



# 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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**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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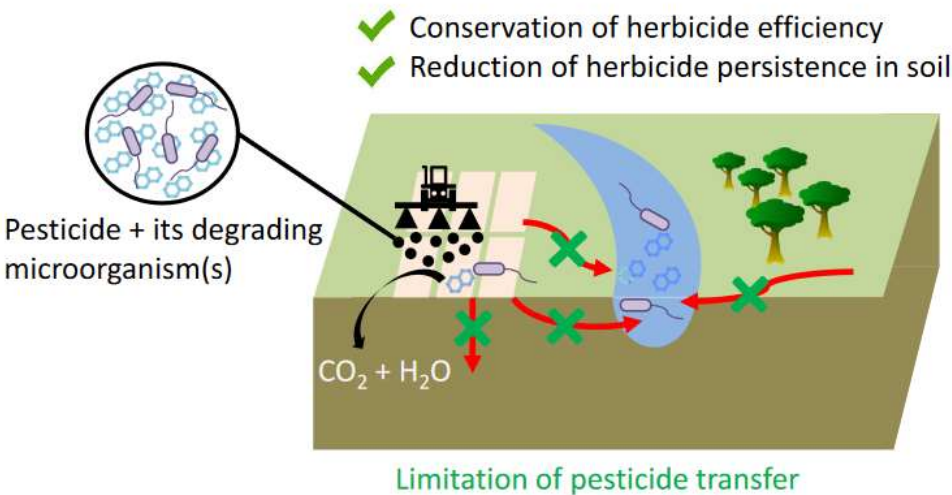
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**Graphical abstract**



37 **Highlights**

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

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## 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<sup>®</sup>) at agricultural recommended doses with  $10^5$  cells.g<sup>-1</sup> 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  $\alpha$ - and  $\beta$ -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 21<sup>st</sup> 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),

inoculum load and survival rate of exogenous microorganisms, or pesticide concentration and bioavailability (Lebeau, 2011; Vandermaesen *et al.*, 2016; Cycoń *et al.*, 2017). To overcome some of these limitations, this approach has sometimes been combined with bio-stimulation (e.g. Raimondo *et al.* (2020)) and other mitigation strategies (e.g. grassed buffer strips and constructed wetlands) to reduce pesticide inputs into surface water and groundwater (Reichenberger *et al.*, 2007; Swartjes and Van der Aa, 2020). Nevertheless, the effectiveness of these approaches was highly variable. It would thus be very advantageous to develop new strategies able to decrease the time of pesticide residence in soil in order to avoid its adsorption and to limit its diffusion into the various environment compartments by rapid biodegradation.

The present study focused on the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid), which belongs to the phenoxy-acid chemical family (Mitchell and Hamner, 1944). It is a widely-used herbicide in agricultural and urban areas around the world for efficient control of broad-leaved weeds in cereal crops and lawns (Peterson *et al.*, 2016). 2,4-D is an analogue of the phytohormone auxin (indole-3-acetic acid, IAA) resulting in uncontrolled growth and death for plants prone to the molecule. It targets dicots and its recommended agronomic field rate is 750 g of active ingredient (a.i.) ha<sup>-1</sup> (EFSA, 2014). Taking its water solubility into account and K<sub>d</sub> values (547 mg L<sup>-1</sup> and 1.81 – 4.28 L kg<sup>-1</sup>, respectively), 2,4-D is classified as having a moderate potential for ground water contamination, which is also proven by GUS (Groundwater Ubiquity Score) values of 2 – 2.7 (Farenhorst *et al.*, 2008; Pfeiffer, 2010; EFSA, 2014). Nevertheless, it has been quantified worldwide in ground water, sometimes reaching concentrations exceeding 1 µg/L (Brauns *et al.*, 2018; ADES, 2019; Muszyński *et al.*, 2020). 2,4-D is also frequently detected in surface water: in 50% of the samples in France in 2018 (NAIADES, 2019) and also all around the world (Ensminger *et al.*, 2013; Brauns *et al.*, 2018; Horn *et al.*, 2019; de Castro Lima *et al.*, 2020; Muszyński *et al.*, 2020). As 2,4-D was shown to lead to numerous adverse effects on non-target aquatic organisms, plants and human life (Smith *et al.*, 2017; Dehnert *et*

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

Microorganisms are the main drivers of 2,4-D degradation in soil (Peterson *et al.*, 2016). The model strain *Cupriavidus necator* JMP134 (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Waustersia eutropha*), which is able to use 2,4-D as the sole carbon source (Don and Pemberton, 1981), has been the most studied. It was notably used to investigate the biodegradation pathways of this herbicide (You and Ghosal, 1995; Leveau *et al.*, 1999; Laemmli *et al.*, 2000). The *C. necator* JMP134 strain possesses the well-described plasmid *pJP4* that contains the degrading-gene cluster *tfd* (Plumeier *et al.*, 2002; Trefault *et al.*, 2004). Each *tfd* gene encodes an enzyme involved in each of the 7 steps of 2,4-D mineralization. Among these genes, *tfdA* encodes for 2,4-D/ $\alpha$ -ketoglutarate dioxygenase (TfdA) involved in the first step of the herbicide degradation. Hence its frequent use as a molecular marker for the degradation potential of 2,4-D in the environment (Stibal *et al.*, 2012; Mierzejewska *et al.*, 2019). Curative 2,4-D bioaugmentation assays, using between  $10^5$  to  $10^8$  CFU/g<sup>-1</sup> soil of various degrading-strains, were carried out in soil slurry (Inoue *et al.*, 2012; Chang *et al.*, 2015), in reactor (Pepper *et al.*, 2002), in sieved or amended soil microcosms (Dejonghe *et al.*, 2000; Xia *et al.*, 2017) or more recently in combination with other mitigation strategies (Yang *et al.*, 2018; Barba *et al.*, 2021). The removal of 2,4-D has also been investigated in soil microcosms following simultaneous application of the herbicide and its degrading-strain (Önneby *et al.*, 2010). This interesting approach increases the direct contact between the pesticide and its degrading-microorganism enhancing its biodegradation in real time, thus avoiding adsorption processes. Moreover, in such an approach, the exogenous degrading-microorganisms must be efficient for only a short period of time.

