycine (2.6 %) and formaldehyde (7.8 %) at the final sampling time.

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Figure 4. Mass balance of glyphosate and metabolites in the glyphosate (A) and AMPA (B)
treatments at initial (0 hours; I), intermediate (1/2 dissipation of glyphosate/AMPA; M) and
final (complete dissipation of glyphosate/AMPA; F) times for the five bacterial strains.



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490 A detailed analysis on metabolite formation by the CNI26 strain subjected to K<sub>2</sub>HPO<sub>4</sub> and AMPA treatments revealed stable and quite low concentrations of sarcosine and glycine 491 throughout the experiment (< 10  $\mu$ M, Figure 5A and B). Formaldehyde concentrations were 492 below the limit of detection regardless of the treatment which contradicts the results obtained 493 in section 3.3, where formaldehyde reached 14 % of mass balance calculations at the final 494 sampling time for CNI26 (Figure 4). In the glyphosate treatment (Figure 5C), sarcosine 495 496 production was observed along with the dissipation of glyphosate to reach a concentration value of  $185 \pm 0.6 \mu M$  after 168 hours of experiment, close to the initial glyphosate 497 concentration supplied. The sarcosine results coincide with those described in section 3.3 for 498 CNI26 and are in keeping with observations made for known β-proteobacteria glyphosate-499 degrading strains in the literature (Figure 2). 500

The relative quantity of C-P lyase and glyphosate oxidase genes (corrected by the 16S 501 rRNA gene) varied among P treatments. Both C-P lyase and the glyphosate oxidase genes 502 503 increased their expression between 36 h and 48 h in the K<sub>2</sub>HPO<sub>4</sub> treatment and between 100 h and 124 h in the glyphosate treatment (Kruskal-Wallis test, P < 0.05; Figure 5D and F), 504 505 although this increase was greater in the presence of glyphosate (30X in average) than in the presence of K<sub>2</sub>HPO<sub>4</sub> (10X in average). The opposite trend was observed in the case of the 506 507 AMPA treatment. The expression of C-P lyase and glyphosate oxidase was overall greater at 36 h than at 48 h, although this difference was only statistically significant for the glyphosate 508 509 oxidase gene (P < 0.05). The expression of C-P lyase at 36 h in the AMPA treatment was extremely variable (Figure 5E). 510

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**Figure 5.** Kinetics of K<sub>2</sub>HPO<sub>4</sub> (A) AMPA (B) or glyphosate (C) dissipation along with metabolites formation (sarcosine, glycine and AMPA) for the CNI26 strain in the culture media exposed to either K<sub>2</sub>HPO<sub>4</sub>, AMPA or glyphosate. The values are means  $\pm$  standard errors (n = 3) of the experimental data. The black rectangle highlights the times at which C - P lyases (grey filled) and glycine oxidase (white filled) genes relative quantities were measured in the culture media exposed to either K<sub>2</sub>HPO<sub>4</sub> (D), AMPA (E) or glyphosate (F). Asterisks indicate significant differences in genes relative expression (Kruskal-Wallis test, *P*< 0.05).



#### 532 4. DISCUSSION

Four out of the five bacterial strains (*Acidovorax* sp. CNI26, *Agrobacterium tumefaciens* CNI28, *Novosphingobium* sp. CNI35 and *Ochrobactrum pituitosum* CNI52) isolated from stream biofilms were able to completely dissipate AMPA between 30 to 120 hours and glyphosate between 125 to 400 hours when no alternative P source was available. These results are consistent with the literature as the utilization of glyphosate and AMPA as P

