# Potential of preventive bioremediation to reduce environmental contamination by pesticides in an agricultural context: A case study with the herbicide 2,4-D

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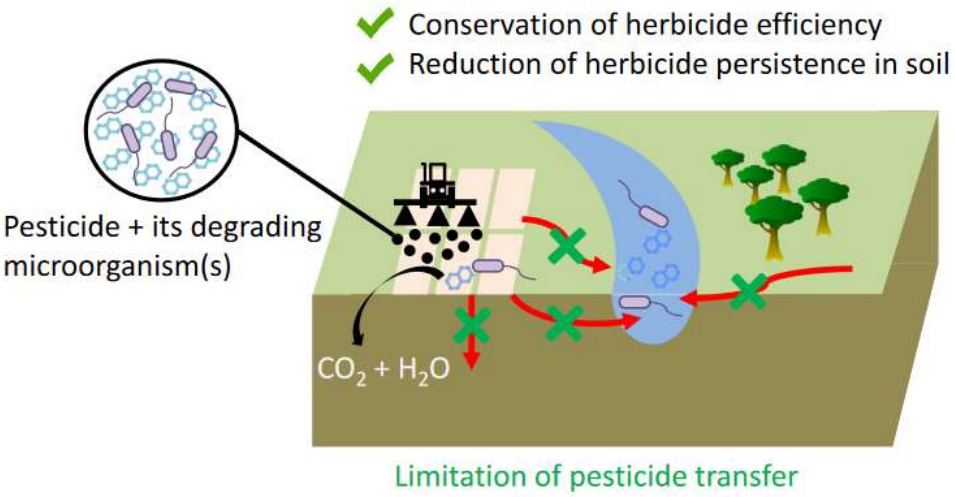
**Graphical abstract**



## Highlights

- The preventive bioremediation is implemented and tested in an agricultural context
- The herbicidal efficiency was conserved in the preventive bioremediation treatment
- The preventive bioremediation reduced the pesticide persistence in soil
- The soil bacterial diversity was not affected by the inoculation of *C. necator*

**Abstract**

One of the major problems with pesticides is linked to the non-negligible proportion of the sprayed active ingredient that does not reach its intended target and contaminates environmental compartments. Here, we have implemented and provided new insights to the preventive bioremediation process based on the simultaneous application of the pesticide with pesticide-degrading microorganisms to reduce the risk of leaching into the environment. This study pioneers such a practice, in an actual farming practice context. The 2,4-dichlorophenoxyacetic acid herbicide (2,4-D) and one of its bacterial mineralizing-strains (*Cupriavidus necator* JMP134) were used as models.

The 2,4-D biodegradation was studied in soil microcosms planted with sensitive (mustard) and insensitive (wheat) plants. Simultaneous application of a 2,4-D commercial formulation (DAM®) at agricultural recommended doses with $10^5$ cells.$g^{-1}$ dw of soil of the JMP134 strain considerably accelerated mineralization of the herbicide since its persistence was reduced threefold for soil supplemented with the mineralizing bacterium without reducing the herbicide efficiency. Furthermore, the inoculation of the *Cupriavidus necator* strain did not significantly affect the α- and β-diversity of the bacterial community.

By tackling the contamination immediately at source, the preventive bioremediation process proves to be an effective and promising way to reduce environmental contamination by agricultural pesticides.

**Keywords:** 2,4-dichlorophenoxyacetic acid; *Cupriavidus necator*; environmental protection; microbial ecotoxicology; mineralization.

## 1. Introduction

One of the greatest challenges of the 21st century is the development of innovative approaches for sustainable agriculture as underlined by the recently launched farm to fork European strategy (European Commission, 2020). Agricultural practices should not only satisfy food demand but also avoid risks for the environment and human health. Accordingly, authorities in some countries have introduced recommendations to reduce pesticide use, which are regularly revised or have postponed their deadlines due to a lack of solutions to reach the targeted quantitative objectives (e.g. the successive Ecophyto and Ecophyto II+ action plans in France (Légifrance, 2019) or the Directives 2009/128/EC and 2019/782 for the European Union). Indeed, alternative solutions to pesticide use, such as biocontrol or bio-stimulation, are the subject of intense research but often their efficiency does not meet expectations (Syed Ab Rahman *et al.*, 2018; Ferreira *et al.*, 2019; Köhl *et al.*, 2019; Marian and Shimizu, 2019; Blake *et al.*, 2020), thus limiting their use in practice for the moment. For that reason, pesticide use cannot yet be completely avoided and the remediation of pesticide-polluted environments is still of great interest.

Biological remediation approaches, including phytoremediation (Del Buono *et al.*, 2020) and microbial remediation (Morillo and Villaverde, 2017; Ortiz-Hernandez *et al.*, 2018; Sun *et al.*, 2018), are the most popular for *in situ* treatments of agricultural soils because of their efficiency, low cost and eco-friendliness compared to physical and chemical remediation. Consequently, they are sustainable techniques and an attractive research area in remediation. By using the powerful diversity of microorganisms, several curative bioremediation methods have been developed in the last decades. Among them, bioaugmentation has proven its efficiency for the removal of pesticides in soil (Lebeau, 2011; Cycoń *et al.*, 2017; Morillo and Villaverde, 2017). However, several factors influence the success rate of this strategy, such as environmental parameters (soil moisture content, pH, temperature, organic matter content),

109 inoculum load and survival rate of exogenous microorganisms, or pesticide concentration and
110 bioavailability (Lebeau, 2011; Vandermaesen *et al.*, 2016; Cycoń *et al.*, 2017). To overcome
111 some of these limitations, this approach has sometimes been combined with bio-stimulation
112 (e.g. Raimondo *et al.* (2020)) and other mitigation strategies (e.g. grassed buffer strips and
113 constructed wetlands) to reduce pesticide inputs into surface water and groundwater
114 (Reichenberger *et al.*, 2007; Swartjes and Van der Aa, 2020). Nevertheless, the effectiveness
115 of these approaches was highly variable. It would thus be very advantageous to develop new
116 strategies able to decrease the time of pesticide residence in soil in order to avoid its adsorption
117 and to limit its diffusion into the various environment compartments by rapid biodegradation.
118 The present study focused on the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid), which
119 belongs to the phenoxy-acid chemical family (Mitchell and Hamner, 1944). It is a widely-used
120 herbicide in agricultural and urban areas around the world for efficient control of broad-leaved
121 weeds in cereal crops and lawns (Peterson *et al.*, 2016). 2,4-D is an analogue of the
122 phytohormone auxin (indole-3-acetic acid, IAA) resulting in uncontrolled growth and death for
123 plants prone to the molecule. It targets dicots and its recommended agronomic field rate is 750
124 g of active ingredient (a.i.) $ha^{-1}$ (EFSA, 2014). Taking its water solubility into account and $K_d$
125 values (547 mg $L^{-1}$ and 1.81 – 4.28 L $kg^{-1}$, respectively), 2,4-D is classified as having a moderate
126 potential for ground water contamination, which is also proven by GUS (Groundwater Ubiquity
127 Score) values of 2 – 2.7 (Farenhorst *et al.*, 2008; Pfeiffer, 2010; EFSA, 2014). Nevertheless, it
128 has been quantified worldwide in ground water, sometimes reaching concentrations exceeding
129 1 µg/L (Brauns *et al.*, 2018; ADES, 2019; Muszyński *et al.*, 2020). 2,4-D is also frequently
130 detected in surface water: in 50% of the samples in France in 2018 (NAIADES, 2019) and also
131 all around the world (Ensminger *et al.*, 2013; Brauns *et al.*, 2018; Horn *et al.*, 2019; de Castro
132 Lima *et al.*, 2020; Muszyński *et al.*, 2020). As 2,4-D was shown to lead to numerous adverse
133 effects on non-target aquatic organisms, plants and human life (Smith *et al.*, 2017; Dehnert *et*

134 *al.*, 2018; Islam *et al.*, 2018; Zuanazzi *et al.*, 2020), considerable efforts must be made to find
135 remediation solutions.