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



the 2,4-D contamination in agricultural soils and the risk of leaching and runoff in aquatic ecosystems. This strategy consists of simultaneously applying the formulated 2,4-D herbicide as DAM<sup>®</sup> and its mineralizing *Cupriavidus necator* JMP134 strain in a planted soil, in order to optimize the conditions for its biodegradation, just after its action and before its diffusion to the surrounding ecosystems. The experiment was carried out with a laboratory microcosm approach under controlled conditions. Herbicide dissipation and mineralization but also herbicidal efficiency, survival of the added degrading strain and impact on the structure of edaphic bacterial community were assessed.

## 2. Materials and methods

### 2.1. Model herbicide and soil

The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid,  $\geq 98$  % purity) and [ring-<sup>14</sup>C]-2,4-D (analytical-grade purity  $> 99\%$ , specific activity  $4.6 \times 10^8$  Bq mmol<sup>-1</sup>) were purchased from Sigma Aldrich (France). The formulated 2,4-D (DAM<sup>®</sup>, 391 g 2,4-D L<sup>-1</sup>) was kindly provided by Agriphar (now UPL OpenAg, France). The OECD sandy loam standard soil 5M was purchased from LUFA Speyer (Germany). The physico-chemical properties and characteristics of the soil are indicated in Table 1.

### 2.2. Bacterial strain

The edaphic 2,4-D-degrader *Cupriavidus necator* JMP134 strain was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM number: 4058). Prior to the experiment, we checked for rifampicin-resistant microorganisms on Rif-LB plates (must be absent) from a suspension of the OECD standard soil 5M. Then, as already used to specifically enumerate a bacterial strain in soil (Schreiter *et al.*, 2014; Strauss *et al.*, 2015;

Sharma *et al.*, 2017), a chromosomal rifampicin resistance (Rif<sup>r</sup>) mutation was induced in the *C. necator* strain (Karunakaran and Davies, 2000; Lyu and Zhao, 2014). To achieve this goal, *C. necator* JMP134 strain was cultivated in 5 mL LB broth overnight at 28 °C. After centrifugation (8,000 g, 15 min), the pellet was washed in 5 mL of NaCl 0.8 % and spread on LB agar plate supplemented with 150 µg mL<sup>-1</sup> of rifampicin (Sigma Aldrich, France, ≥ 97 % purity). After 48 h of incubation at 28 °C, spontaneous Rif<sup>r</sup> mutants were obtained. Their ability to mineralize pure and formulated 2,4-D (DAM<sup>®</sup>) was tested and found to be the same as that of the parent strain.

### 2.3. Preparation of inoculum

The Rif<sup>r</sup> *C. necator* JMP134 mutant (called later *C. necator* Rif<sup>r</sup>) was cultivated overnight at 28 °C in 25 mL of LB broth containing 50 µg mL<sup>-1</sup> rifampicin and 50 mg L<sup>-1</sup> 2,4-D under orbital agitation at 150 rpm in 50 mL Erlenmeyer flasks. The bacterial culture was then centrifuged (8,000 g, 15 min) and the pellet was washed twice in 20 mL of sterile NaCl 0.8 % before being resuspended in 10 mL sterile Volvic<sup>®</sup> water. In parallel, the colony forming units enumeration (CFU mL<sup>-1</sup>) was carried out to ensure that the theoretical expected bacterial concentration was spread on microcosms (see below). The correlation between OD<sub>600 nm</sub> measurements and CFU mL<sup>-1</sup> (one OD<sub>600 nm</sub> unit corresponds to 6 10<sup>8</sup> CFU mL<sup>-1</sup>) was performed by spreading serial dilutions of culture on LB agar plates and counting the colonies after 48 h of incubation at 28 °C.

### 2.4. Microcosm setup and sampling

Ninety-six microcosms were prepared by adding 100 g dry weight (dw) of OECD soil in each crystal polystyrene box (length: 90 mm, width: 60 mm and height: 50 mm), previously 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<sup>2</sup>) 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<sup>-1</sup> 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 only (10<sup>5</sup> CFU g<sup>-1</sup> 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 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<sub>2</sub>O (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  $89 \pm 2$  %.

### 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  $\lambda = 222$  and 285 nm. The mobile phase was composed of aqueous H<sub>3</sub>PO<sub>4</sub> (0.01 % v/v, pH = 2.9) (A) and acetonitrile (B) at a flow rate of 1 mL.min<sup>-1</sup>. 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:  $C_t = (C_0 - C_f) / [1 + 10^{DT_{50} - t(p)}]$ .  $t$  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<sup>r</sup> 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  $\mu\text{g mL}^{-1}$  of both rifampicin and cycloheximide. The plates were then incubated during 48 h at 28 °C for *C. necator* Rif<sup>r</sup> enumeration. The number of *C. necator* Rif<sup>r</sup> was expressed as CFU  $\text{g}^{-1}$  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  $\text{mg kg}^{-1}$  dw) and [ring- $^{14}\text{C}$ ]-2,4-D (1.7 K bq), and incubated in a radiorespirometer at 20 °C in the dark during one month.  $^{14}\text{C}$ -carbon dioxide resulting from the mineralization of  $^{14}\text{C}$ -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  $^{14}\text{C}$ -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  $^{14}\text{C}$ -2,4-D. The slope of the 1<sup>st</sup> regression line is considered to be the maximum mineralization potential rate.