sources have been reported as the most common metabolic pathway within bacteria in various 538 539 environments (including soils, rhizosphere, and industrial effluents or activated sludge; Table S1). For instance, 23 out of 29 strains isolated metabolized glyphosate as P source, against 9 540 strains as C source and 4 strains as N source (Figure 2). Such generalized use of glyphosate 541 and/or AMPA as P source could be explained by the widespread presence of the C-P lyase 542 enzyme system encoded by the phn operon in bacteria (Hove-Jensen et al. 2014) which is 543 responsible for C-P bond cleavage and inorganic phosphorus (Pi) transport and assimilation 544 by cells (Chen et al. 1990, Stosiek et al. 2019). The only exception in our study was strain 545 546 CNII15 which was not able to degrade glyphosate but completely dissipated AMPA in 200 547 hours. Indeed, the strain (*Ensifer sp.*CNII15) was neither capable to dissipate nor to grow on 548 glyphosate suggesting: i) lack of adequate enzymatic machinery to metabolize glyphosate despite being able to metabolize AMPA, or ii) slow growth dynamics in the presence of 549 550 glyphosate within 330 hours. It must be noted that the start of growth for CNII15 in the glyphosate treatment ( $OD_{600} = 0.18$ ) was observed at 334 hours (Rossi F. personal 551 552 observation), without glyphosate dissipation, reinforcing the validity of our second hypothesis. When linking the 5 bacterial strains to their environmental origin, four out of the 553 554 five strains (CNI26, CNI28, CNI35 and CNI52) were isolated from P-poor biofilms from an upstream section of the Artière River, whereas CNII15 was isolated from a P-rich biofilm 555 from the downstream section of the same river (Carles et al. 2019). Since the Artière River is 556 characterized by a strong gradient of pesticide contamination between its upstream and 557 downstream sections (including glyphosate and AMPA, Rossi et al. 2019), the specific 558 AMPA metabolism of CNII15 may result from site-adaptation mechanisms. For instance, 559 Ensifer sp. CNII 15 could benefit from AMPA, which may result from glyphosate 560 biodegradation by other microorganisms in biofilms such as the four other bacterial strains 561 studied. 562

Dissipation was faster overall for AMPA than for glyphosate as suggested in our 563 initial hypothesis. As expected, the time required for glyphosate/AMPA concentrations to 564 decline 50 % ( $DT_{50}$ ) was closely related to the time required for the bacterial density to reach 565 50 % increase (Max<sub>50</sub>). The AMPA treatment showed lower values of both  $DT_{50}$  and  $Max_{50}$ 566 compared to the glyphosate treatment. These results are consistent with the literature, since 567 568 AMPA was already reported as an easier substrate for degradation compared to glyphosate. 569 The review of Hove-Jensen et al. (2014) reported that among a total of 133 environmental bacterial strains isolated from glyphosate-treated as well as non-treated soil, 26 strains were 570 571 able to utilize glyphosate as a P source while 55 strains were capable to use AMPA as P

source. The remaining 52 strains were able to degrade neither glyphosate nor AMPA. Hence, 572 573 the faster dissipation of AMPA over glyphosate can likely be explained by i) a greater affinity of C-P lyase enzymes for AMPA than glyphosate molecules (Hove-Jensen et al. 2014), ii) 574 575 low affinity of glyphosate oxidase for glyphosate (Pedotti et al. 2009), or iii) a purely 576 chemical difference between glyphosate and AMPA, the latter being less complex and consequently being much more easily cleaved to release P. The molecular analysis of 577 Acidovorax sp. CNI26 revealed that the highest expression of glycine oxidase and CP-lyase 578 579 genes was observed before reaching  $Max_{50}$  in the presence of AMPA suggesting a decoupling 580 between the expression of these genes and the increase of bacterial density. In contrast, the 581 expression of glycine oxidase and CP-lyase genes was higher after reaching Max<sub>50</sub> in the presence of glyphosate, hypothesizing that the degrading gene expression and their activity 582 could be linked to the bacterial cell number. Moreover, the faster dissipation of AMPA is 583 584 favoured by the lower toxicity for aquatic microorganisms of AMPA compared to glyphosate (Bonnet et al. 2007). 585

586 Differences in AMPA and glyphosate dissipation were observed among the five tested strains. The fastest AMPA dissipation  $(DT_{50})$  was found for CNI26 and CNI28 (29 and 24 587 588 hours, respectively), while the fastest dissipation for glyphosate was found for CNI26 and 589 CNI52 (114 and 113 hours, respectively). To our knowledge, our study describes for the first 590 time the ability of the Acidovorax genus (Acidovorax sp. CNI26) to dissipate both glyphosate and AMPA. The genome sequencing of Acidovorax sp. CNI26 revealed the presence of one 591 glycine oxidase gene copy and two copies of the *phn* operon. A study reveals that bacterial 592 glycine oxidase is capable to efficiently cleave glyphosate, producing AMPA and glyoxylate 593 (Pedotti et al. 2009). Sviridov et al. (2015) speculate that there exists a whole range of 594 bacterial flavin oxidases differing in structure, substrate specificity, and the extent of mutual 595 596 homology, which can be generically termed glyphosate oxidoreductases (referring to their 597 ability to oxidize glyphosate). Besides, the phn operon encodes transport proteins, phosphonate catabolism enzymes and auxiliary polypeptides involved in phosphonate 598 599 catabolism, among others. Within this operon, seven genes (phnG, phnH, phnI, phnJ, phnK, phnL and phnM) are supposed to encode the core components of the membrane-bound C-P 600 601 lyase, metabolizing phosphonates to phosphate by *PhnJ* catalyst (Metcalf and Wanner 1993). Both glycine oxidase and phnG to M genes confer to Acidovorax sp. CNI26 the potential to 602 603 metabolize glyphosate either through the C-P lyase or the glyphosate oxidase degradation pathways. The genome analysis of several Agrobacterium species (including Agrobacterium 604 605 vitis S4, A. tumefaciens strain C58, A. radiobacter K84) revealed identical phn gene orders