136 Microorganisms are the main drivers of 2,4-D degradation in soil (Peterson *et al.*, 2016).
137 The model strain *Cupriavidus necator* JMP134 (formerly *Alcaligenes eutrophus*, *Ralstonia*
138 *eutropha* and *Waustersia eutropha*), which is able to use 2,4-D as the sole carbon source (Don
139 and Pemberton, 1981), has been the most studied. It was notably used to investigate the
140 biodegradation pathways of this herbicide (You and Ghosal, 1995; Leveau *et al.*, 1999;
141 Laemmli *et al.*, 2000). The *C. necator* JMP134 strain possesses the well-described plasmid
142 *p*JP4 that contains the degrading-gene cluster *tfd* (Plumeier *et al.*, 2002; Trefault *et al.*, 2004).
143 Each *tfd* gene encodes an enzyme involved in each of the 7 steps of 2,4-D mineralization.
144 Among these genes, *tfdA* encodes for 2,4-D/α-ketoglutarate dioxygenase (TfdA) involved in
145 the first step of the herbicide degradation. Hence its frequent use as a molecular marker for the
146 degradation potential of 2,4-D in the environment (Stibal *et al.*, 2012; Mierzejewska *et al.*,
147 2019). Curative 2,4-D bioaugmentation assays, using between $10^5$ to $10^8$ CFU/$g^{-1}$ soil of various
148 degrading-strains, were carried out in soil slurry (Inoue *et al.*, 2012; Chang *et al.*, 2015), in
149 reactor (Pepper *et al.*, 2002), in sieved or amended soil microcosms (Dejonghe *et al.*, 2000; Xia
150 *et al.*, 2017) or more recently in combination with other mitigation strategies (Yang *et al.*, 2018;
151 Barba *et al.*, 2021). The removal of 2,4-D has also been investigated in soil microcosms
152 following simultaneous application of the herbicide and its degrading-strain (Önneby *et al.*,
153 2010). This interesting approach increases the direct contact between the pesticide and its
154 degrading-microorganism enhancing its biodegradation in real time, thus avoiding adsorption
155 processes. Moreover, in such an approach, the exogenous degrading-microorganisms must be
156 efficient for only a short period of time.

157 Thus here, we implemented this preventive bioremediation method in the actual context of
158 a realistic farming practice, allowing sustainable agriculture to be promoted and reducing both

159 the 2,4-D contamination in agricultural soils and the risk of leaching and runoff in aquatic
160 ecosystems. This strategy consists of simultaneously applying the formulated 2,4-D herbicide
161 as DAM® and its mineralizing *Cupriavidus necator* JMP134 strain in a planted soil, in order to
162 optimize the conditions for its biodegradation, just after its action and before its diffusion to the
163 surrounding ecosystems. The experiment was carried out with a laboratory microcosm
164 approach under controlled conditions. Herbicide dissipation and mineralization but also
165 herbicidal efficiency, survival of the added degrading strain and impact on the structure of
166 edaphic bacterial community were assessed.
167
168 **2. Materials and methods**
169
170 2.1. Model herbicide and soil
171 The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid, ≥ 98 % purity) and [ring-$^{14}$C]-2,4-D
172 (analytical-grade purity > 99%, specific activity $4.6 \times 10^8$ Bq mmol$^{-1}$) were purchased from
173 Sigma Aldrich (France). The formulated 2,4-D (DAM®, 391 g 2,4-D L$^{-1}$) was kindly provided
174 by Agriphar (now UPL OpenAg, France). The OECD sandy loam standard soil 5M was
175 purchased from LUFA Speyer (Germany). The physico-chemical properties and characteristics
176 of the soil are indicated in Table 1.
177
178 2.2. Bacterial strain
179 The edaphic 2,4-D-degrader *Cupriavidus necator* JMP134 strain was purchased from the
180 German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM number: 4058). Prior
181 to the experiment, we checked for rifampicin-resistant microorganisms on Rif-LB plates (must
182 be absent) from a suspension of the OECD standard soil 5M. Then, as already used to
183 specifically enumerate a bacterial strain in soil (Schreiter *et al.*, 2014; Strauss *et al.*, 2015;

184 Sharma *et al.*, 2017), a chromosomal rifampicin resistance (Rif$^r$) mutation was induced in the
185 *C. necator* strain (Karunakaran and Davies, 2000; Lyu and Zhao, 2014). To achieve this goal,
186 *C. necator* JMP134 strain was cultivated in 5 mL LB broth overnight at 28 °C. After
187 centrifugation (8,000 g, 15 min), the pellet was washed in 5 mL of NaCl 0.8 % and spread on
188 LB agar plate supplemented with 150 µg $mL^{-1}$ of rifampicin (Sigma Aldrich, France, ≥ 97 %
189 purity). After 48 h of incubation at 28 °C, spontaneous Rif$^r$ mutants were obtained. Their ability
190 to mineralize pure and formulated 2,4-D (DAM®) was tested and found to be the same as that
191 of the parent strain.
192
193 2.3. Preparation of inoculum
194 The Rif$^r$ *C. necator* JMP134 mutant (called later *C. necator* Rif$^r$) was cultivated overnight
195 at 28 °C in 25 mL of LB broth containing 50 µg $mL^{-1}$ rifampicin and 50 mg $L^{-1}$ 2,4-D under
196 orbital agitation at 150 rpm in 50 mL Erlenmeyer flasks. The bacterial culture was then
197 centrifuged (8,000 g, 15 min) and the pellet was washed twice in 20 mL of sterile NaCl 0.8 %
198 before being resuspended in 10 mL sterile Volvic® water. In parallel, the colony forming units
199 enumeration (CFU $mL^{-1}$) was carried out to ensure that the theoretical expected bacterial
200 concentration was spread on microcosms (see below). The correlation between $OD_{600\ nm}$
201 measurements and CFU $mL^{-1}$ (one $OD_{600\ nm}$ unit corresponds to 6 $10^8$ CFU $mL^{-1}$) was
202 performed by spreading serial dilutions of culture on LB agar plates and counting the colonies
203 after 48 h of incubation at 28 °C.
204
205 2.4. Microcosm setup and sampling
206 Ninety-six microcosms were prepared by adding 100 g dry weight (dw) of OECD soil in
207 each crystal polystyrene box (length: 90 mm, width: 60 mm and height: 50 mm), previously
208 checked for their absence in 2,4-D adsorption. The humidity of each soil microcosm was then

209 adjusted to 20 % (corresponding to 50 % of its maximal Water Holding Capacity (WHC))
210 before the sowing of 4 seeds of 2,4-D-insensitive wheat (Récital variety, kindly provided by
211 INRAe Crouël, France) per microcosm. After the germination of wheat (two days at 20 °C ± 2
212 °C), 24 seeds of 2,4-D-sensitive white mustard (*Sinapis alba*) were planted in each microcosm.
213 Four replicates were carried out for each sampling time. Microcosms were randomly placed in
214 a unique enclosure of 150 mm height, covered with porous plastic film allowing air exchange
215 while limiting soil dehydration and incubated for 2 weeks in an experimental room at 20 °C ±
216 2 °C, with a day/night cycle (14/10 h) ensured by an artificial light (Osram-L-36W/964, 750
217 lux). The treatments were applied on each microcosm surface (5,400 $mm^2$) 5 days after the
218 beginning of sowing.

219 Four different treatments were carried out by spreading 2 mL of Volvic® water
220 supplemented with herbicide at the agronomic recommended dose (4.23 µg of 2,4-D $g^{-1}$ dw of
221 soil, corresponding to 1.08 µL of DAM® per microcosm) (**24D**) or not (control, **H2O**), and
222 inoculated with *C. necator* Rif$^r$ only ($10^5$ CFU $g^{-1}$ dw of soil) (**INOC**) or not (**NI**). The sprays
223 were performed using a TG-470 airbrush (Fraulein 3°8; flume diameter = 0.3 mm). Soil water
224 content was adjusted every 2-3 days with Volvic® water all along the experiment.

225 Four randomly chosen replicates from each treatment were immediately sacrificed (n = 4)
226 after the spray (day 0) to quantify the initial doses of 2,4-D and bacteria applied. Then, the
227 sampling was performed on days 2, 4, 7, 9 and 15. For each sampling date, the number of living
228 plants was counted and the herbicidal efficiency was evaluated by visual observation of plant
229 survival. Photos were also taken on days 0, 1, 2, 4, 7, 9, 15 to calculate the total leave surface
230 coverage after treatment by using the Fiji software (Schindelin *et al.*, 2012). The plants and the
231 roots were removed, and the soil was mixed before being sub-sampled for further analyses (see
232 below). Enumeration of *C. necator* Rif$^r$ and 2,4-D mineralization kinetics were carried out on

fresh soil samples. Quantitative PCR, 16S metabarcoding and herbicide quantification were obtained from -20 °C soil frozen aliquots.