## 2.8. Quantitative PCR analysis

### 2.8.1. DNA extraction

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

### 2.8.2. Quantitative PCR analysis

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

## 2.9. 16S metabarcoding

### 2.9.1. Amplicon generation and MiSeq sequencing

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

### 2.9.2. Sequencing analysis

The sequence data were analysed using an in house developed Jupyter Notebooks (Kluyver *et al.*, 2016) piping together different bioinformatics tools. Briefly, R1 and R2 sequences were assembled using PEAR (Zhang *et al.*, 2014) with default settings. Further quality checks were conducted using the QIIME pipeline (J Gregory Caporaso *et al.*, 2010) and short sequences (< 400 bp) were removed. Reference based and *de novo* chimera detection, as well as clustering in OTUs were performed using VSEARCH (Rognes *et al.*, 2016) and the adequate reference databases (Greengenes' representative set of sequences). The identity thresholds were set at 94 % for 16S rRNA gene data based on replicate sequencing of a bacterial mock community containing 40 bacterial species. Representative sequences for each OTU were aligned using PyNAST (J. G. Caporaso *et al.*, 2010) and a 16S rRNA gene phylogenetic tree was constructed using FastTree (Price *et al.*, 2010). Taxonomy was assigned using UCLUST (Edgar, 2010) and the latest released Greengenes database v.05/2013 (McDonald *et al.*, 2012).  $\alpha$ -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 H<sub>2</sub>O, INOC H<sub>2</sub>O, 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<sub>50</sub>),  $\alpha$ -diversity indexes of soil microbial communities and abundance of *C. necator* Rif<sup>r</sup> 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  $\beta$ -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<sup>r</sup> (**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 H<sub>2</sub>O and INOC H<sub>2</sub>O 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<sup>r</sup> 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<sup>r</sup> 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<sup>r</sup> strain (INOC 24D), experienced the lowest surface coverage. Furthermore, no significant difference was observed between NI H<sub>2</sub>O vs. INOC H<sub>2</sub>O, and NI 24D vs. INOC 24D. These results indicate that the presence of exogenous *C. necator* Rif<sup>r</sup> 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 H<sub>2</sub>O and INOC H<sub>2</sub>O 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).

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

### 3.2.1. 2,4-D dissipation in soil

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

The inoculation of the *C. necator* Rif<sup>r</sup> strain, previously grown in LB medium supplemented with 2,4-D, concomitantly to the herbicide in soil (INOC 24D), suppressed the lag phase before herbicide dissipation. Under these conditions, the 2,4-D dissipation rate was increased ( $0.47 \pm 0.06 \text{ day}^{-1}$ ) and its  $DT_{50}$  was reduced to  $2.34 \pm 0.19$  days compared to the NI 24D soil. Thus, 4 days after treatment when the 2,4-D dissipation just started in the NI 24D condition, a decrease of about 85% of the 2,4-D initial concentration was already observed, leading to a complete dissipation of the herbicide within 7 days (Figure 3 and Table 2). Therefore, the INOC 24D treatment reduced the persistence of 2,4-D by a 3-fold factor compared to the NI 24D treatment ( $P < 0.05$ ). Note that comparison of the 2,4-D mineralizing kinetics between the natural *C. necator* strain and its mutant showed no significant difference. This preventive bioremediation process thus reduced the risk of 2,4-D leaching and runoff into water bodies where it is currently detected (Brauns *et al.*, 2018; Islam *et al.*, 2018; Horn *et al.*, 2019; NAIADES, 2019; de Castro 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  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -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  $^{14}\text{C}$ -2,4-D to this end and the mineralization percentage was monitored by radiorespirometry. The maximum potential rate of  $^{14}\text{C}$ -2,4-D mineralization was calculated from modelled mineralization kinetics. It was found to be rather low ( $< 5\%$  of  $^{14}\text{C}$ -2,4-D per day) and stable throughout the experiment in the control (NI H<sub>2</sub>O) (Figure 4). It was always significantly higher in the inoculated microcosms (INOC H<sub>2</sub>O and INOC 24D) than in the control (NI H<sub>2</sub>O) ( $P < 0.001$ ). As compared to the NI H<sub>2</sub>O treatment, the inoculation of *C. necator* Rif<sup>r</sup> led to a 4- and 5-fold increase in the rate of mineralization of INOC H<sub>2</sub>O 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 H<sub>2</sub>O (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

also shown to be moderately persistent ( $DT_{50} = 45$  days) in aerobic aquatic environments and highly persistent ( $DT_{50} = 231$  days) in anaerobic terrestrial and aquatic environments (Reregistration Eligibility Decision (RED) 2,4-D, 2005), it is of great importance to mineralize it as soon as possible after spraying onto the soil before it reaches the surrounding environments.

### 3.3. Abundance of *C. necator* Rif<sup>r</sup> in soil

*C. necator* Rif<sup>r</sup> was inoculated at  $10^5$  cfu g<sup>-1</sup> dw soil. In both inoculated soil microcosms, *C. necator* Rif<sup>r</sup> strain was maintained around  $10^4$  -  $10^5$  cfu g<sup>-1</sup> dw soil during the first four days of incubation (Figure 5). *C. necator* Rif<sup>r</sup> was enumerated at a significantly higher value in the INOC 24D microcosms than in the inoculated INOC H<sub>2</sub>O ones at days 7 ( $P < 0.01$ ), 9 and 15 ( $P < 0.001$ ). We could hypothesize that the exposure to 2,4-D provides a temporary selective advantage for microorganisms capable of using the herbicide as carbon and energy source and led to the growth of *C. necator* Rif<sup>r</sup> population as long as it is bioavailable (Macur *et al.*, 2007; Zabaloy *et al.*, 2010). Then, a plateau of the *C. necator* Rif<sup>r</sup> population was observed from day 10 in the INOC 24D treatment (Figure 5) probably because of complete mineralization of the 2,4-D already at day 7. Thus, it seems that these conditions of preventive bioremediation with a simultaneous spray of 2,4-D and the *C. necator* Rif<sup>r</sup> strain can overcome some of the limiting factors influencing the success of curative bioaugmentation processes in soil such as i) sorption of pesticides on soil particles and formation of bound residues leading to a decrease of bioavailability and biodegradability, and ii) competition between the inoculated strain and indigenous microorganisms and its survival (Cycoń *et al.*, 2017), even when the 2,4-D herbicide was removed. Indeed, at day 15, no growth was observed anymore but the strain is still able to survive. This is not surprising since *Cupriavidus necator* can easily adapt its growth to nutrient availability in the environment. Indeed, this strain is described as an optional predator for other bacteria and is able to switch from autotrophic to heterotrophic metabolism depending the