conferring the potential to use a variety of phosphonates as P sources (Hove-Jensen et al. 606 2014). Similarly, Ochrobactrum species (i. e. O. anthropi strain GPK3) phylogenetically 607 close to Ochrobactrum pituitosum CNI52 have also been shown able to grow with glyphosate 608 609 as P source. Both the C-P lyase and the glyphosate oxidase degradation pathways have been 610 suggested for O. anthropi strain GPK3 (Sviridov et al. 2012). Despite the fact that the genetic potential for successful glyphosate/AMPA degradation seems rather generalized among 611 environmental bacteria, the differences found among bacterial strains could be attributed to a 612 range of factors including differences in i) cellular P requirements and P storage or ii) enzyme 613 614 affinities and/or catalytic efficiencies for glyphosate and/or AMPA.

615 Metabolite concentration analyses shed some light on degradation pathways used by 616 bacteria to degrade glyphosate and/or AMPA. On the one hand, the AMPA degradation pathway was similar among all the strains. After complete AMPA dissipation, the five strains 617 618 accumulated very low concentrations of metabolites, mostly formaldehyde. Also, formaldehyde did not accumulate in the media probably because of incorporation in the 619 620 microbial biomass via the tetrahydrofolate cycle (Wang et al. 2016b). On the other hand, strains CNI28, CNI35 and CNI52 accumulated formaldehyde and glycine after glyphosate 621 622 transformation. Since no intermediary AMPA or sarcosine metabolites were detected, we can suggest that both C-P lyase and/or glyphosate oxidase degradation pathways could take place. 623 Very low concentrations of easily degradable metabolites are detected at the end of 624 exponential decay phases for glyphosate in CNI28, CNI35 and CNI52 suggesting the 625 incorporation of these metabolites in the microbial biomass or release in the form of CO<sub>2</sub>. 626

The glyphosate degradation pathway was different in the strain Acidovorax sp. CNI26, 627 compared to CNI28, CNI35 and CNI52, since it accumulated sarcosine. Sarcosine results 628 from the direct cleavage of the C-P bond by C-P lyases yielding sarcosine and Pi (Figure 1). 629 The detection of this metabolite proves, at least, the existence of the C-P lyase degradation 630 pathway by CNI26 but does not exclude that both the C-P lyase and oxidative pathways were 631 occurring in parallel. This is especially true when comparing the different accumulations of 632 633 sarcosine and formaldehyde by CNI26 at the end of experiments I and II (see section 3.3 and 3.4). Unfortunately, not all glyphosate/AMPA metabolites were measured in this experiment 634 (glyoxylate and methylamine were missing) to be able to better refine glyphosate/AMPA 635 636 degradation pathways employed by the different bacterial strains.

The specific analysis of C-P lyase and glycine oxidase genes in *Acidovorax* sp. CNI26
revealed different expression patterns depending on P treatments. The increased expression of
C-P lyase and glycine oxidase genes at 124 hours in the glyphosate treatment showed that i)

C-P lyase certainly contributed to sarcosine accumulation in the media and ii) glycine oxidase 640 could contribute to parallel oxidation of sarcosine. Indeed, glycine oxidase from Bacillus 641 subtilis (Martínez-Martínez et al. 2006) and/or other microbial species (Sviridov et al. 2015) 642 has been proven to be capable of oxidative deamination of various amines, including 643 sarcosine. Consequently, the differences in sarcosine accumulation between the first and 644 second experiments suggest that oxidation of sarcosine by glycine oxidase may become 645 relevant in later stages of glyphosate decomposition (Job et al. 2002). In the AMPA treatment, 646 expression levels of both C-P lyase and glycine oxidase were high at 36 hours indicating 647 648 AMPA degradation by C-P lyase as well as methylamine degradation by glycine oxidase. The 649 expression level of these two enzymes decreased at 48 h when neither AMPA nor metabolites 650 remained in the media.