## 2.5. Quantification of 2,4-D in soil

### *2.5.1. 2,4-D extraction from soil*

The herbicide was extracted from 5 g of thawed soil by adding 20 mL of MeOH/$H_2O$ (4/1 v/v) in centrifuge tubes. Samples were stirred for 24 h using an orbital shaker at 50 rpm and room temperature. After centrifugation (15,000 g for 15 min), the supernatant was filtered on a 0.45 µm syringe PVDF filter (after checking the lack of sorption on 2,4-D on this filter) and concentrated in a Thermo Scientific SpeedVac concentrator without heating. The dried residue was then dissolved in 500 µL methanol, vortexed and injected on HPLC. The soil was spiked with standard 2,4-D solutions at different concentrations and submitted to the same protocol. The recovery percentage was 89 ± 2 %.

### *2.5.2. Analysis by HPLC*

The herbicide concentrations of soil liquid extracts were determined by HPLC on an Agilent 1100 apparatus (Courtaboeuf, France) equipped with a reverse-phase column (C18 Zorbax Eclipse Plus column, 75 mm × 4.6 mm, 3.5 µm) at 22 °C and a diode array detector set at λ= 222 and 285 nm. The mobile phase was composed of aqueous $H_3PO_4$ (0.01 % v/v, pH = 2.9) (A) and acetonitrile (B) at a flow rate of 1 mL.min$^{-1}$. Gradient (linear): 0-1 min: 20 % B; 1-7 min: 20-70 % B; 7-8 min: 70-100 % B; 8-9 min: 100-20 % B; 9-10.5 min: 20% A. Injection volume: 30 µL. Each sample was analyzed twice.

### *2.5.3. Modelling of 2,4-D dissipation kinetics*

The kinetics of 2,4-D dissipation in soil were fitted with the DoseResp function of the OriginPro V8 software (Origin Lab Corporation, USA) characterized by the following equation: $C_t=(C_0-C_f)/[1+10^{DT50-t(p)}]$. $t$ is the incubation time, $C_t$ the 2,4-D concentration at

time *t*, $C_0$ the initial concentration of 2,4-D, $C_f$ the final concentration of 2,4-D, *p* the dissipation rate of 2,4-D and *DT50* the time required to reduce the concentration by 50%.

### 2.6. Survival of *C. necator* Rif$^r$ in soil

One gram of mixed soil was taken and diluted in 1 mL of sterile NaCl 0.8 %. After homogenization, serial dilutions were sprayed on LB agar plate containing 50 µg $mL^{-1}$ of both rifampicin and cycloheximide. The plates were then incubated during 48 h at 28 °C for *C. necator* Rif$^r$ enumeration. The number of *C. necator* Rif$^r$ was expressed as CFU $g^{-1}$ of dry weight (dw) soil.

### 2.7. 2,4-D mineralization kinetics in soil

The ability of soil microorganisms to mineralize 2,4-D was determined at each sampling date by radiorespirometry analysis (Soulas, 1993). Briefly, 20 g of the various previously collected fresh soil samples, adjusted to 80 % of its WHC, were treated with 2,4-D (2.5 mg $kg^{-1}$ dw) and [ring$^{14}$C]-2,4-D (1.7 Kbq), and incubated in a radiorespirometer at 20 °C in the dark during one month. $^{14}$C-carbon dioxide resulting from the mineralization of $^{14}$C-2,4-D was trapped in 5 mL of 0.2 M sodium hydroxide solution placed in the respirometer. The sodium hydroxide trapping solutions were regularly changed and the amount of the trapped $^{14}$C-carbon dioxide was determined with a liquid scintillation counter (Packard 1900 TR-Tricarb) after adding 10 mL of scintillation fluid (ACSII, Amersham). A segmented regression model (Muggeo, 2003) was used to estimate the maximum mineralization potential rate of the $^{14}$C-2,4-D. The slope of the 1$^{st}$ regression line is considered to be the maximum mineralization potential rate.

### 2.8. Quantitative PCR analysis

283 *2.8.1. DNA extraction*

284 DNA was directly extracted from 250 mg of soil samples collected after different times of
285 incubation as recommended by ISO 11063 derived from the method described by Martin-
286 Laurent *et al.* (2001). Briefly, soil samples were submitted to mechanical and chemical lysis.
287 Soil and cell debris were discarded by centrifugation and proteins were eliminated by sodium
288 acetate precipitation. The nucleic acids were precipitated with cold isopropanol, washed with
289 70% ethanol and then purified by passage through affinity and exclusion columns. The soil
290 DNA extracts were quantified with the Quant-iT™ PicoGreen® dsDNAassay kit according to
291 manufacturer' recommendations and stored at - 20 °C until use.

292 *2.8.2. Quantitative PCR analysis*

293 Prior to run qPCR assay, the absence of PCR inhibitors in the DNA extracts was assessed
294 as described previously in ISO 17601 (ISO 17601, 2006, 17). The abundance of total bacterial
295 community was estimated by qPCR targeting 16S rDNA with 341_F and 534_R universal
296 primers (López-Gutiérrez *et al.*, 2004). The abundance of 2,4-D degrading bacterial community
297 was measured by qPCR targeting *tfd*A gene with tfdA_F and tfdA-R primers described by
298 Bælum *et al*. (2008). qPCR assays were done in a ViiA7™ thermocycler (life Technologies,
299 Carlsbad, CA, USA) in a 15 µL volume containing 250 ng of T4 Gene 32 product (Qbiogene,
300 UK), 2 ng of soil DNA and 1 µM of each primer. The amplification conditions were as follows:
301 15 min at 95 °C; 30 cycles of 15 s at 95 °C, 30 s at 60 °C for 16S rDNA or 54 °C for *tfdA* and
302 30 s at 72 °C; one melting cycle was realized from 55 to 95 °C by incremental temperature of
303 0.2 °C $s^{-1}$. Standard curves were obtained using serial dilutions of linearized plasmids
304 containing appropriated cloned targeted gene sequence. Ct values outside the range of the
305 standard curve or for which melting curves differed from the standard were removed. Four
306 independent replicates for each treatment and three NTCs (non-template control) were used for
307 both qPCR assays.

308

309 2.9. 16S metabarcoding

310 *2.9.1. Amplicon generation and MiSeq sequencing*

311 Amplicons were generated and sequenced as previously described (Romdhane *et al.*, 2019).
312 Briefly, the bacterial 16S rRNA gene V3–V4 hyper variable regions were firstly amplified with
313 overhang adapters to allow the subsequent addition of Illumina Nextera indexes sequences.
314 First-step PCR amplicons were then used as template for a second step PCR using unique
315 multiplex primers pair combinations for each sample. The amplicons were cleaned-up using
316 sequalPrep$^{TM}$ Normalization plate kit 96-well (Invitrogen) and followed by equimolar pooling.
317 Sequencing was performed on MiSeq (Illumina, 2×250 bp) using the MiSeq reagent kit v2 (500
318 cycles). Demultiplexing and trimming of Illumina adaptors and barcodes was done with
319 Illumina MiSeq Reporter software (version 2.5.1.3).

320 *2.9.2. Sequencing analysis*

321 The sequence data were analysed using an in house developed Jupyter Notebooks (Kluyver
322 *et al.*, 2016) piping together different bioinformatics tools. Briefly, R1 and R2 sequences were
323 assembled using PEAR (Zhang *et al.*, 2014) with default settings. Further quality checks were
324 conducted using the QIIME pipeline (J Gregory Caporaso *et al.*, 2010) and short sequences (<
325 400 bp) were removed. Reference based and *de novo* chimera detection, as well as clustering
326 in OTUs were performed using VSEARCH (Rognes *et al.*, 2016) and the adequate reference
327 databases (Greengenes' representative set of sequences). The identity thresholds were set at 94
328 % for 16S rRNA gene data based on replicate sequencing of a bacterial mock community
329 containing 40 bacterial species. Representative sequences for each OTU were aligned using
330 PyNAST (J. G. Caporaso *et al.*, 2010) and a 16S rRNA gene phylogenetic tree was constructed
331 using FastTree (Price *et al.*, 2010). Taxonomy was assigned using UCLUST (Edgar, 2010) and
332 the latest released Greengenes database v.05/2013 (McDonald *et al.*, 2012). α-Diversity metrics

and UniFrac distance matrices (Lozupone *et al.*, 2011) were calculated based on rarefied OTU tables (21 000 sequences per sample). Sequences were deposited to the SRA at NCBI under the accession number PRJNA566399.