environmental conditions (Makkar and Casida Jr, 1987; Holt *et al.*, 1994). Similar results have already been observed, the 2,4-D degraders being able to survive and remain at high abundance for about 1 month after herbicide exposure has stopped (Merini *et al.*, 2007; Zabaloy *et al.*, 2010).

These results were confirmed by the 16S rRNA sequence affiliated to *C. necator* Rif<sup>r</sup> (similarity above 95%) which were retrieved in both inoculated microcosms but in higher abundances in the INOC 24D treatment ( $4.4 \pm 2.0$  reads per 100,000) than in the INOC H<sub>2</sub>O one ( $0.7 \pm 0.3$  reads per 100,000). No sequences affiliated to *C. necator* Rif<sup>r</sup> were found in the non-inoculated microcosms (NI H<sub>2</sub>O and NI 24D) confirming that no cross-contamination by *C. necator* Rif<sup>r</sup> occurred during the experiment.

#### 3.4. Abundance of 2,4-D bacterial degraders

The abundance of 2,4-D microbial degraders was monitored throughout the incubation by estimating the relative abundance of *tfdA* (coding for an enzyme involved in 2,4-D mineralization) per 16S rRNA sequence (Figure 6). We observed that 2,4-D bacterial degraders were present in rather large amount in the control soil NI H<sub>2</sub>O averaging 5.5 *tfdA* sequences per thousand 16S rRNA and remained stable during the 15 day-experimentation (Figure 6). For the non-inoculated soils, the abundance of *tfdA* gene copy number increased in the presence of 2,4-D (NI 24D) after 9 days of incubation and was still higher after 15 days than in the non-treated soils (NI H<sub>2</sub>O). These results indicate that the indigenous 2,4-D degrader populations need to be stimulated by the herbicide before being able to grow and activate their 2,4-D mineralization capacity as shown in Figures 3 and 4. This induction of 2,4-D degrading gene expression has already been described (Trefault and Guzmán, 2009) and may contribute to the accelerated biodegradation observed with edaphic microbial communities chronically exposed 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 H<sub>2</sub>O) was similar to the NI H<sub>2</sub>O and remained stable throughout the experiment but increased after only 4 days after inoculation in the treated soils (INOC 24D). The *C. necator* Rif<sup>R</sup> 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<sup>r</sup> 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.  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\beta$ -diversity was affected by the time ( $P_{uw}$  and  $P_w < 0.01$ ) but not by the pesticide treatment ( $P_{uw} = 0.119$ ,  $P_w = 0.129$ ) nor the strain inoculation ( $P_{uw} = 0.253$ ,  $P_w = 0.181$ ) (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 necator* 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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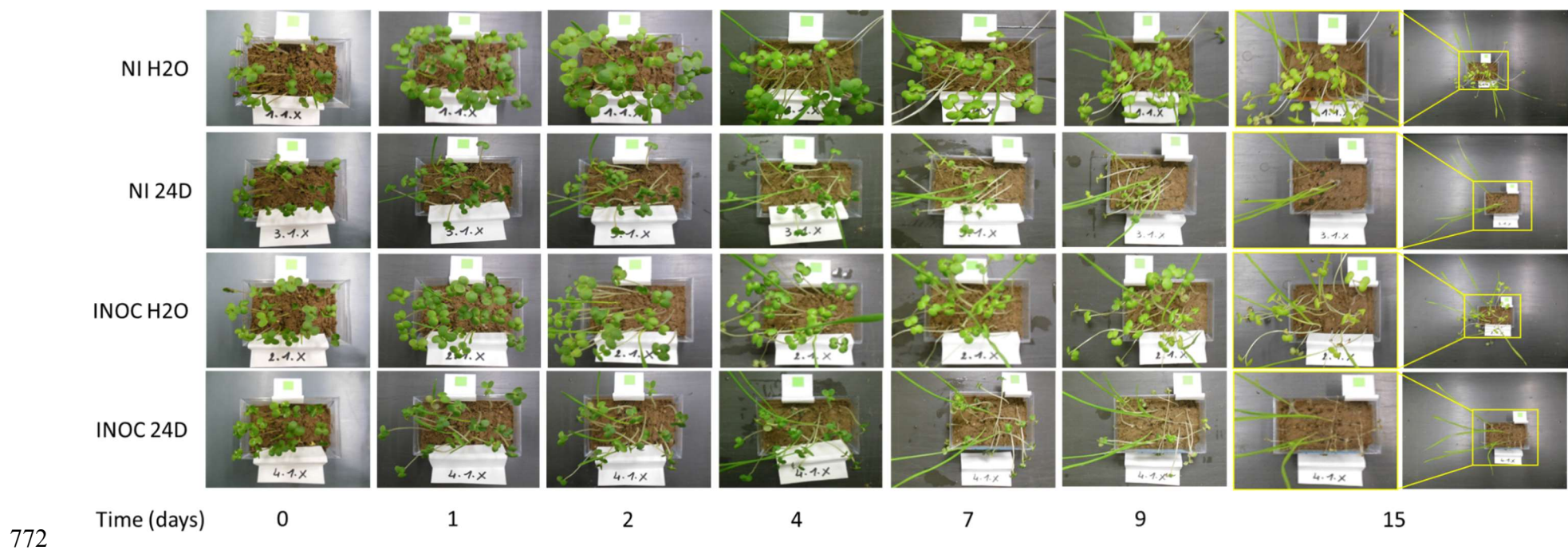