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## 652 **5.** CONCLUSION

The present study show that stream biofilms can host bacterial species capable to grow and 653 654 metabolize both glyphosate and AMPA and other species only capable to metabolize AMPA. The close interactions between microbial species in biofilms are expected to guarantee the 655 656 survival of species such as Ensifer sp. CNII15 tolerant to glyphosate but only able to metabolize AMPA. It must be noted that this study constrained each bacterial strain to use P 657 from glyphosate or AMPA, although other P sources may be available for bacteria in stream 658 biofilms. Hence, glyphosate mitigation by microbial biofilms still depends on P concentration 659 levels in stream waters. 660

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663

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## 827 SUPPLEMENTARY INFORMATION

Figure S1. Bacterial density evolution in  $K_2HPO_4$  (A), AMPA (B) and glyphosate (C) treatments are shown. Lines correspond to fittings to the Gompertz growth model (SGompertz) with mean parameters values for each condition. The values are means  $\pm$ standard errors (n = 3) of the experimental data.

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**Table S1**. Bacterial isolates known for their glyphosate-degrading activity. In addition to the 16S rDNA gene sequences of the 5 newly isolated

838 strains (present study), sequences from previously isolated glyphosate-degrading strains were included in the phylogenetic analysis (Figure 2).

839 These sequences were taken from the studies listed by the review of Sviridov et al. (2015), as well as from more recent studies.

Strain	Source	Metabolite	Metabolism	Reference	NCBI Accession Number	Alternative strain	NCBI Accession Number	Reference
Arthrobacteratrocyaneus ATCC 13752	Cell-collection	AMPA	Р	(Pipke and Amrhein, 1988)	X80746.1			
Arthrobactersp. GLP-1	Unknown	Sarcosine	NP	(Pipke et al., 1987)	NA	Arthrobactersp. R-II-1	AB017541.1	Unpublished
Streptomyceslusitanus	Cell-collection	Unknown	NP	(Lipok et al., 2009)	NA	Streptomyceslusitanus strain OUCT 16-27	MH532549.1	Unpublished
Streptomycessp. StC	Activ-sludge	Sarcosine	NP	(Obojska et al., 1999)	NA	Streptomycessp. strain YP127	MF102228.1	Unpublished
Flavobacteriumsp. GD1	Activ-sludge	AMPA	Р	(Balthazor and Hallas, 1986)	NA	Flavobacteriumsp. CC-JY-6	DQ239767.1	Unpublished
Spirulinaspp.	Cell-collection	Unknown	NP	(Lipok et al., 2007)	NA	Spirulina laxissima SAG 256.80	DQ393278.1	Unpublished
Bacillus cereus CB4	GP-Soil	AMPA	С	(Fan et al., 2012)	JN887351.1			
Bacillus subtilis Bs-15	Rhizosphere	Unknown	СР	(Yu et al., 2015)	KX783560.1			
Geobacillus caldoxylosily ticus T20	Industrial	AMPA	Р	(Obojska et al., 2002)	NA	Geobacilluscaldoxylosilyticus strain S1812	NR_028708.1	(Ahmad et al., 2000)
Ochrobactrumanthropi GDOS	GP-Soil	AMPA	Р	(Hadi et al., 2013)	JF831448.1			
Ochrobactrumanthropi GPK 3	GP-Soil	AMPA	Ρ	(Sviridov et al., 2012)	NA			
Ochrobactrumanthropi LBAA	Soil	AMPA	Р	(Gard et al., 1997; Obojska et al., 2002)	NA			
Ochrobactrumanthropi S5	Soil	AMPA	Р	(Gard et al., 1997)	NA			
Ochrobactrumpituitosum CNI52	River-biofilm	Unknown	Р	This study	MN017830			
Agrobacteriumradiobacter	Activ-sludge	Sarcosine	Ρ	(Wackett et al., 1987)	NA	Agrobacteriumtumefaciens P5	KX255008.1	Unpublished
Agrobacteriumradiobacter SW9	Activ-sludge	AMPA	С	(Mcauliffe et al., 1990)	NA			
Agrobacteriumtumefaciens CNI28	River-biofilm	Unknown	Р	This study	MN017828			