### 2.10. Statistical analyses

Statistical analyses were carried out using RStudio (Version 1.1.456). The factors (modalities) were Treatment (NI H2O, INOC H2O, NI 24D, INOC 24D) and Time (D0, D1, D2, D4, D7, D9, D15). Statistical differences in relative leave surface coverage, 2,4-D persistence in soil ($DT_{50}$), α-diversity indexes of soil microbial communities and abundance of *C. necator* Rif$^r$ in soil were assessed using one-way ANOVA followed by separate post hoc comparisons (Tukey's test, $P < 0.05$). Normality and homogeneity of variance were checked prior to ANOVA analysis (Shapiro's and Levene's tests, respectively, $P < 0.05$) and data that were not normally distributed were transformed using logarithmic, square, square root or Box-Cox functions. The effect of the treatments on β-diversity was assessed by performing permutational multivariate analysis of variance on the unweighted and weighted unifrac distance matrices (PERMANOVA), using the Adonis function of the R package Vegan (version 2.5-5).

## 3. Results and Discussion

### 3.1. Herbicide efficiency of 2,4-D

The soil microcosms containing wheat (insensitive to 2,4-D) and mustards, chosen as a model of sensitive plant (dicot) and as a potential competitor of wheat (Haghighi, 2019), were exposed to the formulated 2,4-D (**24D**) or not (control, **H2O**), and inoculated with *C. necator* Rif$^r$ (**INOC**) or not (**NI**). At the beginning of the experiment, the development and appearance of wheat and mustard plants were similar whatever the treatments (Figure 1). A visual

observation indicated an effect of 2,4-D from day 1, mustards being completely killed in the corresponding microcosms (NI 24D treatment) while these plants were still alive after 15 days in the NI H2O and INOC H2O treatments (Figure 1). As expected, these results confirmed the efficiency of the NI 24D treatment used in the present study to remove mustards (sensitive species). Interestingly, the inoculation of the 2,4-D-mineralizing *C. necator* Rif$^r$ strain concomitantly to the herbicide did not reduce the efficiency of the herbicide. Indeed, similarly to the NI 24D treatment, all mustard plants were killed within 15 days in the INOC 24D treatment (Figure 1). The kinetic of 2,4-D mineralization by *C. necator* Rif$^r$ strain was therefore slow enough to permit the foliar uptake of the herbicide in the sensitive plants which takes place in just a few hours (Barrier and Loomis, 1957; Knoche and Bukovac, 1999). These results were confirmed by the analyses of total leave surface coverage (Figure 2). After only one day of incubation, significant differences in leaf-coverage were observed between treatments ($P < 0.001$), the mustard leaves showing a slight curling effect in the treated 2,4-D soils. Both treatments receiving 2,4-D, alone (NI 24D) or in mixture with the *C. necator* Rif$^r$ strain (INOC 24D), experienced the lowest surface coverage. Furthermore, no significant difference was observed between NI H2O *vs*. INOC H2O, and NI 24D *vs*. INOC 24D. These results indicate that the presence of exogenous *C. necator* Rif$^r$ strain i) did not impact the growth of mustard and wheat and ii) did not reduce the efficiency of 2,4-D. Besides, the visual observation revealed a slight negative effect of 2,4-D on wheat plants (less developed and less robust leaves compared to NI H2O and INOC H2O treatments). This is not surprising because, although wheat is not sensitive to 2,4-D, it has already been shown that this herbicide can impact wheat yield at all growth stages, mainly due to a decrease in the number of kernels per spikelet and a reduction of the numbers of spikes per plant and spikelets per spike (Pinthus and Natowitz, 1967; Islam *et al.*, 2018; Oliveira *et al.*, 2019; Scholtes *et al.*, 2019).

383 3.2. 2,4-D dissipation and mineralization potential in soil

384 *3.2.1. 2,4-D dissipation in soil*

385 After a lag phase of about 4 days, apparently necessary to activate the degrading genes of
386 the indigenous microflora, a significant dissipation of 2,4-D was observed in soil treated with
387 the herbicide (NI 24D), with a dissipation rate of 0.33 ± 0.11 $day^{-1}$ and a $DT_{50}$ value of 7.40 ±
388 0.64 days (Figure 3 and Table 2), leading to a 94 % decrease of the initial 2,4-D amount within
389 15 days. The half-life obtained is similar to that observed in aerobic soils ($DT_{50}$ = 6.2 days)
390 (Reregistration Eligibility Decision (RED) 2,4-D, 2005) but lower than the mean half-life
391 average values (12.6 days) collected from the datasets from laboratory studies (Wang *et al.*,
392 2018). Indeed, the 2,4-D dissipation in soil can be very variable depending on the indigenous
393 degrader concentration, organic matter content, moisture and temperature (Bouseba *et al.*, 2009;
394 Yang *et al.*, 2018).

395 The inoculation of the *C. necator* $Rif^r$ strain, previously grown in LB medium supplemented
396 with 2,4-D, concomitantly to the herbicide in soil (INOC 24D), suppressed the lag phase before
397 herbicide dissipation. Under these conditions, the 2,4-D dissipation rate was increased (0.47 ±
398 0.06 $day^{-1}$) and its $DT_{50}$ was reduced to 2.34 ± 0.19 days compared to the NI 24D soil. Thus, 4
399 days after treatment when the 2,4-D dissipation just started in the NI 24D condition, a decrease
400 of about 85% of the 2,4-D initial concentration was already observed, leading to a complete
401 dissipation of the herbicide within 7 days (Figure 3 and Table 2). Therefore, the INOC 24D
402 treatment reduced the persistence of 2,4-D by a 3-fold factor compared to the NI 24D treatment
403 ($P < 0.05$). Note that comparison of the 2,4-D mineralizing kinetics between the natural *C.*
404 *necator* strain and its mutant showed no significant difference. This preventive bioremediation
405 process thus reduced the risk of 2,4-D leaching and runoff into water bodies where it is currently
406 detected (Brauns *et al.*, 2018; Islam *et al.*, 2018; Horn *et al.*, 2019; NAIADES, 2019; de Castro
407 Lima *et al.*, 2020). Faster pesticide dissipation accordingly reduced its transfer into the

environment. The preventive bioremediation could also provide the means to better protect the culture and avoid a decrease of productivity. Some studies have evidenced 2,4-D causing many injuries to crops, despite its theoretical selectivity (Islam *et al.*, 2018; Oliveira *et al.*, 2019; Scholtes *et al.*, 2019).

### *3.2.2. 2,4-D mineralization potential in soil*

The evolution of $^{14}CO_2$ from $^{14}$C-2,4-D was monitored to estimate mineralization potential of 2,4-D microbial guild extemporarily. Each previous fresh soil sample for each date was supplemented by $^{14}$C-2,4-D to this end and the mineralization percentage was monitored by radiorespirometry. The maximum potential rate of $^{14}$C-2,4-D mineralization was calculated from modelled mineralization kinetics. It was found to be rather low (< 5 % of $^{14}$C-2,4-D per day) and stable throughout the experiment in the control (NI H2O) (Figure 4). It was always significantly higher in the inoculated microcosms (INOC H2O and INOC 24D) than in the control (NI H2O) ($P < 0.001$). As compared to the NI H2O treatment, the inoculation of *C. necator* Rif$^r$ led to a 4- and 5-fold increase in the rate of mineralization of INOC H2O and INOC 24D, respectively. Overall, these results show for the first time that the mineralization potential of 2,4-D in soil was enhanced by simultaneous application of the degrading strain. This is a major advantage of preventive bioremediation, which aims at reducing the soil contamination by mineralization (i.e. without producing degradation products).