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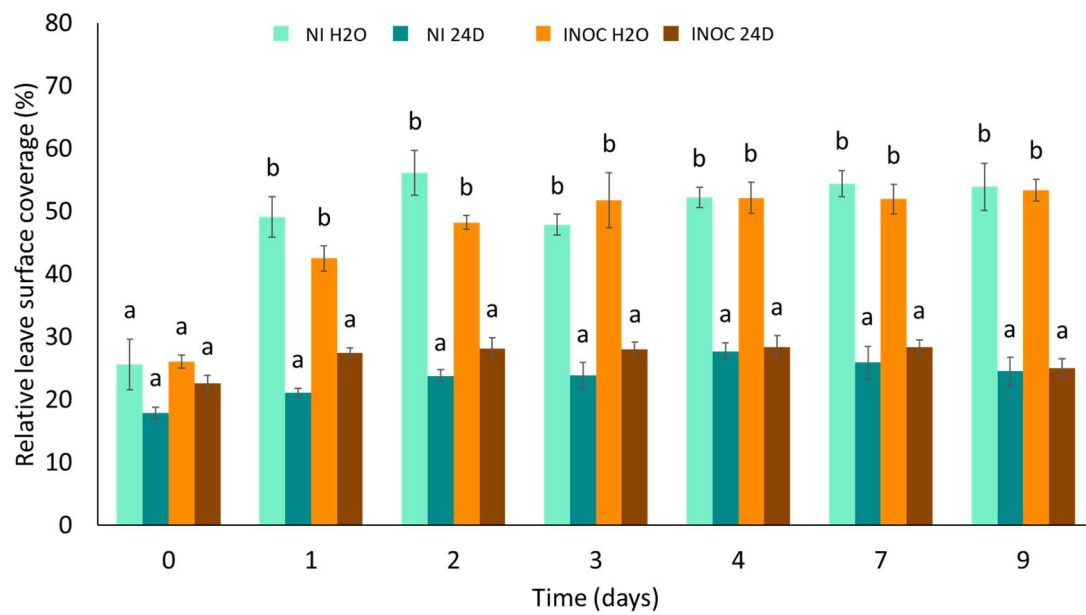
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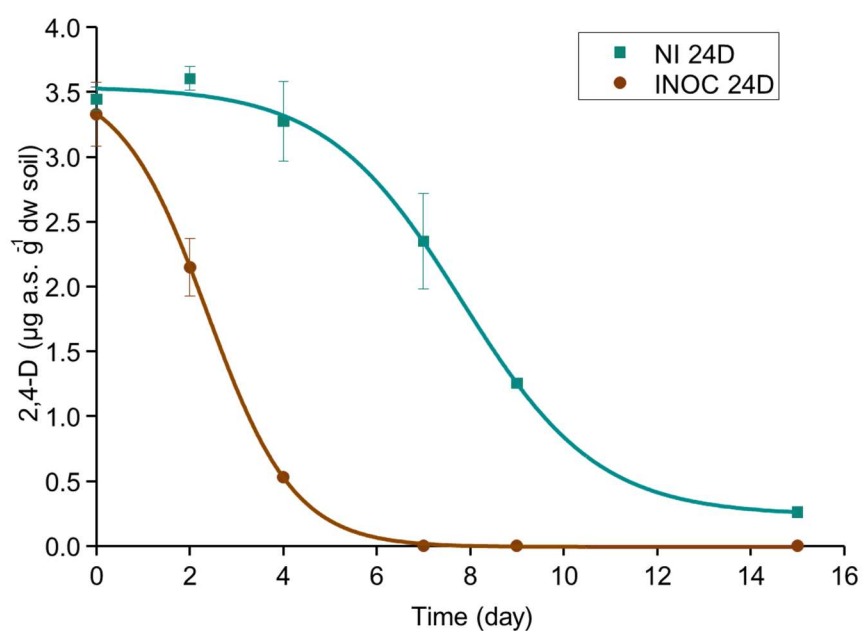
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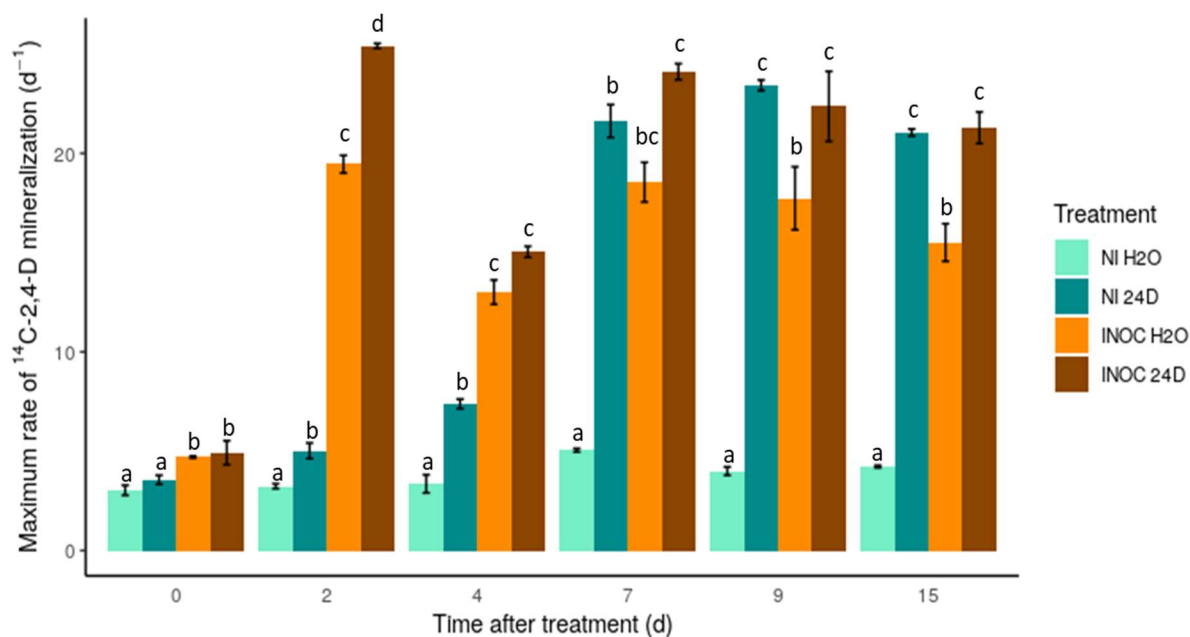
773 **Figure 1.** Top view of soil microcosms inoculated with *C. necator* Rif at  $10^5$  cfu g<sup>-1</sup> dw soil (INOC) or not (NI) and treated with the agronomic  
 774 dose of formulated 2,4-D (24D) or not (H<sub>2</sub>O) along the experiment (0-15 days). One replicate per treatment has been 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 leaf surface coverage over time expressed as a percentage of the surface of soil microcosms inoculated with *C. necator* Rif<sup>r</sup> at 10<sup>5</sup> cfu g<sup>-1</sup> dw soil (INOC) or not (NI) and treated with the agronomic dose of formulated 2,4-D (24D) or not (H<sub>2</sub>O). For each date, statistical differences were indicated by lowercase letters (a < b), Tukey's test (P < 0.05).