Rhizobium meliloti 1021	Unknown	Sarcosine	Р	(Liu et al., 1991)	NA	Sinorhizobiummeliloti strain LU12	EU182657.2	Unpublished
Sinorhizobiumsp. CNII15	River-biofilm	Unknown	Р	This study	MN017826			
Novosphingobiumsp. CNI35	River-biofilm	Unknown	Р	This study	MN017829			
Achromobacter sp. Kg 16	GP-Soil	Unknown	Ρ	(Ermakova et al., 2010; Shushkova et al., 2016)	NA	Achromobacter sp. CH1	HQ619222.1	Unpublished
Achromobacter sp. LW9	Activ-sludge	AMPA	с	(Mcauliffe et al., 1990)	NA			
Achromobacter sp. MPK 7	GP-Soil	Sarcosine	Р	(Ermakova et al., 2017)	NA			
Achromobacter sp. MPS 12A	phosphonate- Soil	Sarcosine	Р	(Sviridov et al., 2012)	NA			
Alcaligenessp. GL	Cyanobacteria	Sarcosine	Р	(Lerbs et al., 1990)	NA	Alcaligenessp. PGBS001	EU622578.1	Unpublished
Burkholderiasp. AQ5-13	GP-Soil	Unknown	Р	(Manogaran et al., 2017)	KX792234.1			
Burkholderiaviet namiensis AQ5-12	GP-Soil	Unknown	Р	(Manogaran et al., 2017)	KX792233.1			
Acidovoraxsp. CNI26	River-biofilm	Unknown	Р	This study	MN017827			
Enterobactercloacae K7	Rhizosphere	Sarcosine	Р	(Kryuchkova et al., 2014)	NA	Enterobactercloacae strain ST23	KU049660.1	Unpublished
Klebsiellaoxytoca SAW-5	GP-Soil	Unknown	с	(Sabullah et al., 2016)	NA	Klebsiellaoxytoca strain ATCC 13182	NR_119277.1	(Boye and Hansen, 2003)
Providenciaalcalifaciens	GP-Soil	AMPA	С	(Nourouzi et al., 2011)	NA	Providenciaalcalifaciens strain Cl P82.90	NR_042053.1	(Dauga, 2002)
Pseudomonas fluorescens	Soil	AMPA	Р	(Zboińska et al., 1992)	NA	Pseudomonas fluorescens strain SG- 1	KU291443.1	(Carles et al., 2017)
Pseudomonas pseudomallei 22	GP-Soil	AMPA	Р	(Peñaloza-Vazquez et al., 1995)	NA	Burkholderiapseudomallei strain ATCC 23343	NR_043553.1	(Glass et al., 2006)
Pseudomonas putida T5	HC-Soil	Sarcosine	Р	(Selvi and Manonmani, 2015)	NA	Pseudomonas putida strain IAM 1236	NR_043424.1	(Anzay et al., 1997)
Pseudomonas sp. 4ASW	GP-Soil	Sarcosine	Р	(Dick and Quinn, 1995)	NA			
Pseudomonas sp. GA07	GP-Soil	AMPA	С	(Zhao et al., 2015)	KP671491			
Pseudomonas sp. GA09	GP-Soil	AMPA	С	(Zhao et al., 2015)	KP671492			
Pseudomonas sp. GC04	GP-Soil	AMPA	С	(Zhao et al., 2015)	KP671493			
Pseudomonas sp. GLC11	Unknown	Sarcosine	Р	(Selvapandiyan and Bhatnagar, 1994)	NA			

Pseudomonas sp. LBr	Activ-sludge	AMPA	Р	(Jacob et al., 1988)	NA			
Pseudomonas sp. PG2982	Unknown	Sarcosine	Р	(Moore et al., 1983)	NA			
Pseudomonas sp. SG-1	Activ-sludge	AMPA	Р	(Talbot et al., 1984)	NA			
Stenotrophomonasmaltophilia	GP-Soil	AMPA	С	(Nourouzi et al., 2011)	NA	Stenotrophomonasmaltophilia strain IAM 12423	NR_041577.1	Unpublished