The maximum rate of mineralization was significantly higher in the INOC 24D treatment than in the INOC H2O (after 2, 7, 9 and 15 days) ($P < 0.001$). In addition, after a four-day lag phase, the rate of mineralization in the NI 24D microcosm increased dramatically to reach values similar to those of INOC 24D treatment. This natural mineralization for the NI 24D treatment could be explained by the presence of indigenous degraders as already reported in other soils (Bælum *et al.*, 2008; Zabaloy *et al.*, 2010). Furthermore, knowing that the 2,4-D was

433 also shown to be moderately persistent ($DT_{50}$ = 45 days) in aerobic aquatic environments and
434 highly persistent ($DT_{50}$ = 231 days) in anaerobic terrestrial and aquatic environments
435 (Reregistration Eligibility Decision (RED) 2,4-D, 2005), it is of great importance to mineralize
436 it as soon as possible after spraying onto the soil before it reaches the surrounding environments.
437
438 3.3. Abundance of *C. necator* Rif$^r$ in soil
439 *C. necator* Rif$^r$ was inoculated at $10^5$ cfu $g^{-1}$ dw soil. In both inoculated soil microcosms, *C.*
440 *necator* Rif$^r$ strain was maintained around $10^4$ - $10^5$ cfu $g^{-1}$ dw soil during the first four days of
441 incubation (Figure 5). *C. necator* Rif$^r$ was enumerated at a significantly higher value in the
442 INOC 24D microcosms than in the inoculated INOC $H_2O$ ones at days 7 ($P < 0.01$), 9 and 15
443 ($P < 0.001$). We could hypothesize that the exposure to 2,4-D provides a temporary selective
444 advantage for microorganisms capable of using the herbicide as carbon and energy source and
445 led to the growth of *C. necator* Rif$^r$ population as long as it is bioavailable (Macur *et al.*, 2007;
446 Zabaloy *et al.*, 2010). Then, a plateau of the *C. necator* Rif$^r$ population was observed from day
447 10 in the INOC 24D treatment (Figure 5) probably because of complete mineralization of the
448 2,4-D already at day 7. Thus, it seems that these conditions of preventive bioremediation with
449 a simultaneous spray of 2,4-D and the *C. necator* Rif$^r$ strain can overcome some of the limiting
450 factors influencing the success of curative bioaugmentation processes in soil such as i) sorption
451 of pesticides on soil particles and formation of bound residues leading to a decrease of
452 bioavailability and biodegradability, and ii) competition between the inoculated strain and
453 indigenous microorganisms and its survival (Cycoń *et al.*, 2017), even when the 2,4-D herbicide
454 was removed. Indeed, at day 15, no growth was observed anymore but the strain is still able to
455 survive. This is not surprising since *Cupriavidus necator* can easily adapt its growth to nutrient
456 availability in the environment. Indeed, this strain is described as an optional predator for other
457 bacteria and is able to switch from autotrophic to heterotrophic metabolism depending the

458 environmental conditions (Makkar and Casida Jr, 1987; Holt *et al.*, 1994). Similar results have
459 already been observed, the 2,4-D degraders being able to survive and remain at high abundance
460 for about 1 month after herbicide exposure has stopped (Merini *et al.*, 2007; Zabaloy *et al.*,
461 2010).
462 These results were confirmed by the 16S rRNA sequence affiliated to *C. necator* Rif$^r$ (similarity
463 above 95%) which were retrieved in both inoculated microcosms but in higher abundances in
464 the INOC 24D treatment (4.4 ± 2.0 reads per 100,000) than in the INOC H2O one (0.7 ± 0.3
465 reads per 100,000). No sequences affiliated to *C. necator* Rif$^r$ were found in the non-inoculated
466 microcosms (NI $H_2O$ and NI 24D) confirming that no cross-contamination by *C. necator* Rif$^r$
467 occurred during the experiment.
468
469 3.4. Abundance of 2,4-D bacterial degraders
470 The abundance of 2,4-D microbial degraders was monitored throughout the incubation by
471 estimating the relative abundance of *tfdA* (coding for an enzyme involved in 2,4-D
472 mineralization) per 16S rRNA sequence (Figure 6). We observed that 2,4-D bacterial degraders
473 were present in rather large amount in the control soil NI H2O averaging 5.5 *tfdA* sequences
474 per thousand 16S rRNA and remained stable during the 15 day-experimentation (Figure 6). For
475 the non-inoculated soils, the abundance of *tfdA* gene copy number increased in the presence of
476 2,4-D (NI 24D) after 9 days of incubation and was still higher after 15 days than in the non-
477 treated soils (NI H2O). These results indicate that the indigenous 2,4-D degrader populations
478 need to be stimulated by the herbicide before being able to grow and activate their 2,4-D
479 mineralization capacity as shown in Figures 3 and 4. This induction of 2,4-D degrading gene
480 expression has already been described (Trefault and Guzmán, 2009) and may contributes to the
481 accelerated biodegradation observed with edaphic microbial communities chronically exposed
482 to 2,4-D (Arbeli and Fuentes, 2007).

For the inoculated soils, the quantity of degrader in untreated soil i.e. without 2,4-D (INOC H2O) was similar to the NI H2O and remained stable throughout the experiment but increased after only 4 days after inoculation in the treated soils (INOC 24D). The *C. necator* RifR strain being already acclimatised to the presence of 2,4-D (see 2.3), the delay for the increase of 2,4-D degrading community size was shortened as shown by the enumeration of *C. necator* Rif$^r$ in soil samples (Figure 5). Lack of lag phase observed for the dissipation of 2,4-D in the soil (Figure 3) and its mineralization potential (Figure 4) corroborate the former facts.

### 3.5. Impact of 2,4-D on soil bacterial communities

NGS analysis of the soil bacterial community revealed that the soil was composed of about 3200 OTUS (similarity threshold of 0.94). Proteobacteria (34 %), Acidobacteria (31 %), Gemmatimonadetes (8 %) and Bacteroidetes (7 %) were the 4 most abundant phyla representing up to 80 % of sequences in all. α-diversity was estimated by a richness (observed species), a phylogenetic diversity (PD whole tree), and a composite evenness (Simpson reciprocal) index (Figure S1). We found that the soil bacterial α-diversity was significantly affected by time in the microcosm study however none of the treatments (INOC, 24D), nor their interaction (preventive bioremediation) had a significant effect on α-diversity indices of the bacterial community (Figure S1). In addition, as revealed by PERMANOVA performed on PCoAs using both unweighted (uw) and weighted (w) unifrac distance matrices, the β-diversity was affected by the time (Puw and Pw < 0.01) but not by the pesticide treatment (Puw = 0.119, Pw = 0.129) nor the strain inoculation (Puw = 0.253, Pw = 0.181) (Figure S2). Our results are consistent with several studies showing that the 2,4-D herbicide treatment, used at the recommended agricultural dose, has little or no impact on bacterial communities (Bouseba *et al.*, 2009; Zabaloy *et al.*, 2010; Inoue *et al.*, 2012) and that the inoculation of a 2,4-D degrading-*Cupriavidus* sp. strain did not disturb the soil indigenous microorganisms (Chang *et al.*, 2015).

## Conclusion

The present work demonstrates that preventive bioremediation is a promising tool for a new agricultural strategy to reduce the persistence of pesticides in soil and their subsequent risk of transfer into the environment. Moreover, our results show that the simultaneous application of the 2,4-D herbicide and its degrader, *Cupriavidus necotor* JMP134, did not compromise herbicide efficiency and led to a threefold reduction of its persistence in soil without significantly affecting the indigenous bacterial community. Further studies (e.g. other herbicide/degrading strains, other soil types and climatic conditions, larger scale) are under investigation to validate the "proof of concept" of this technique in agriculture.

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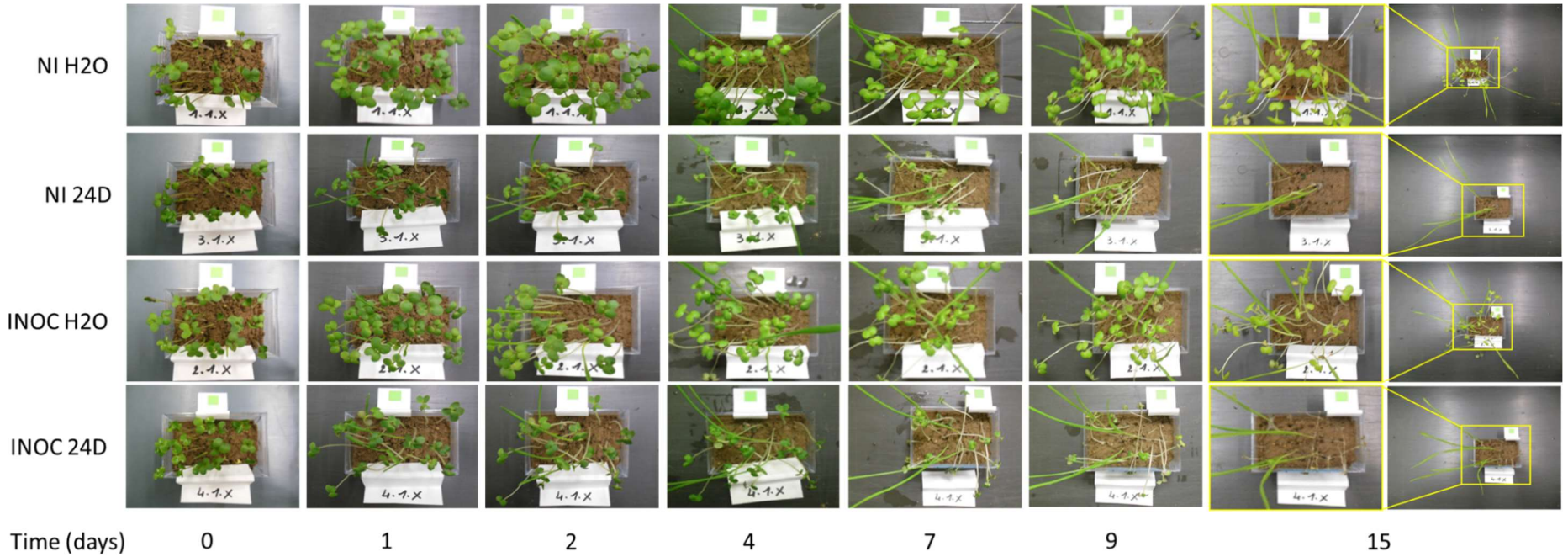


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773 **Figure 1**. Top view of soil microcosms inoculated with *C. necator* Rif$^r$ at $10^5$ cfu $g^{-1}$ dw soil (INOC) or not (NI) and treated with the agronomic

774 dose of formulated 2,4-D (24D) or not ($H_2O$) along the experiment (0-15 days). One replicate per treatment has been selected as a representative

775 example for this figure. The green square served as an internal scale for total surface leave coverage calculation (n = 4).