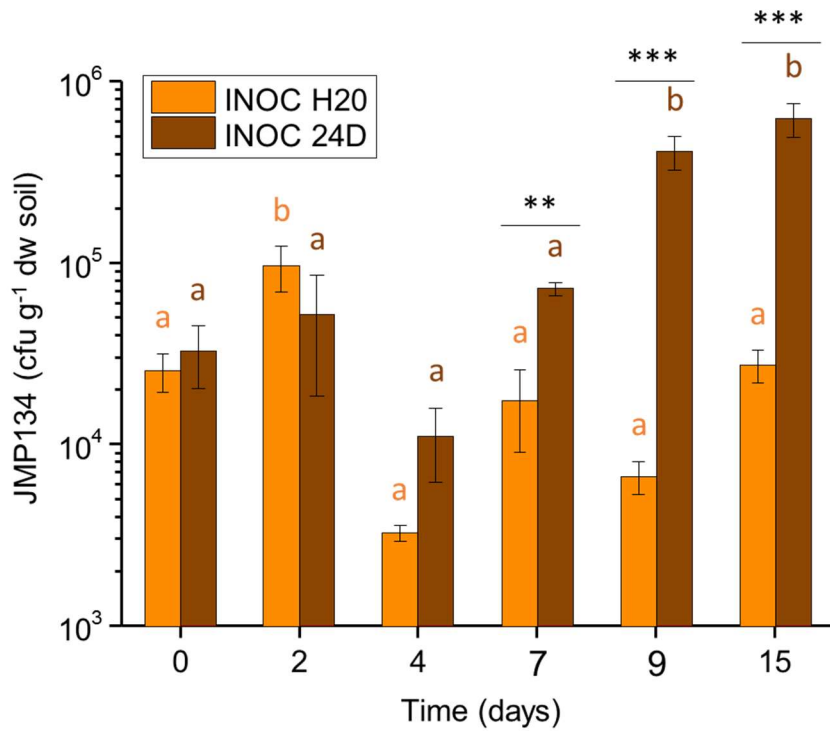


**Figure 3.** Dissipation kinetics of 2,4-D in soil. Microcosms were treated with the agronomical dose of formulated 2,4-D and either inoculated with *C. necator* Rif<sup>r</sup> at  $10^5$  cfu g<sup>-1</sup> dw soil (INOC 24D) or not (NI 24D). The values are means  $\pm$  standard errors of experimental data (n = 4).

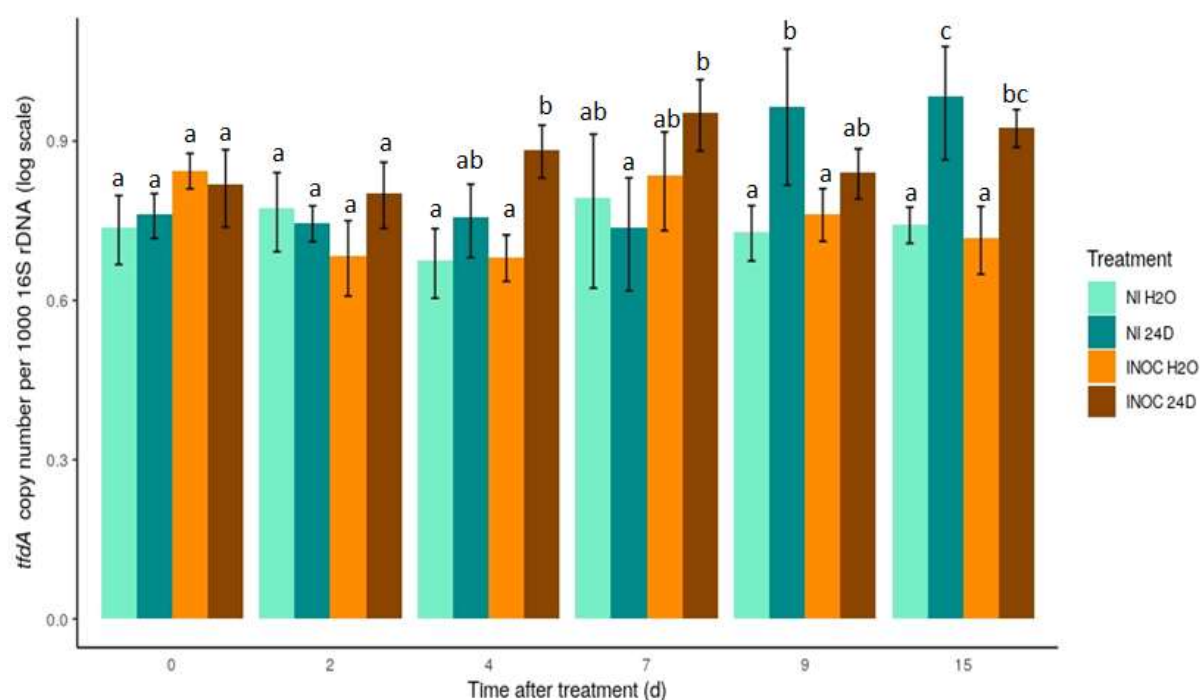


**Figure 4.** Maximum mineralization potential rate of the  $^{14}\text{C}$ -2,4-D at 0, 2, 4, 7, 9 and 15 days after treatment in soil microcosms inoculated with *C. necator* Rif<sup>r</sup> at  $10^5$  cfu g<sup>-1</sup> dw soil (INOC) or not (NI) and treated with the agronomic dose of formulated 2,4-D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





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



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

**Table 1.** Physico-chemical properties of 5M OECD standard soil (LUFA Speyer, Germany).

Values are means of different batch analyses  $\pm$  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 <sub>2</sub> )	Maximum water holding capacity (g / 100 g)	Particule size (mm)	Particule size distribution (%)
17.7 $\pm$ 3.7	1.02 $\pm$ 0.08	0.13 $\pm$ 0.01	7.3 $\pm$ 0.1	40.1 $\pm$ 2.4	< 0.002	10.9 $\pm$ 0.9
					0.002 – 0.05	30.9 $\pm$ 1.4
					0.05 – 2.0	58.2 $\pm$ 1.6