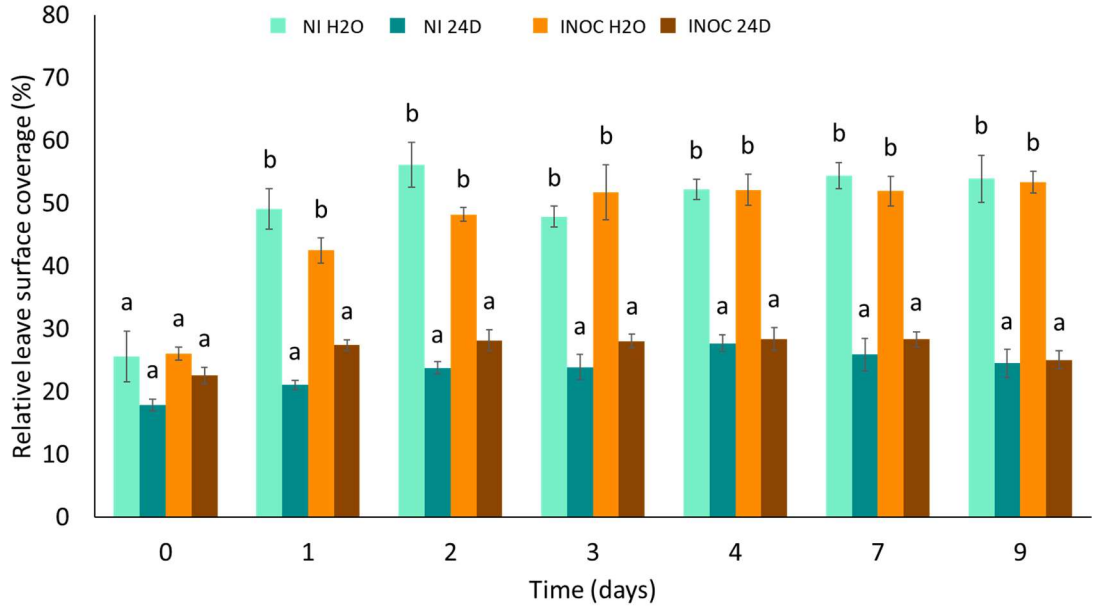
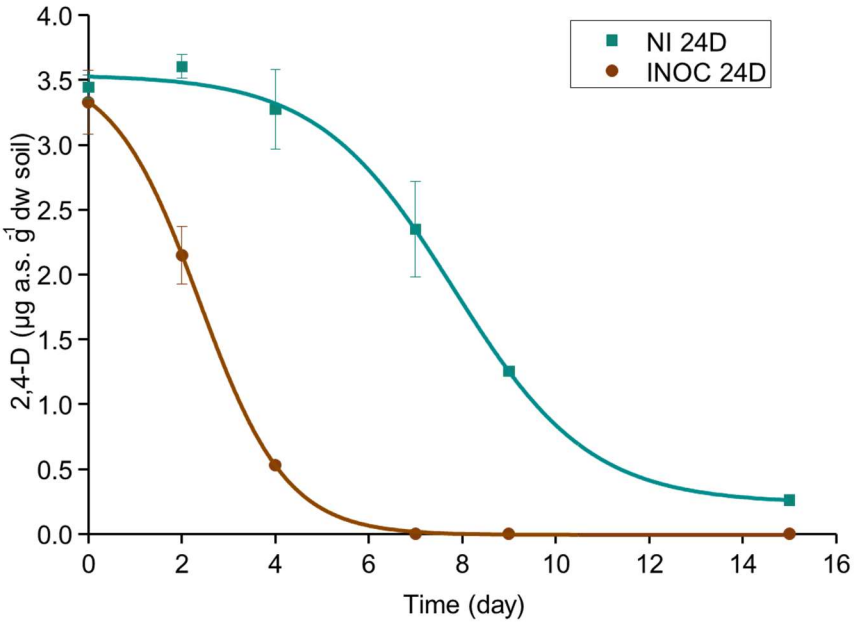



**Figure 2**. Evolution of total leave surface coverage over time expressed as a percentage of the surface of soil microcosms inoculated with *C. necator* Rif$^r$ at $10^5$ cfu $g^{-1}$ dw soil (INOC) or not (NI) and treated with the agronomic dose of formulated 2,4-D (24D) or not ($H_2O$). For each date, statistical differences were indicated by lowercase letters (a < b), Tukey's test ($P < 0.05$).

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793 **Figure 3**. Dissipation kinetics of 2,4-D in soil. Microcosms were treated with the agronomical

794 dose of formulated 2,4-D and either inoculated with *C. necator* Rif$^r$ at $10^5$ cfu $g^{-1}$ dw soil (INOC

795 24D) or not (NI 24D). The values are means ± standard errors of experimental data (n = 4).

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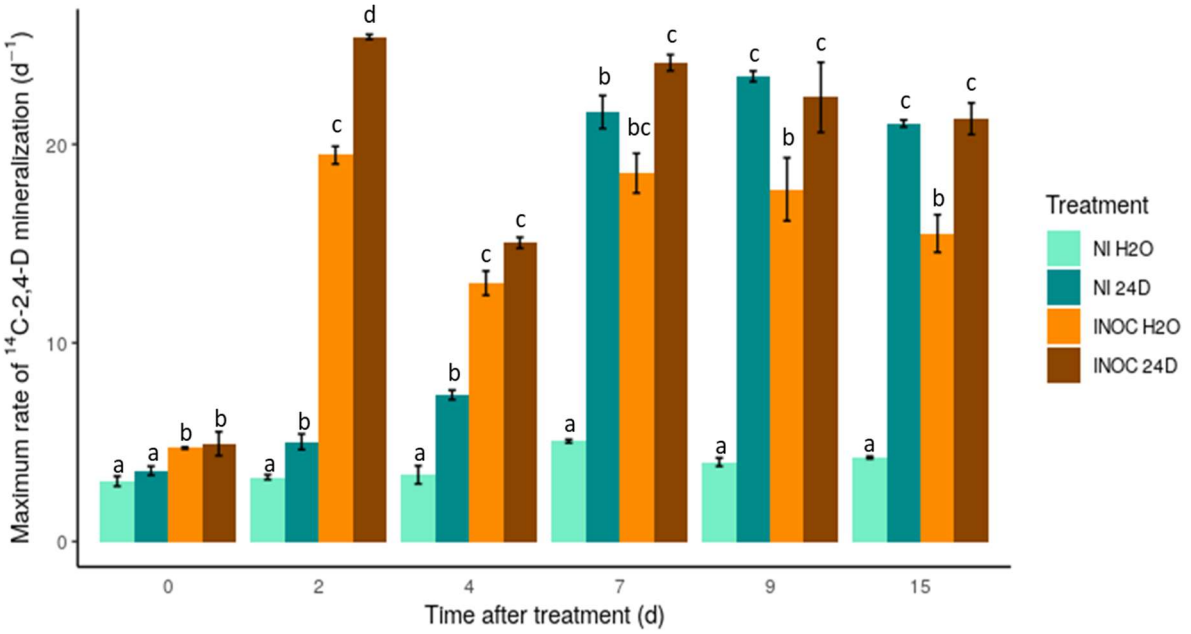
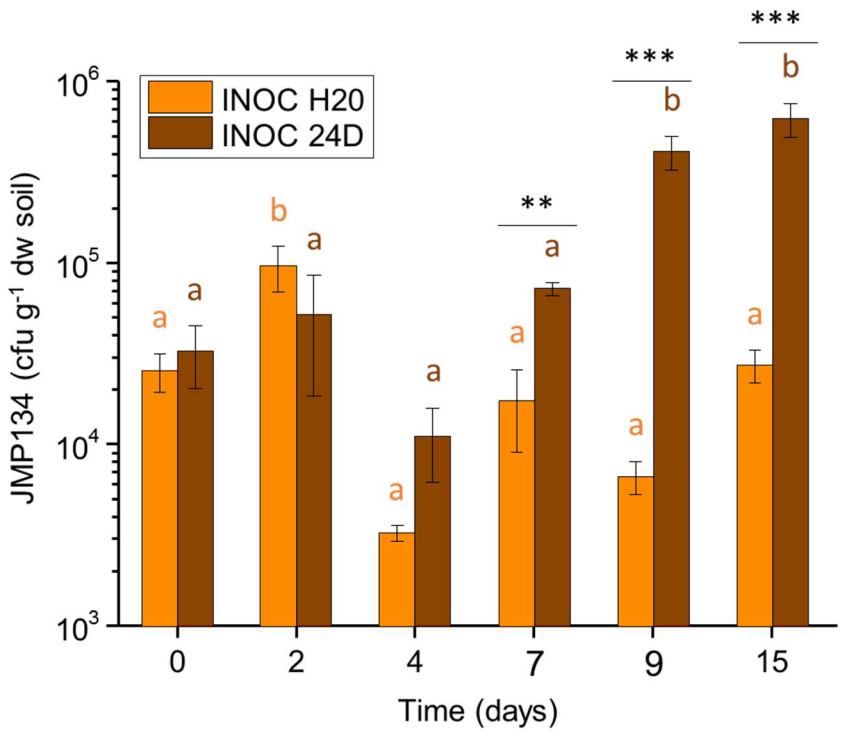



**Figure 4**. Maximum mineralization potential rate of the $^{14}$C-2,4-D at 0, 2, 4, 7, 9 and 15 days after treatment in soil microcosms inoculated with *C. necator* Rif$^r$ at $10^5$ cfu g$^{-1}$ dw soil (INOC) or not (NI) and treated with the agronomic dose of formulated 2,4-D (24D) or not ($H_2O$). The values are means ± standard errors (n = 4). For a given date, different letters indicate significant differences between treatments ($p < 0.001$).

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828 **Figure 5**. Abundance of *C. necator* Rif$^r$ in the soil microcosms at 0, 2, 4, 7, 9 and 15 days after
829 treatment with $H_2O$ (INOC H2O) and 2,4-D (INOC 24D). The values are means ± standard
830 errors (n = 4) of experimental data. Statistical differences (Tukey's test) were indicated for each
831 date by stars (** $P < 0.01$, *** $P < 0.001$) and for each treatment by lowercase letters ($a < b$, $P$
832 $< 0.05$).

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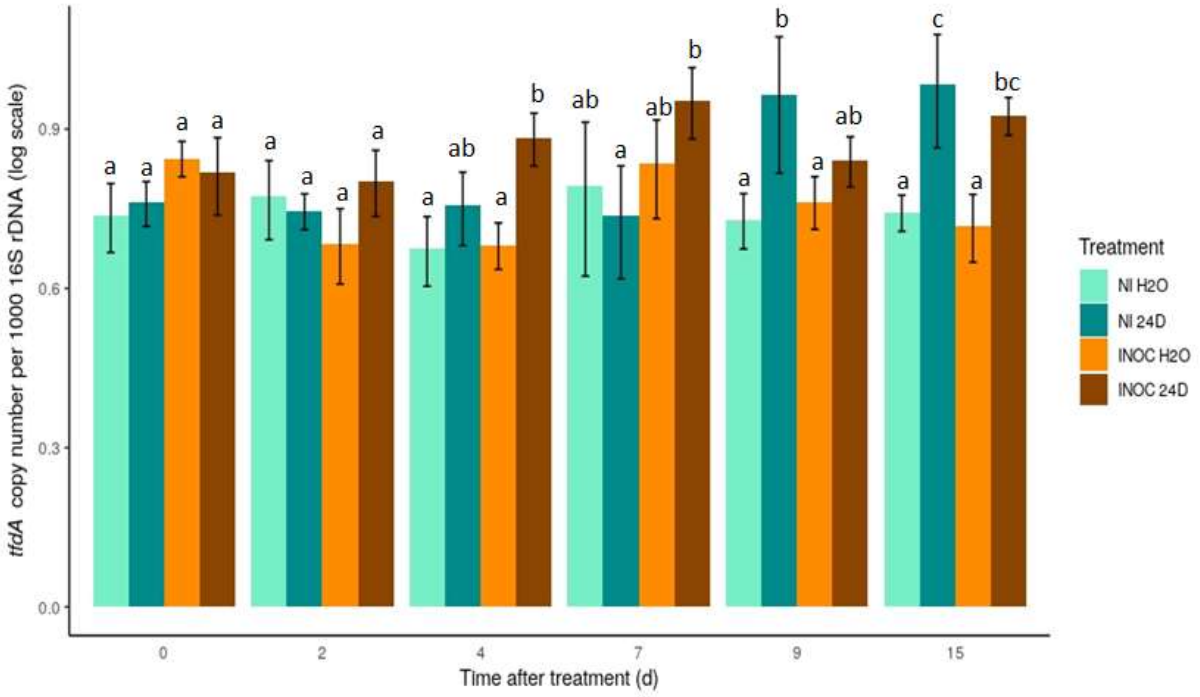


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843 **Figure 6**. Relative abundance of 2,4-D degraders in the overall bacterial community at 0,
844 2, 4, 7, 9 and 15 days after treatment in soil microcosms inoculated with *C. necator* $Rif^r$ at $10^5$
845 cfu. $g^{-1}$ dw soil (INOC) or not (NI) and treated with the agronomic dose of formulated 2,4-D
846 (24D) or not ($H_2O$). The values are means ± standard errors (n = 4). At each time, different
847 letters indicate significant differences in *tfdA* gene copy number ($P < 0.001$).

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**Table 1**. Physico-chemical properties of 5M OECD standard soil (LUFA Speyer, Germany). Values are means of different batch analyses ± standard deviation. All values refer to dry matter.

| Cationic exchange capacity (meq / 100 g) | Organic carbon % C | Nitrogen % N | pH-value (0.01 M $CaCl_2$) | Maximum water holding capacity (g / 100 g) | Particule size (mm) | Particule size distribution (%) |
|---|---|---|---|---|---|---|
| 17.7 ± 3.7 | 1.02 ± 0.08 | 0.13 ± 0.01 | 7.3 ± 0.1 | 40.1 ± 2.4 | < 0.002<br>0.002 – 0.05<br>0.05 – 2.0 | 10.9 ± 0.9<br>30.9 ± 1.4<br>58.2 ± 1.6 |

878 **Table 2**. Parameters of 2,4-D dissipation kinetics in soil microcosms treated with agronomical
879 dose of formulated 2,4-D and either inoculated with *C. necator* Rif$^r$ (INOC 24D) or not (NI
880 24D). Experimental data were fitted with the DoseResp model (OriginPro 8). The values are
881 means ± standard errors of experimental data (n = 4). For each parameter, statistical differences
882 were indicated by lowercase letters (a < b), Tukey's test (P < 0.01).

| | Model parameters | | | | |
|---|---|---|---|---|---|
| Treatment | $C_0$ (µg a.s. $g^{-1}$ dw soil) | $C_f$ (µg a.s. $g^{-1}$ dw soil) | $p$ ($day^{-1}$) | $DT_{50}$ (day) | $r^2$ |
| NI 24D | 3.77 ± 0.18 (a) | 0.19 ± 0.11 (a) | 0.33 ± 0.11 (a) | 7.40 ± 0.64 (b) | 0.992 ± 0.004 |
| INOC 24D | 3.61 ± 0.24 (a) | 0.00 ± 0.00 (a) | 0.47 ± 0.06 (a) | 2.34 ± 0.19 (a) | 0.999 ± 0.001 |

883 $C_0$: initial concentration of 2,4-D; $C_f$ : final concentration of 2,4-D; $p$: dissipation rate of 2,4-D; $DT_{50}$: time
884 required to reduce the concentration by 50%
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**Supplementary Information**

# Potential of preventive bioremediation to reduce environmental contamination by pesticides in an agricultural context: a case study with the herbicide 2,4-D