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

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

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

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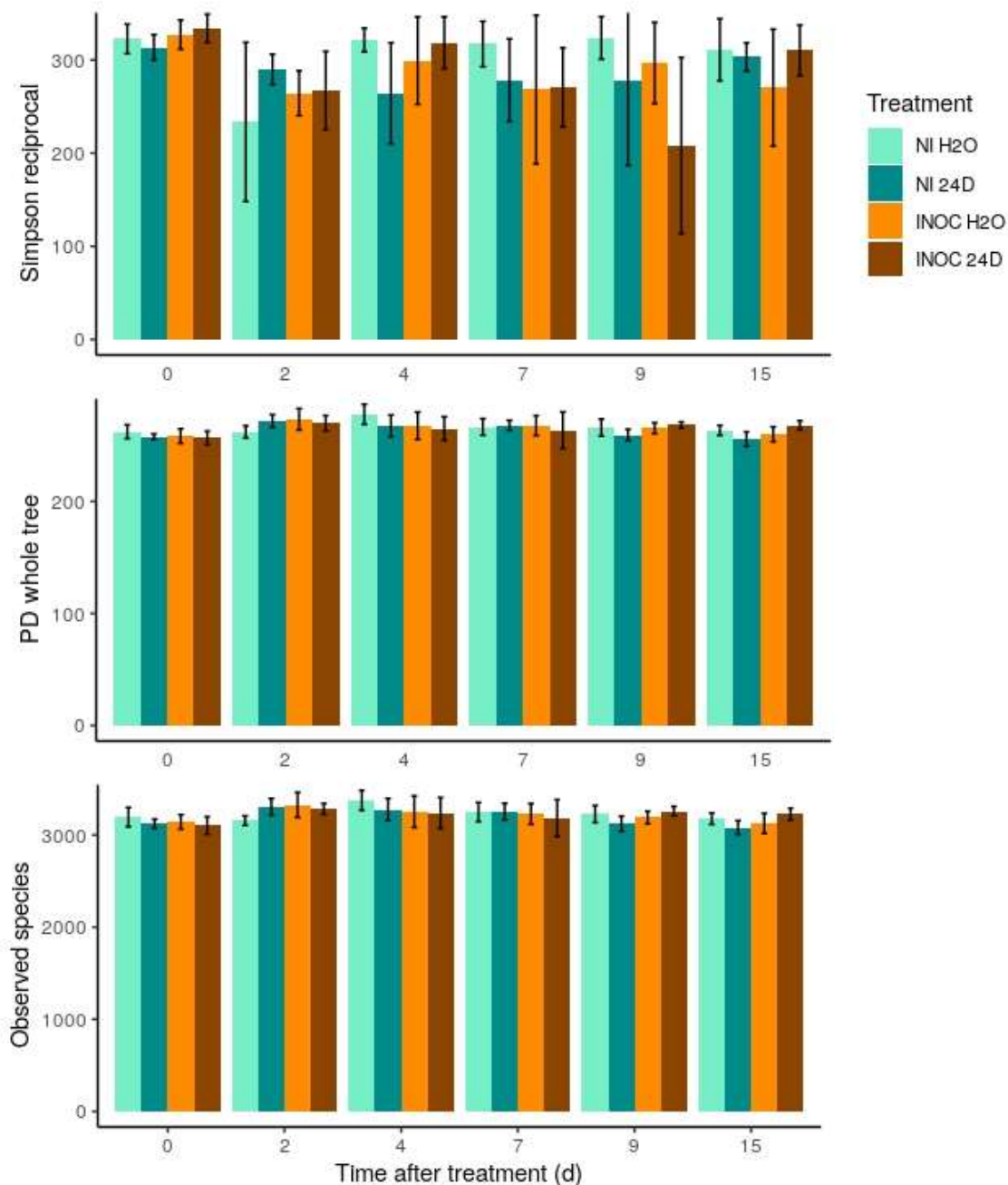
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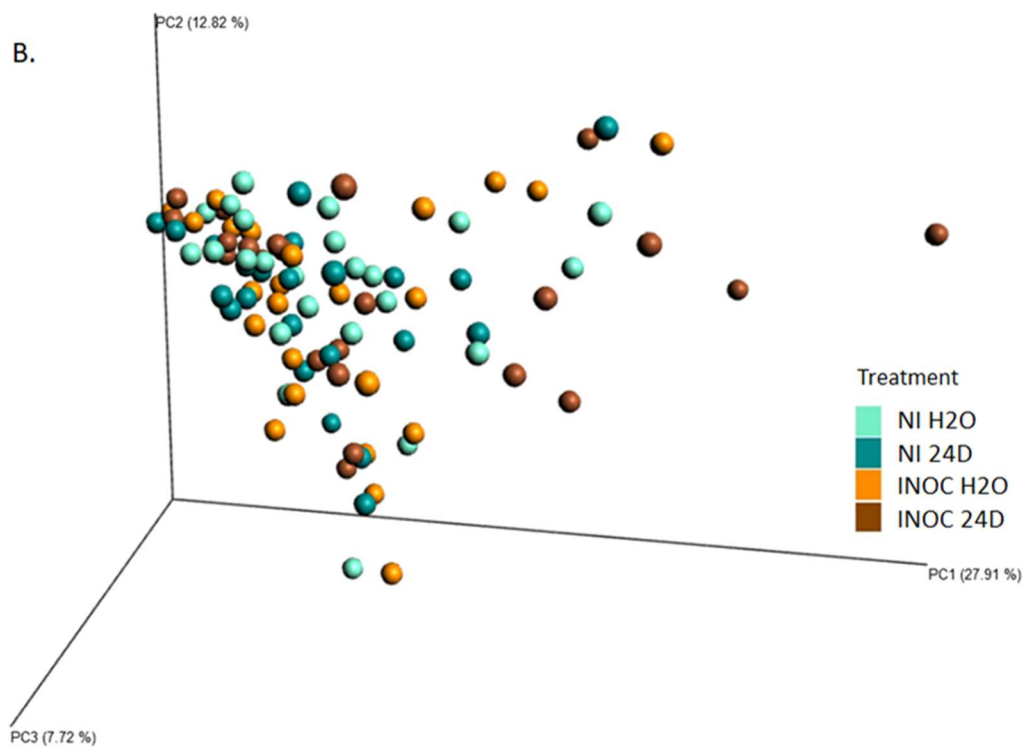
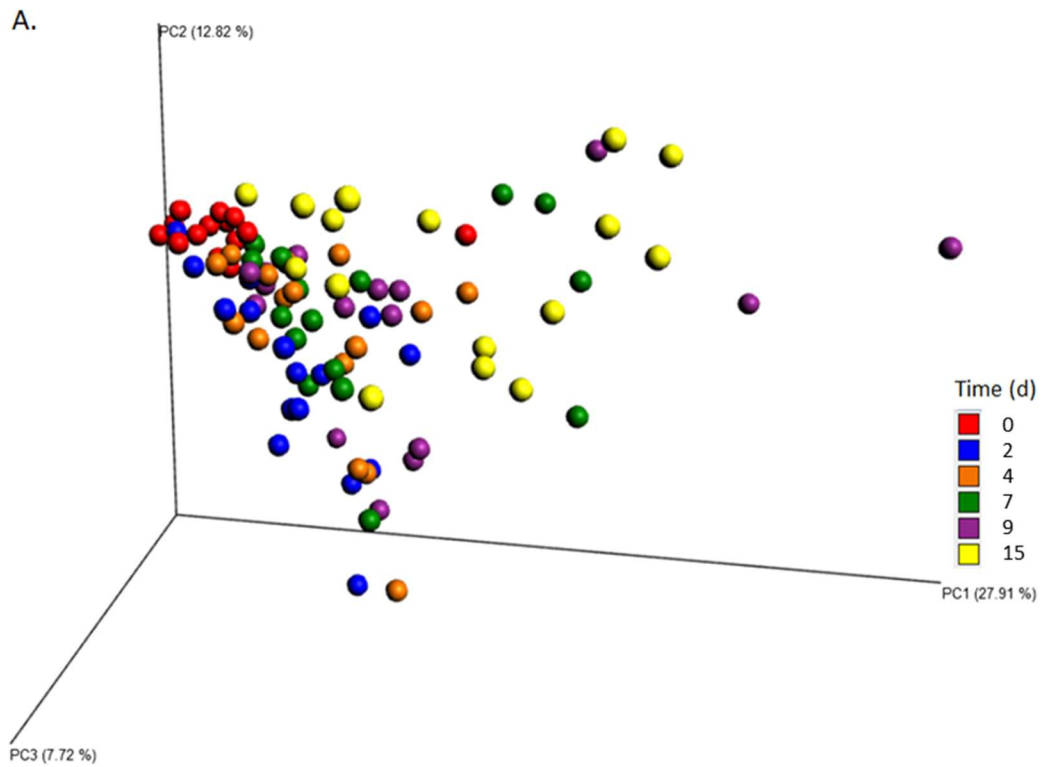
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**Figure S1.** Bacterial  $\alpha$ -diversity indices. Simpson reciprocal, PD whole tree and observed species indices were estimated at 0, 2, 4, 7, 9 and 15 days after treatment in soil microcosms inoculated with *C. necator* Rif<sup>r</sup> at  $10^5$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



**Figure S2.** Bacterial  $\beta$ -diversity. PcoA of the weighted unifrac distance matrix obtained from 16S metabarcoding. A. The different colors represent the different sampling times. B. The different colours represent the different treatments.