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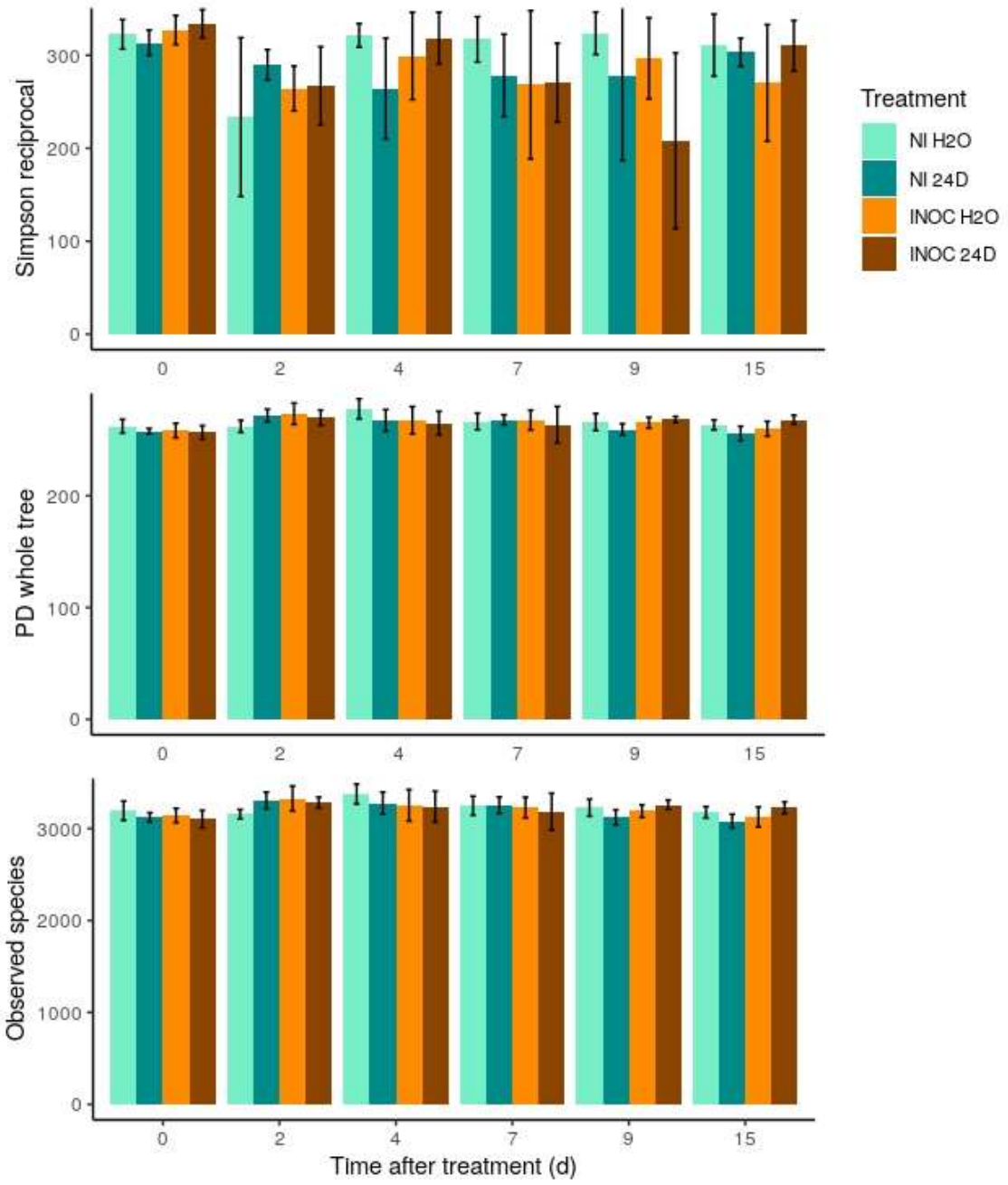


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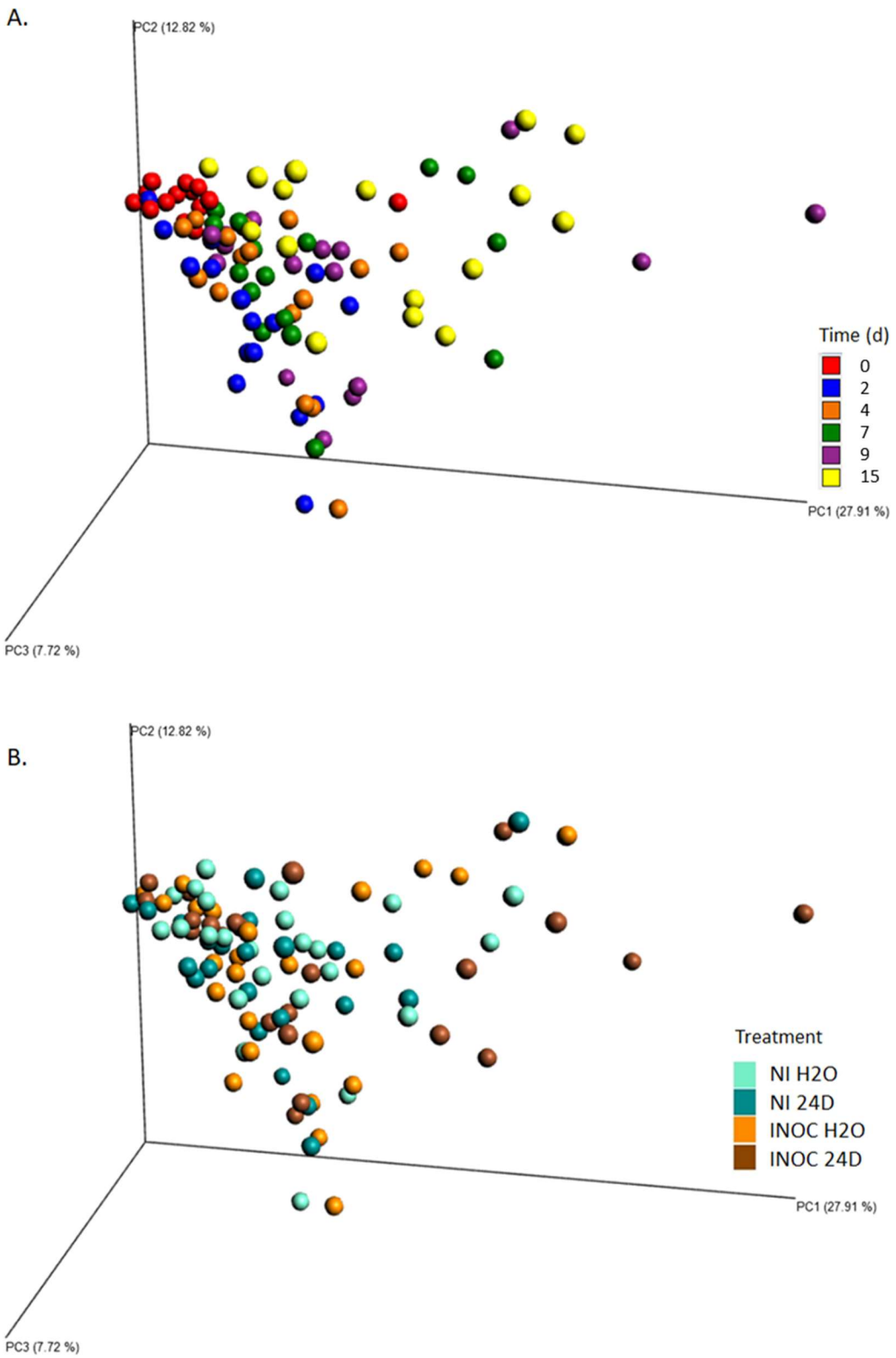
927 **Figure S1**. Bacterial α-diversity indices. Simpson reciprocal, PD whole tree and observed
928 species indices were estimated at 0, 2, 4, 7, 9 and 15 days after treatment in soil microcosms
929 inoculated with *C. necator* Rif$^r$ at $10^5$ cfu $g^{-1}$ dw soil (INOC) or not (NI) and treated at the
930 agronomic dose of formulated 2,4-D (24D) or not ($H_2O$). The values are means ± standard
931 errors (n = 4). ANOVA indicated that neither inoculation nor 2,4-D treatment had impact on
932 the measured indices (n = 4, $p < 0.01$).

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934 **Figure S2.** Bacterial β-diversity. PcoA of the weighted unifrac distance matrix obtained from

935 16S metabarcoding. A. The different colors represent the different sampling times. B. The dif-

936 ferent colours represent the different treatments.